Detection of Adenoviral Genome in the Myocardium of Adult Patients With Idiopathic Left Ventricular Dysfunction

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Background—The use of molecular biological techniques has demonstrated the importance of enteroviral infection of the myocardium in the pathogenesis of myocarditis and dilated cardiomyopathy in adults and adenovirus and enterovirus infection in children. The aim of this study was to determine the frequency of adenoviral infection of the myocardium of adults with impaired left ventricular function of unknown origin.

Methods and Results—Nested polymerase chain reaction (nPCR) was used to determine the frequency of detection of adenoviral DNA and enteroviral RNA in myocardial tissue samples from 94 adult patients with idiopathic left ventricular dysfunction and 14 control patients. Histological and immunohistological analyses were performed to detect myocardial inflammation. Adenoviral genomic DNA was detected by nPCR in 12 of the 94 patients with left ventricular dysfunction (in each case, adenovirus type 2), whereas enteroviral RNA was detected in another 12 patients. All control samples were negative for both viruses. In all patients, active myocarditis was excluded according to the Dallas criteria. However, there was significantly decreased CD2, CD3, and CD45RO T lymphocyte counts in the adenovirus-positive group compared with the adenovirus-negative group (P<0.05), whereas no differences were associated with enterovirus infection.

Conclusions—Although enteroviruses are an important causative agent in the pathogenesis of myocarditis and dilated cardiomyopathy, this study shows that adenovirus infection is also important in the pathogenesis of left ventricular failure in adults. However, the pathogenetic basis of disease associated with adenovirus infection may be different than that after infection with other agents, particularly with respect to activation of the host immune response. (Circulation. 1999;99:1348-1354.)

Key Words: cardiomyopathy □ myocarditis □ viruses

Myocarditis and its sequela, dilated cardiomyopathy (DCM), are believed to be initiated by a number of different agents, including viruses, parasitic organisms, and drugs; viral infection is believed to be most common. Since the development of molecular biological techniques, several studies have established a role for persistent enterovirus infection of the myocardium in the pathogenesis of myocarditis and DCM with molecular hybridization or polymerase chain reaction (PCR) methods. We have previously reported the prevalence of viral sequences, detected by PCR and reverse-transcription PCR (RT-PCR), within myocardial samples from pediatric patients with myocarditis or DCM.9,10 In these studies, adenoviral and enteroviral sequences were commonly identified in the myocardium of children with these diseases, with adenovirus detected in 28% and enterovirus detected in 16%.9 Since then, we have studied >300 samples from such patients and detected adenovirus in 19%, enterovirus in 13%, herpes simplex virus or cytomegalovirus (CMV) in ~1%, and parvovirus or Epstein-Barr virus in <1%.9,10 These findings contrast somewhat with the studies reported in adult cases of myocarditis or DCM with which enterovirus1–6 or CMV11 infections have been most commonly associated. However, detection of adenoviral DNA in myocardial samples from adult patients with myocarditis has been reported in a very small number of patients. Both adenoviral DNA and enteroviral RNA were detected in 4 of 7 patients with myocarditis (57%) and in 0 of 6 control subjects.12

The aim of this study was to determine whether adenoviral genomic DNA can be detected in endomyocardial biopsies of patients with idiopathic left ventricular dysfunction by use of nested PCR (nPCR) and compare the frequency of detection with that for enteroviral RNA in the same patient population. Previously, Martin et al7 reported that in an number of adenovirus-associated pediatric cases of myocarditis, the level of inflammation was less than in enterovirus-positive cases. Therefore, we studied each of the endomyocardial biopsy samples histologically and immunohistologically for the presence of active or chronic inflammatory processes to determine whether a similar association exists in adult patient samples.

Received June 22, 1998; revision received November 11, 1998; accepted November 30, 1998.

