End-Stage Dilated Cardiomyopathy
Persistence of Enterovirus RNA in Myocardium at Cardiac Transplantation and Lack of Immune Response

Neil E. Bowles, Marlene L. Rose, Pat Taylor, Nicholas R. Banner, Peter Morgan-Capner, Louise Cunningham, Leonard C. Archard, and Magdi H. Yacoub

Tissue from the explanted hearts of 21 patients with idiopathic dilated cardiomyopathy or 19 patients with other specific heart muscle diseases were investigated for presence of enterovirus-specific RNA with an enterovirus group–specific cDNA probe. This was complementary to coxsackievirus B2 RNA sequences between nucleotide numbers 6,550 and 7,400, which are highly conserved between enteroviruses. Hearts from six patients with dilated cardiomyopathy and one patient with ischemic heart disease were found to contain virus RNA. Serology revealed that only one patient (dilated cardiomyopathy group) was positive for coxsackievirus B–specific IgM but negative for virus RNA in the myocardium. Quantitation of leukocytes and T-lymphocytes in the myocardium and expression of major histocompatibility locus antigens revealed no significant differences associated with persistence of virus RNA. These data demonstrate that enterovirus RNA persists in myocardium of a significant proportion of patients with end-stage dilated cardiomyopathy in the absence of a continuing cell-mediated or humoral immune response. (Circulation 1989;80:1128–1136)

Dilated cardiomyopathy has been defined as a heart muscle disease of unknown origin with dilation of the ventricles.1 A study of the population of Malmo, Sweden, has suggested that it occurs as frequently as 10 per 100,000,2 which would suggest an occurrence of approximately 5,500 cases a year in the United Kingdom. A survey in the United States suggested that up to 10,500 deaths a year were due to cardiomyopathies (five per 100,000) and that of these, about 87% were due to idiopathic dilated cardiomyopathy.3

A potential role for coxsackievirus B in the pathogenesis of dilated cardiomyopathy was proposed several years ago.4 In that study, patients with dilated cardiomyopathy had significantly higher neutralizing antibody titers than did controls, a feature more obvious among patients with a short medical history. There have been many animal and human studies demonstrating the etiologic role of enteroviruses in myocarditis, and several groups have proposed that dilated cardiomyopathy is a long-term sequela of myocarditis.5,6 A recent long-term study of patients with myocarditis7 reported direct evidence for such a link. Of 23 patients originally diagnosed as having myocarditis, 12 developed dilated cardiomyopathy during the follow-up period (average, 43 months). A feature common to these two diseases has been the consistent failure of conventional virologic techniques to demonstrate the presence of viruses in the myocardium by either growth and isolation or by immunofluorescent staining for virus-specific antigens.8

We have previously reported investigations using enterovirus group–specific molecular hybridization probes.5,9,10 We have demonstrated the presence of virus RNA in endomyocardial biopsy samples not only from patients with various grades of histologically proven myocarditis but also from 22 of 54 patients (41%) with healed myocarditis–dilated cardiomyopathy.11 These data show that enteroviruses are capable of persisting in myocardium beyond the acute phase of myocarditis and suggest a role in the etiology of dilated cardiomyopathy, supporting the hypothesis that enterovirus-induced myocarditis disposes to the development of dilated cardiomyopathy.

Those patients with dilated cardiomyopathy we studied previously6,9 and in whom we demonstrated enterovirus-specific RNA sequences in endomyocardial biopsy samples had not reached the end-stage of the disease process requiring cardiac trans-
plantation. We now report a study of tissue from the explanted hearts from a separate group of patients who underwent transplantation: this investigation examines persistence of enteroviruses in myocardium at end-stage disease. In addition, we have investigated whether the persistence of enterovirus RNA is associated with a continuing immune response by quantitating leukocytic and T-lymphocytic infiltrates in the heart and by measuring virus-specific antibodies in serum. We have also investigated whether there is abnormal expression of major histocompatibility locus (MHC) antigens, as occurs in persistent viral hepatitis or acute cardiac allograft rejection.

