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ASSOCIATION OF VIRAL GENOME WITH GRAFT LOSS IN CHILDREN AFTER CARDIAC TRANSPLANTATION

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ABSTRACT

Background The survival of recipients of cardiac allografts is limited by rejection and coronary vasculopathy. The purpose of this study in children who had received heart transplants was to evaluate the cardiac allografts for myocardial viral infections and to determine whether the presence of viral genome in the myocardium correlates with rejection, coronary vasculopathy, or graft loss.

Methods We enrolled heart-transplant recipients 1 day to 18 years old who were undergoing evaluation for possible rejection and coronary vasculopathy. Endomyocardial-biopsy specimens were evaluated for evidence of rejection with the use of standard criteria and were analyzed for the presence of virus by the polymerase chain reaction (PCR).

Results PCR analyses were performed on 553 consecutive biopsy samples from 149 transplant recipients. Viral genome was amplified from 48 samples (8.7 percent) from 34 patients (23 percent); adenovirus was found in 30 samples, enterovirus in 9 samples, parvovirus in 5 samples, cytomegalovirus in 2 samples, herpes simplex virus in 1 sample, and Epstein–Barr virus in 1 sample. In 29 of the 34 patients with positive results on PCR (85 percent), an adverse cardiac event occurred within three months after the positive biopsy, and 9 of the 34 patients had graft loss due to coronary vasculopathy, chronic graft failure, or acute rejection. In 39 of the 115 patients with negative results on PCR (34 percent), an adverse cardiac event occurred within three months of the negative PCR finding; graft loss did not occur in any of the patients in this group. The odds of graft loss were 6.5 times as great among those with positive results on PCR ($P=0.006$). The detection of adenovirus was associated with considerably reduced graft survival ($P=0.002$).

Conclusions Identification of viral genome, particularly adenovirus, in the myocardium of pediatric transplant recipients is predictive of adverse clinical events, including coronary vasculopathy and graft loss. (N Engl J Med 2001;344:1498-503.)

CARDIAC transplantation in children is a lifesaving procedure aimed at sustaining long-term, productive survival. The major short-term and long-term risks associated with transplantation that limit survival include allograft rejection, coronary vasculopathy in the transplant, and lymphoproliferative disease, but the underlying causes of these disorders are not completely understood. The diagnosis of allograft rejection relies on histopathological criteria described by the International Society for Heart and Lung Transplantation (ISHLT).[1] We speculated that there might be an association between allograft rejection and viral infection in these patients.[2] Classically, viral infections are diagnosed by means of serologic tests and viral cultures, which are time-consuming and have limited sensitivity and specificity.[3] Today, molecular hybridization[4-6] and polymerase-chain-reaction (PCR) analysis[7-18] can be used to detect viral nucleic acid in body fluid and tissue samples. Numerous studies of patients with myocarditis have demonstrated the usefulness of PCR analysis for etiologic diagnosis.[7-17,19-22]

It is known that there is an association between the detection by PCR of viral genome in the myocardium and concomitant rejection,[2] but the long-term implications of this association are unclear. Our study addressed the intermediate-term follow-up of pediatric cardiac-transplant recipients who underwent serial PCR analysis as part of the routine surveillance for rejection, as well as patients with clinical evidence of rejection and those who underwent repeated biopsy after receiving treatment for rejection. The principal hypotheses that we tested were that graft survival is worse among patients who have positive PCR results for viral genome than among those who have negative results and that graft survival is worse among patients who have more episodes of rejection. We also investigated the relation between the number of episodes of rejection and the presence or absence of viral ge-

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nome on PCR, graft survival, or both, as well as whether the type of virus detected by PCR influences the risk of graft loss and coronary vasculopathy in the transplant.

METHODS

Between January 1993 and June 1998, serial PCR analysis was performed prospectively on all right ventricular endomyocardial-biopsy samples obtained from children who were cardiac-transplant recipients at Loma Linda University Children's Hospital. These children were enrolled in the study at the time of transplantation. Biopsy samples were fresh-frozen in liquid nitrogen and stored at −80°C for up to one month before the analysis and then analyzed in a batch. All samples were analyzed by PCR for adenovirus, cytomegalovirus, enterovirus, herpes simplex virus, parvovirus, and Epstein–Barr virus. The PCR analysis was performed at a distant site, the Baylor College of Medicine, in a prospective, blinded fashion.

Viral cultures were obtained when they were clinically indicated, but only a small percentage of samples were cultured. Histopathological grading of biopsy specimens was performed according to ISHLT criteria.[1] The standardized ISHLT criteria for the diagnosis of cardiac-allograft rejection ranged from grade 0 (no rejection) to grade 4 (severe acute rejection with a diffuse, aggressive, polymorphous, inflammatory infiltrate and myocyte necrosis and damage associated with edema, hemorrhage, and vasculitis). Rejection scores between 0 and 4 represent an increasing gradient of inflammation, with grade 3A representing multifocal inflammatory infiltrates (multifocal moderate rejection) with some myocyte damage and grade 3B representing a diffuse inflammatory process with myocyte damage and edema.[23] Echocardiograms were obtained and graded for rejection prospectively by means of a previously described system of multivariable computerized analysis.[24,25]

Patients and Samples

We studied all children who had undergone heart transplantation for whom right ventricular endomyocardial-biopsy samples were available. All patients were treated with a standard regimen of cyclosporine and azathioprine. Rejection was treated with steroids, other antirejection medications, or both, on the basis of a clinical examination. Serial biopsy samples were obtained after the parents of the patient had given written informed consent and during routine cardiac catheterization that was part of surveillance for rejection (according to the protocol), as well as from patients with clinical evidence of rejection and as a follow-up procedure after a patient was treated for rejection.