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Methods

Study Design

In this study, 95 patients with idiopathic left ventricular dysfunction were enrolled. Fifty-six patients were randomly and blindly selected from a pool of patients who had undergone cardiac catheterization in 1995 and 1996. The remaining 39 samples were from consecutive patients enrolled prospectively during 1997. All patients underwent cardiac catheterization for further evaluation of impaired global or regional left ventricular function documented by echocardiography, which was performed on the basis of clinical presentation or history of palpitations, reduced exercise tolerance, atypical chest pain, left or right bundle-branch block, or cardiac arrhythmias. In addition, right ventricular endomyocardial biopsies were obtained from all patients.

Endomyocardial biopsies from 14 patients with coronary heart disease (n=1), toxic cardiomyopathy (n=3), primary arrhythmia (n=1), or ejection fraction >60% and without histological and immunohistological evidence of myocardial inflammation (n=9) were used as a control group. None of these 14 patients had a history of recent viral illness.

For evaluation of myocardial inflammation, histological examinations according to the Dallas classification13 and immunohistological staining for T lymphocytes, activated macrophages, and major histocompatibility complex antigens (MHC I and MHC II) were performed. All samples were coded before nucleic acid extractions and nPCR analysis, and detection of adenoviral DNA and enteroviral RNA was performed blinded.

Cardiac Catheterization

In all patients, catheterization of the left and right sides of the heart, hemodynamic measurements, coronary angiography, and left ventricular angiogram were carried out before endomyocardial biopsies were obtained. Endomyocardial biopsy samples were obtained from the right ventricle by standard percutaneous transvenous right pigtail catheter, whereas the cardiac index and stroke volume index were determined by use of a flow-directed catheter. Echocardiographic analysis was performed in all patients to evaluate left ventricular end-diastolic diameter and ejection fraction.

Patient Design and Synthesis

Primer pairs were designed and synthesized (GIBCO-BRL) to amplify the genomic sequence of adenoviruses encoding the hexon protein and the 5' untranslated region of the enteroviruses (Table 1). The adenovirus-specific primers were designed to amplify all adenovirus serotypes for which sequence data are available in GenBank; the enterovirus-specific primers should amplify most enterovirus types. Primers corresponding to sequences in the β-actin gene were used as a positive control for the isolation of intact DNA and RNA (Table 1).16

RNA and DNA Template Preparation

Tissue samples were first homogenized in RNAzol by use of disposable RNase-free pestles (PGC Scientific). Total RNA and genomic/viral DNA were isolated simultaneously from patient specimens with Tris-saturated phenol (pH 6.6) RNAzol in a modification of the method described17 as previously described16 and resuspended in 25 μL of diethyl pyrocarbonate (DEPC)-treated water.17 Adenovirus type 5 DNA and coxsackievirus B3 RNA, isolated from infected cultured cells, were used as positive viral controls for PCR analysis after nucleic acid extraction.

RT and PCR

For detection of enteroviral genomic nucleic acid of the RNA viruses, RT-PCR was used.18 For synthesis of cDNA, 3 μL extracted total nucleic acid was mixed with 0.5 μL (2 μM) of random primers (GIBCO-BRL) and 6.2 μL DEPC-treated water in the presence of 20 U (0.5 μL) of the RNase inhibitor RNasin (Promega). This mixture was heated to 95°C for 5 minutes and then snap-cooled on ice. To this, 4 μL of 5 RT buffer (GIBCO-BRL), 2 μL of 100 mM dithiothreitol, 0.8 μL of 25 mM dNTPs, another 0.5 μL RNasin, and 200 U (1 μL) Moloney murine leukemia virus RT (GIBCO-BRL) were added. The samples were incubated at 37°C for 1 hour, followed by 5 minutes at 95°C to inactivate the enzyme. Then, 2 μL of this first-strand cDNA was subjected to PCR amplification to detect enteroviral RNA (nPCR) or β-actin RNA (single PCR).