Methods

Patients

We examined tissue from 40 consecutive patients undergoing orthotopic cardiac transplantation for end-stage cardiac failure. Before transplantation, each patient had undergone full cardiac evaluation including cardiac catheterization and coronary angiography. The diagnosis of dilated cardiomyopathy was made by exclusion of recognized causes for cardiac failure. The 21 patients classified as having dilated cardiomyopathy (group A) included two patients with a history of heavy alcohol consumption (more than five pints of beer a day) and one woman (46 years old) who had undergone a successful closure for atrial septum defect at the age of 21 years. Her symptoms had begun a few weeks after the delivery of a healthy infant. The 19 patients with identifiable causes for their cardiac failure (group B) included 17 with angiographically proven coronary artery disease, one with congenital heart disease (double inlet ventricle with pulmonary atresia), and one with amyloid heart disease. The characteristics of the two groups are shown in Table 1. During the transplant procedure, samples of right ventricular myocardium were obtained from the explanted heart, snap-frozen, and stored in liquid nitrogen for subsequent analysis.

Hybridization Studies

We used an enterovirus group-specific complementary DNA (cDNA) probe copied from the conserved 3' terminus of the coxsackievirus B2 genome. The probe is complementary to sequences between nucleotide numbers approximately 6,550 and 7,400

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*History of excessive alcohol consumption.
†Amyloid.
‡Congenital heart disease.
Cardiomyopathy group comprises 15 men and six women. Control group comprises 15 men and four women.
of the virus genome and includes the oligo (dT) sequence used to prime the cDNA synthesis. This region of the genome encodes the virus RNA-dependent RNA polymerase and includes the 3’ nontranslated region, both of which are highly conserved between enterovirus serotypes.

The specificity of this probe was demonstrated by quantitative slot blot hybridizations to nucleic acid from a range of RNA viruses. Prototype strains of coxsackievirus A or B, echoviruses, yellow fever virus, or measles virus were propagated in continuous monkey kidney cells (VERO), and infectivity was assayed by plaque titration. Hepatitis A virus was propagated in human diploid cells (MRC5), and infectivity of partially purified suspensions was assayed by enzyme-linked immunosorbant assay (ELISA). Crude virus pellets were prepared by high-speed centrifugation of the clarified supernatants from homogenates of infected or mock-infected cell cultures. RNA was isolated by proteinase K digestion and phenol-chloroform extraction and quantitated by spectrophotometry, and aliquots containing 50 ng total RNA were selectively denatured and immobilized on nitrocellulose membranes as described previously. This filter was hybridized with the enterovirus group–specific probe.

Multiple samples (up to four portions, each less than 5 mg wet wt) were excised from right ventricular tissue specimens of the explanted hearts and processed independently. Total nucleic acids were isolated from these and normal pig heart muscle negative controls as described previously for skeletal muscle. Briefly, tissue was digested overnight at 37°C in the presence of 1 mg/ml proteinase K, 0.5% sodium dodecyl sulfate (SDS), 50 mM Tris (pH 8.0), 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, and 1 unit/μl human placental ribonuclease inhibitor. After deproteinization and ethanol precipitation, total nucleic acids were redissolved, denatured, and immobilized on duplicate nitrocellulose membranes together with purified coxsackievirus B2 genomic RNA as a positive control. One filter was hybridized with the enterovirus group–specific probe and the duplicate filter with a cDNA probe termed 7B6. The function of mRNA from which 7B6 is derived is unknown but its expression is cell-cycle independent, allowing the quantitation of cell mRNA immobilized.

To establish that the extent of hybridization and subsequent detection of labeled probes is proportional to the amount of RNA immobilized, serial dilutions of a preparation of total cellular RNA were applied to filters. These were hybridized with the 7B6 probe and the autoradiographic response quantitated by scanning densitometry.

For routine determinations, the cDNA inserts were isolated from recombinant plasmids by endonuclease cleavage, separated from the vector by electrophoresis and then radiolabeled with 32P by the random oligomeric primer extension method. Filters were pretreated by incubation for 6 hours at 65°C in 2×standard saline citrate (SSC), 0.5% SDS, 5×Denhardt’s, and 100 μg/ml denatured, sonicated salmon sperm DNA. For hybridization, 32P-labeled probes (specific activity, 1–2×107 dpm/μg) were added to a final concentration of approximately 2 ng/ml. After hybridization, the filters were washed to high stringency (30-minute washes each in 2×SSC, 0.1% SDS followed by 0.5×SSC, 0.1% SDS, and 0.1×SSC, 0.1% SDS, all at 65°C) and autoradiographed with presensitized Amersham Hyperfilm-MP radiographic film. The subsequent autoradiographic development was quantitated by scanning densitometry (Chromoscan III, Joyce-Loebl). The data for each sample were expressed as a ratio of the numeric integral of the hybridization signal from the virus-specific probe to that of the signal from the control probe and termed hybridization index (HI). The entire set of determinations is thus internally controlled for the specificity of the probes, the amount of total RNA immobilized from each sample, and variation in the specific activities of the probes.