To verify the isolation of both RNA and DNA from the endomyocardial biopsy samples, 2 μL of cDNA or total nucleic acid was subjected to PCR. The template was amplified in a 20-μL reaction containing 1× PCR buffer (GIBCO-BRL), 2.5 mM magnesium chloride, 0.25 mM dNTPs, 0.5 μM oligonucleotide primers, and 2.5 U Taq DNA polymerase (GIBCO-BRL). After an initial 5-minute incubation at 94°C, 35 rounds of amplification were

### Table 1. PCR Primers Used to Detect Adenoviral DNA, Enteroviral RNA, and β-Actin Sequences (Y=C+T, K=T+G, M=A+C, B=T+C+G)

<table>
<thead>
<tr>
<th>Virus</th>
<th>Primer Sequences</th>
<th>Primer</th>
<th>PCR Product Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus (hexon gene)</td>
<td>Primary PCR</td>
<td>5'-ACTACAAYATTGGCTACAGG-3'</td>
<td>ADH-01</td>
</tr>
<tr>
<td></td>
<td>Secondary PCR</td>
<td>5'-CAAAACATAAAAGAAGKGGGTCG-3'</td>
<td>ADH-02</td>
</tr>
<tr>
<td>Enterovirus (5' untranslated region)</td>
<td>Primary PCR</td>
<td>5'-CGGTACCTTTGTGCGCTGT-3'</td>
<td>ENT-01</td>
</tr>
<tr>
<td></td>
<td>Secondary PCR</td>
<td>5'-GGTCCGCTGCGAGATTGCCC-3'</td>
<td>ENT-02</td>
</tr>
<tr>
<td>β-Actin gene</td>
<td>Primary PCR</td>
<td>5'-TACATGGCTGGGGGTGTTGGA-3'</td>
<td>BA1</td>
</tr>
<tr>
<td></td>
<td>Secondary PCR</td>
<td>5'-AAGAGGCGCATCCTACCCCT-3'</td>
<td>BA2</td>
</tr>
</tbody>
</table>

tricular end-diastolic pressure was determined with a left ventricular pigtail catheter, whereas the cardiac index and stroke volume index were determined by use of a flow-directed catheter. Echocardiographic analysis was performed in all patients to evaluate left ventricular end-diastolic diameter and ejection fraction.
performing a Stratagene Robocycler under the following conditions: 94°C for 45 seconds, 64°C for 45 seconds, and 72°C for 45 seconds. This was followed by a 72°C incubation for 5 minutes.

For detection of viral genomic sequences, 2 μL cDNA or total nucleic acid was subjected to nPCR. The primary reaction was performed under the same conditions as described for amplification of β-actin. For the secondary amplification, 2 μL of the primary reaction was diluted in 98 μL TE (10 mmol/L Tris, pH 7.5, 1 mmol/L EDTA), and then 2 μL diluted product was subjected to 30 cycles of PCR amplification as described for the primary amplification.

Analysis of PCR Products
The products of each reaction were analyzed by 1.75% agarose gel electrophoresis containing 0.5 μg/mL ethidium bromide (Sigma Chemical Co), and the DNA product was visualized by UV transillumination. In all cases, positive (purified viral nucleic acid) and multiple negative (water or nucleic acid from a tissue sample known to be negative) control reactions were performed simultaneously with the test samples. All samples were analyzed without prior knowledge of clinical or histological data for each patient, and all PCR-positive samples were required to have duplicate results. Any sample not giving a signal with the β-actin primers was excluded.

DNA Sequencing
The adenovirus amplimers were reamplified and the PCR products were purified with a PCR purification kit (Qiagen) according to the manufacturer’s instructions and resuspended in 30 μL TE. The DNA sequence was determined by cycle sequencing by use of primer ADH-I3 according to the kit manufacturer’s instructions (Stratagene) with a 8% denaturing polyacrylamide gel.

Histology and Immunohistology
For evaluation of myocardial inflammation, histological examinations of sections of formalin-fixed, paraffin-embedded endomyocardial biopsies were performed according to the Dallas classification. For immunohistochemical staining, endomyocardial biopsies were directly embedded in OCT (Miles Laboratories, Inc) and frozen at −70°C. Sections (5 μm) were fixed in acetone for 10 minutes and then incubated with monoclonal antibodies directed against CD2, CD3, CD4, CD8, and CD45RO T lymphocytes. In addition, monoclonal antibodies against activated macrophages and MHC I and II antigens were used. Unbound antibodies were removed by washing twice with PBS. Peroxidase-conjugated rabbit-anti-mouse antibody (Dianova GmbH), diluted at 1:200 in PBS containing 10% FCS, was then added to each section. Quantification of T lymphocytes, activated macrophages, and cells expressing MHC I and MHC II antigens was performed by 2 independent observers, as described by Kühler et al.

Statistical Analysis
The SPSS statistical software package was used for statistical analysis. Statistical significance was determined by use of Student’s t test, with a confidence level of $P=0.05$.

Results

Clinical Presentation
One enrolled patient was determined to be a child (age, 14 years) with myocarditis and was excluded from data analysis. All enrolled adult patients (age ≥17 years; n=94) had global left ventricular dysfunction with an ejection fraction <55%
or regional left ventricular dysfunction with wall motion disturbances in ≥2 wall segments. Detailed hemodynamic data of these and the control patients are listed in Table 2. Coronary, hypertensive, valvular, restrictive, and constrictive heart diseases were excluded in all patients by cardiac catheterization of both sides of the heart. With respect to clinical data, the adenovirus-positive group was significantly older than the adenovirus-negative group (*P*, 0.05) (Table 3). There were no statistically significant differences in any other clinical criteria between the enterovirus-positive and enterovirus-negative patients (Table 4).

**PCR Analysis**

The sensitivity of nPCR compared with single PCR had previously been determined to be ≥10-fold greater in our hands. In the case of adenovirus-specific PCR, nPCR achieved an estimated sensitivity of 5 to 50 genomes or 1 adenovirus genome per 1000 cells. A similar sensitivity was achieved for the enterovirus-specific PCR, representing a detection limit of 1 to 10 genomes or 10^2 cfu.

All samples were positive for the presence of β-actin sequences by PCR and RT-PCR, indicating the successful isolation of both RNA and DNA, respectively. Of the 94 enrolled adult patients, 12 were adenovirus positive by nPCR (Figure 1), and another 12 were enterovirus positive (Figure 2). None of the 14 control samples was positive for either virus. For 18 of the 94 enrolled patients, 2 biopsies were analyzed; for the other 76, single biopsy samples were studied. Of these 18 patients, 3 had ≥1 sample positive for adenoviral DNA (in 1 patient, both were positive). Of these 18 patients, 5 had at least 1 sample positive for enteroviral DNA.

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### Table 3. Comparison of Adenovirus-Positive and Adenovirus-Negative Patients With Left Ventricular Dysfunction

|                          | PCR Adenovirus-Negative Patients (n=82) | PCR Adenovirus-Positive Patients (n=12) | *P*
|--------------------------|----------------------------------------|----------------------------------------|-----
| Mean age, y              | 43±12 (17–67)                         | 52±12 (32–76)                         | 0.04^* |
| Sex, F/M                 | 28/54                                  | 1/11                                   | 0.14 |
| Echocardiographic parameter |                                        |                                        | 0.55 |
| LV ED D, mm              | 61±11 (43–97)                         | 64±14 (48–89)                         | 0.55 |
| Left ventriculographic variables |                                 |                                        | 0.55 |
| Ejection fraction, %     | 47±13 (21–73)                         | 46±16 (22–72)                         | 0.72 |
| Cardiac index, L·min^-1·m^-2 BSA | 3.7±0.9 (1.3–5.8) | 3.7±1.1 (2.3–5.9) | 0.94 |
| End-diastolic volume index, mL/m² BSA | 125±36 (67–229) | 133±66 (67–224) | 0.73 |
| Stroke volume index, mL/m² BSA | 57±11 (35–84) | 55±14 (35–81) | 0.77 |
| Left ventricular end-diastolic pressure, mm Hg | 11±7 (2–40) | 12±7 (4–27) | 0.61 |
| Histology                |                                        |                                        | 0.55 |
| Active myocarditis (Dallas criteria) | 0/82                               | 0/12                                   | NS  |
| Immunohistochemistry     |                                        |                                        | 0.55 |
| CD2 T-lymphocyte counts/HPF | 2.3±1.7 (0–9.5)          | 1.5±0.9 (0.5–3.5)                | 0.03^* |
| CD3 T-lymphocyte counts/HPF | 2.3±1.7 (0–10)            | 1.5±0.8 (0.3–3)                   | 0.02^* |
| CD4 T-lymphocyte counts/HPF | 1.3±1.1 (0–5.4)          | 0.9±0.7 (0.2–2.7)                | 0.14 |
| CD8 T-lymphocyte counts/HPF | 1.2±0.9 (0–4.7)          | 0.9±0.7 (0.3–2.6)                | 0.27 |
| CD45RO T-lymphocyte counts/HPF | 1.0±1.0 (0–4.7)      | 0.5±0.5 (0.1–1.4)                | 0.01^* |
| Macrophages 102-27 counts/HPF | 1.3±0.7 (0.3–4.2)     | 1.1±0.9 (0–2.5)                  | 0.38 |
| HLA1/vessels/HPF         | 1.6±0.5 (0.5–2.5)         | 1.6±0.4 (1.0–2.0)                | 0.57 |
| HLA1/interstitial/HPF    | 1.4±0.5 (0.5–3.0)         | 1.2±0.3 (1.0–2.0)                | 0.13 |
| HLA2DR/vessels/HPF       | 1.4±0.5 (0.5–2.5)         | 1.6±0.4 (1.0–2.0)                | 0.15 |
| HLA2DR/interstitial/HPF  | 1.3±0.4 (0.5–3.0)         | 1.2±0.4 (1.0–2.0)                | 0.47 |