**Serology**

Coxsackievirus B–specific neutralizing titers of blood samples obtained immediately preoperatively were determined by a microtiter method. Coxackievirus B–specific IgM was quantitated by an IgM capture ELISA in a search for evidence of a continuing humoral immune response.

**Quantitation of Leukocytes and T-Cells and Expression of MHC Antigens in Cardiac Tissue**

Frozen sections of right ventricle were stained for the determination of T-cells and leukocytes by an immunoperoxidase technique described previously. Briefly, 6-μm sections were fixed in acetone and incubated with monoclonal antibodies against the leukocyte common antigen (F10-89-4), against T-cells (Leu-4; Becton Dickinson, Mountain View, California), and against common determinants of class I MHC only (W6/32). After 30 minutes of incubation, the sections were washed and incubated with biotinylated goat anti-mouse IgG and IgM (Dakopatts) followed by a solution containing avidin–biotin–horseradish peroxidase complexes (Sears Labs). Immersion in diaminobenzidine tetrahydrochloride (0.3 mg/ml) and hydrogen peroxidase (0.1%) allowed visualization of peroxidase activity. The sections were counterstained in Harris’s hematoxylin and examined under a Zeiss microscope. With a ×40 objective, the number of positively stained cells were counted in 10 fields (each field corresponding to 0.048 mm2) with an eyepiece graticule, and the results were expressed as mean numbers (cells/0.048 mm2).

Myocardium was examined for expression of MHC class antigen on the plasma membrane: myocardium does not normally express class I antigens.

**Results**

Details of the 40 patients studied, including the results of the enterovirus-specific RNA and serology studies, are shown in Table 1.
**Figure 1.** Autoradiograph showing the specificity of the coxsackievirus B2–derived cDNA probe. Hybridization to a slot blot of virus RNA isolated from infected cells or to cellular RNA from noninfected cells (vero). CB, coxsackievirus B; CA, coxsackievirus A; E, echovirus; HAV, hepatitis A virus; MV, measles virus; YFV, yellow fever virus; CB2 RNA, coxsackievirus B2–specific RNA isolated by density gradient centrifugation.

**Hybridization Studies**

*Specificity of the enterovirus probe.* We used a coxsackievirus B2 cDNA clone, which is a copy of a subgenomic sequence highly conserved between different enteroviruses. This probe gives significant hybridization signals with RNA from representative serotypes of coxsackievirus A, coxsackievirus B, or echovirus but not with RNA from hepatitis A, yellow fever, or measles viruses or from mock-infected cultured cells (Figure 1).

**Quantitation of hybridization and detection.** Hybridization of the 7B6 control probe to graded amounts of total cellular RNA demonstrates by densitometric scanning that the intensity of the subsequent autoradiographic signal is directly proportional to the amount of target RNA immobilized on the filter (Figure 2).

**Detection of enterovirus RNA in clinical samples.** Figure 3 is a representative autoradiograph showing how variation in the total amount of RNA...
immobilized from individual samples is controlled by densitometric determination of the intensity of the autoradiographic signals with the 7B6 control probe. Calculation of the ratio of the strength of the hybridization signals with the control probe and the enterovirus group-specific probe (HI) allows comparison between tissue samples for the relative abundance of virus RNA.

After hybridization, the clinical samples fell into two distinct groups (Figure 4). The majority of the samples gave low values of HI similar to those of the normal pig heart controls (Figure 4, group C).

**Figure 2.** Left panel: Autoradiograph after hybridization of the 7B6 probe to slot blots of total RNA (immobilized in a twofold serial dilution) and the integrals produced by scanning densitometry. Right panel: Relation of signal intensity to RNA concentration (arbitrary units).