See Table 2 for abbreviations and details. Numbers in parentheses are ranges. *Statistically significant.

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**Figure 1.** Detection of adenoviral genomic DNA by nPCR with primers designed to hexon region. Products were detected by ethidium bromide staining of 1.75% agarose gel. A 100-bp ladder (M) is shown in first and last lanes. Adenovirus-positive control PCR (+) is seen as 330-bp amplimer in penultimate lane (from left to right). In preceding lanes are patient samples (indicated by sample number) and negative controls (−). Note that samples 14 and 22 are positive.
RNA, including 2 patients for whom both were positive. BLAST search analysis of the DNA sequences revealed that in all 12 adenovirus-positive samples from adults, type 2 adenovirus was detected (Figure 3). The single pediatric sample was also positive for adenovirus, type 5 in this case (Figure 3, sample 5).

### Histopathology and Immunohistochemistry

The histopathological analysis of the enrolled adult patients (n=94) and the control group (n=14) excluded active myocarditis in each patient according to the Dallas classification.13 There were significantly fewer CD2 (Figure 4), CD3, and CD45RO T lymphocytes in adenovirus-positive compared with adenovirus-negative patients (Table 3), but no significant differences existed in the number of CD4 and CD8 T lymphocytes or activated macrophages. In addition, the number of MHC I and MHC II antigen-expressing cells was statistically indistinguishable between both subgroups (Table 3). There was no difference in the degree of myocardial inflammation or types of cells present associated with the presence of enterovirus (Table 4).

### Discussion

Application of molecular biological methods (slot-blot technique, in situ hybridization, PCR) has facilitated detection of viral nucleic acid, especially enteroviral RNA, in endomyocardial biopsies of adult patients with myocarditis or DCM. Adenoviral infection of the myocardium in myocarditis or DCM patients has primarily been reported in studies of myocardial samples from children.7,8,20–23 These have revealed that adenoviral infection is at least as common as enterovirus infection. Adenoviral infection of the myocardium has also been reported in a study of a small number of samples from adult myocarditis patients.12 The aim of this
The study was to determine whether adenoviral genomic DNA can be detected in endomyocardial biopsies of adult patients with idiopathic left ventricular dysfunction by use of nPCR. The data presented here demonstrate that in adult patients with idiopathic left ventricular dysfunction (after exclusion of coronary, hypertensive, valvular, restrictive, and constrictive heart diseases), adenoviral DNA can be detected in a significant proportion (12 of 94, 13%). Enteroviral RNA could be detected with a similar incidence (12 of 94). These are similar to the data from pediatric patients with DCM in whom adenoviral DNA and enteroviral RNA have been detected in 16 of 132 patients (12%) and 10 of 132 patients (8%), respectively.7,10 No patients were infected with both adenovirus or enterovirus. In 18 patients, 2 biopsies were studied: In 8 patients, virus was detected in ≥1 sample, but virus was detected in both samples in only 3 patients. These data suggest that viral infection of the myocardium is focal and that multiple samples should be studied to accurately determine the frequency of virus infection. These data are consistent with the pathological data that have shown that histological evidence of myocarditis is not always evident in each sample.23