**Figure 3.** Representative autoradiographs after hybridization of the 7B6 control probe or the enterovirus group-specific probe to slot blots of RNA isolated from tissue from explanted hearts. Ratio of the signal intensity produced by the virus-specific probe to that by the 7B6 probe (hybridization index, HI) is shown for the samples illustrated.
Samples are with 3 SD

FIGURE 4. Plot of detection of enterovirus RNA in right ventricular samples of explanted hearts from patients with end-stage disease. Group A, tissue from patients with dilated cardiomyopathy; group B, tissue from patients with heart disease of specific cause; group C, normal pig myocardium. Samples in shaded area are negative (less than 3 SD more than mean HI of group C), and the remainder are positive for enterovirus RNA.

However, tissue from seven patients had hybridization indexes more than 3 SD from the mean of the controls. Six of these were from the dilated cardiomyopathy group (Figure 4, group A), whereas the seventh was from a patient with ischemic heart disease (Figure 4, group B). In one patient from the cardiomyopathy group, two of four tissue samples processed independently were positive for the presence of enterovirus RNA: in all other positive cases, virus RNA was detected in only one of four samples. Neither of the two patients in the cardiomyopathy group who had histories of excessive alcohol consumption were positive for virus RNA. Even including these two, the result of six of 21 patients with dilated cardiomyopathy positive for virus RNA compared with only one of the control group demonstrates a significant difference between the two groups (p<0.05).

Serology

Serology showed that only one patient (with dilated cardiomyopathy) was positive for coxsackievirus B-

specific IgM: this was directed against the B2 serotype and was a low titer. Samples of myocardium from this patient were negative for enterovirus RNA. Determination of neutralizing antibody titers showed no significant differences between the two groups; six of 21 patients with dilated cardiomyopathy (three of whom were positive for virus RNA) and nine of 19 patients with other specific heart diseases had neutralization titers of 1:320 or more.

Quantitation of Leukocytes and T-Cells and Expression of MHC Antigens

Quantitation of leukocytes and T-cells is shown in Figure 5. There is no significant difference between the patients in groups A and B or between patients positive or negative for enterovirus RNA. There was no obvious aberrant expression of class I MHC antigens on the myocardium from either group.

Discussion

Various other studies have suggested that virus-induced myocarditis predisposes to the development of dilated cardiomyopathy. Although the mechanism of myocardial damage is unknown, an autoimmune response triggered by aberrant induction of MHC antigen expression induced by virus infection, as has been suggested in endocrine autoimmune diseases, could be responsible.

We previously reported data from a pilot study in which enterovirus RNA was detected in endomyocardial biopsy samples from a proportion of patients with either histologically proven myocarditis or idiopathic dilated cardiomyopathy. We subsequently reported data from a larger series in which three of seven patients with histologically proven myocarditis and 11 of 20 patients with dilated cardiomyopathy had endomyocardial biopsy samples positive for enterovirus RNA. No virus-specific sequences were found in biopsy samples from patients with other specific heart muscle diseases. On this basis, we suggested that persistence of virus RNA in myocardium after an enterovirus-induced myocarditis disposes to the subsequent development of dilated cardiomyopathy. In that study, the patients with dilated cardiomyopathy (15 men, five women; age, 10–64 years) had not reached end-stage disease: those with biopsy samples positive for virus RNA had a mean duration of symptoms of 7 months, whereas those negative had a mean duration of 21 months.

In the present study, we present data derived from a study of tissue from the explanted hearts of a separate group of patients undergoing transplantation for either end-stage dilated cardiomyopathy or a control group with heart failure of known cause. We found that enterovirus RNA sequences are present in the myocardium in a higher proportion of patients transplanted for dilated cardiomyopathy than in the control group. We have not excluded from the cardiomyopathy group those patients who have a history of excessive alcohol
Figure 5. Plot of immunocytochemical detection of leukocytes or T-cells in heart tissue from patients with dilated cardiomyopathy (group A) or other heart muscle disease (group B). ○, ■, samples that are positive; ○, □, samples that are negative for enteroviral RNA.

consumption or whose symptoms began after pregnancy. The alcoholic patients and the postpartum patient were negative and positive, respectively, for the presence of enterovirus RNA.