DNA sequence analysis of the PCR products showed that in all 12 adult samples positive for adenovirus, the serotype detected was type 2 adenovirus. This is similar to the data from neonatal and pediatric patients with myocarditis or DCM in whom adenoviral DNA and enteroviral RNA have been detected in 16 of 132 patients (12%) and 10 of 132 patients (8%), respectively.7,10

Despite the considerable evidence that adenoviruses and enteroviruses are the major causative agents of myocarditis and DCM identified to date, it has been something of a conundrum why 2 such distinctly different virus families infect myocytes and cause a similar pathology. The description of the common coxsackievirus B and adenovirus (subgroup C) receptor offers at least a partial explanation,24,25 because the expression of this receptor by cardiomyocytes would facilitate the uptake of either of these viruses.

None of the enrolled patients showed active myocarditis according to the Dallas criteria. Thus, left ventricular function cannot be explained by active myocarditis. Therefore, the clinical data (impaired regional or global left ventricular dysfunction), combined with the histological results, indicate either a diagnosis of DCM or DCM as the precipitating event. The observation of a significant difference between the number of CD2, CD3, and CD45RO T lymphocytes in the adenovirus-positive group compared with the adenovirus-negative group is interesting. We have previously shown that in pediatric patients the inflammatory infiltrate is often less in patients positive for adenovirus than those positive for enterovirus.7 The reduction in the number of activated lymphocytes in the adenovirus-infected myocardium compared with myocardial samples infected with other viruses or of unknown origin may have importance in the pathogenesis of adenovirus-induced myocardial disease. The adenoviruses have a number of strategies for modulating the immune response. Several adenovirus-encoded proteins are capable of interacting with host immune components (for a review, see Reference 26). These include proteins encoded by the E3 region, which can protect cells from tumor necrosis factor–mediated lysis,27 as well as downregulation of MHC class I antigen expression.28 The E1A proteins are capable of pro-
motivating the induction of apoptosis\(^{29}\) and inhibiting IL-6 expression,\(^{30}\) as well as interfering with IL-6 signal transduction pathways.\(^{31}\) These functions of E1A may be particularly pertinent in explanations of the myocardial pathology observed in DCM patients. First, IL-6 promotes lymphocyte activation, which was reduced in the adenovirus-infected patient samples in this study. Second, it has been reported that in a small number of cases, apoptotic cells were detected in myocardial tissue samples from patients with idiopathic DCM by an in situ labeling protocol, including adenovirus-infected samples.\(^{32,33}\)

In this study, we have shown that in adult patients with idiopathic left ventricular dysfunction, including patients with DCM, both adenoviral DNA and enteroviral RNA can be detected with similar frequencies. Furthermore, adenovirus types 2 and 5 (group C adenoviruses) appear to be cardio-viral serotypes of adenovirus in adults and children. However, infection of the myocardium with adenovirus may result in less immune cell activation than with other agents, suggesting the possibility of a different mechanism of pathogenesis of adenovirus-induced chronic myocardial disease.

Acknowledgments

This work was supported by the Texas Children’s Hospital Foundation Chair in Pediatric Cardiac Research (Dr Towbin) and an Averchome Cardiac Fund Grant (Drs Bowles and Towbin). This work was performed in the Phoebe Wilingham Muzzy Pediatric Molecular Cardiology Laboratory at Baylor College of Medicine, Houston, Tex.

References

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Circulation. 1999;99:1348-1354
doi: 10.1161/01.CIR.99.10.1348

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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