While our previous studies revealed the presence of enterovirus RNA in endomyocardial biopsy samples from patients with various grades of myocarditis or dilated cardiomyopathy, the results we present demonstrate that virus can persist in the myocardium until end-stage disease. The hybridization probe used in these studies, although derived from coxsackievirus B2 RNA, is group-specific and, therefore, would detect enteroviruses other than coxsackieviruses. However, no serologic investigations for enteroviruses other than coxsackievirus B serotypes were undertaken.

We examined the relation between the duration of disease and detection of enterovirus RNA in the cardiomyopathy group. Ten of the 21 cardiomyopathy patients had a history of disease of 1 year or longer, and this subset contained five of the six positive for enterovirus RNA.

Our experience with endomyocardial biopsy specimens shows that the occurrence of viral RNA is often focal as are the histopathologic changes of myocarditis. In the present study, the multiple tissue samples studied were obtained from the same region of the myocardium. This may have resulted in false-negatives and, thus, an underestimate of the frequency of virus involvement. Tissue from the right ventricle was studied: comparison of the frequency of histologic diagnosis of myocarditis or dilated cardiomyopathy by endomyocardial biopsy of either left or right ventricle revealed no significant differences, suggesting that this did not affect the results reported here.

Serologic investigations revealed no significant differences between cardiomyopathy patients and controls in the frequency of either neutralizing antibody or coxsackievirus B–specific IgM. The frequency of significant coxsackievirus B–neutralizing titers in both groups of patients was greater than reported for controls in previous studies. One of the patients with cardiomyopathy was positive for coxsackievirus B–specific IgM but negative for virus RNA in the myocardium. Concurrent serologic investigations were done in a separate series of 23 patients with myocarditis or non–end-stage dilated cardiomyopathy: four of 10 patients positive for enterovirus RNA in biopsy specimens were also positive for coxsackievirus B–specific IgM compared with none of the 13 enterovirus RNA–negative patients.

In this present study, the single patient with ischemic heart disease and positive for enterovirus RNA had no history suggestive of an acute enteroviral illness. It is possible that the presence of enterovirus RNA in the myocardium of this patient was due to an undetected cardiomyopathy or viral myocarditis: the fact that dilated cardiomyopathy was diagnosed by exclusion means that disease coexisting with ischemic heart disease would go undetected.

Patients with dilated cardiomyopathy often have lymphocytes in the myocardium. In all cases, the degree of infiltration is considerably less than is diagnostic of myocarditis. In this study, characterization of the infiltrate showed no significant differences either between cardiomyopathy patients and controls or between those patients shown to be positive or negative for virus RNA. It appears that, in contrast to viral myocarditis, persistence of virus in dilated cardiomyopathy is not associated with a cell-mediated immune response.

We have found altered expression of myocardial MHC class I antigens to be a good marker of rejection in cardiac transplantation. Induction of class I antigens on the normally negative myocardium is probably caused by local release of interferon by infiltrating cells. It might be expected that a persistent viral infection would also cause abnormal expression of class I antigens, as has been shown to occur on hepatocytes in persistent viral hepatitis.

In the present study, there was no obvious abnor-
mal expression of class I antigens in either group. However, these are patients with end-stage–dilated cardiomyopathies, and it is known that diluted cardiomyopathy may occur after myocarditis.6,7,23 It is, therefore, our intention to investigate whether there is abnormal MHC expression in myocarditis.

It is not clear how enteroviruses persist in an apparently immunocompetent host, although a carrier state infection of myocytes in vitro28 and persistent infection of a human amnion cell line by defective virus29 have been described. The presence of a virus in the myocardium may result in interference with normal myocyte function in the absence of cell destruction. The perturbation of nonessential gene functions in persistently infected cells has been documented30 and proposed as a pathogenetic mechanism in other diseases associated with persistent enterovirus infection.31

Long-term follow-up studies of patients with acute myocarditis together with the demonstration of virus persistence in the myocardium through all grades of myocarditis have provided evidence for a link between viral myocarditis and subsequent development of diluted cardiomyopathy.6,7,23 The data reported here confirm that enteroviruses are capable of persisting in the myocardium of patients with diluted cardiomyopathy until end-stage disease requiring transplantation. This provides further evidence of a viral etiology for some cases of dilated cardiomyopathy.

Acknowledgments

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