Control Samples

Cardiac-biopsy samples from age- and sex-matched patients with no evidence of myocarditis, dilated cardiomyopathy, or other inflammatory processes were used as negative controls. These included formalin-fixed samples obtained at autopsy and snap-frozen tissue from explanted hearts that had been obtained at the time of transplantation from patients with congenital heart disease or hypertrophic cardiomyopathy.

Template Preparation and PCR

Samples were homogenized in RNAzol with the use of disposable manual homogenizers, and total RNA and DNA were isolated as previously described.[16,17,19] Reverse-transcriptase PCR was used to detect enterovirus RNA, and PCR was used to detect the DNA of adenovirus, cytomegalovirus, herpes simplex virus, parvovirus, and Epstein–Barr virus.[2,16,17,19-21]

All samples were analyzed by technicians who had no knowledge of the clinical or serologic data or the results of cultures, and all assays were performed in duplicate. The presence of amplifiable nucleic acid in each sample was verified with the use of primers designed to amplify cellular nucleic acid (K-*ras* or β-actin).[2,16,17,19-21]

Statistical Analysis

Survival analysis was used to determine the differences in the incidence of graft loss associated with the findings on PCR analysis. The Wilcoxon test was used to assess the statistical significance of the difference in survival between patients with positive results on PCR and those with negative results. Logistic regression was used to determine the association between graft loss and the number of episodes of rejection and to assess the independent associations between the PCR results and graft loss as well as between the number of episodes of rejection and graft loss. Contingency-table analysis was used to determine whether, among those with positive results on PCR, the presence of coronary vasculopathy in the transplant was associated with graft loss. Fisher's exact tests were used to determine the statistical significance of any associations found by the contingency-table analyses. All P values were based on two-sided statistical tests. An alpha level of 0.05 was used to reject the null hypothesis of no difference between the groups. The Bonferroni method was used to correct reported P values for the three statistical tests that addressed the two primary hypotheses. Nominal P values are presented for the statistical tests that were used to address the secondary end points of the study, including virus type, number of episodes of rejection, and association of graft loss with virus type.

RESULTS

Samples and Patients

A total of 553 serial endomyocardial-biopsy samples from 149 patients were studied (range, 1 to 17 per patient during the 5.5 years of study; median, 3 samples per patient). The median age of the patients at the time of transplantation was 2.3 months (range, 0.1 to 216), and 657 patient-years of follow-up were completed after transplantation (range, 4.2 to 145.3 months per patient; median, 47.6).

PCR Results

A viral product was amplified by PCR from 48 of the 553 biopsy samples (8.7 percent) from 34 of the 149 patients (23 percent). In 30 samples (from 24 patients) adenovirus, usually type 2, was amplified (Fig. 1). In addition, enterovirus was detected in nine samples, parvovirus in five samples, cytomegalovirus in two samples, and herpes simplex virus and Epstein–Barr virus in one sample each. In seven patients, more than one virus was detected during follow-up. Patients first had positive results on PCR within 0.4 to 103.4 months after transplantation (median, 15.7). Follow-up continued for 64.8 patient-years after the first positive results were obtained; the median follow-up was 21.5 months (range, 3.1 to 55.6).

Adenovirus was detected during all seasons (Fig. 2), whereas enterovirus appeared primarily in the winter and autumn months. Other viruses (parvovirus, Epstein–Barr virus, or cytomegalovirus) were seen during the spring, summer, and autumn months. A contingency-table analysis was performed to assess the homogeneity of the specific types of these viruses for which patients tested positive and the seasons in which they did so. No significant differences in seasonal variability were identified, although trends were seen ($P=0.09$ by a global test for differences among seasons).

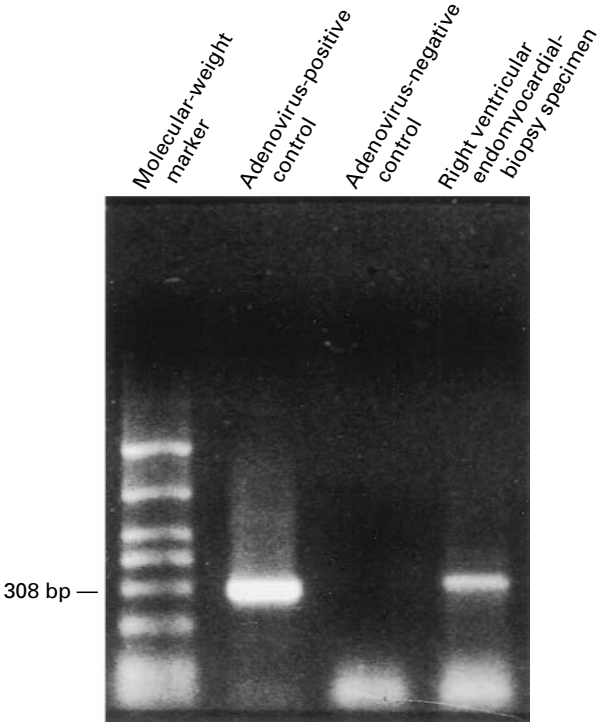

Figure 1. PCR Analysis Showing Adenovirus DNA in a Right Ventricular Endomyocardial-Biopsy Specimen of a Patient with Acute Rejection.

Indications for Biopsy in PCR-Positive Cases

For all patients with positive results on PCR, charts were retrospectively reviewed to determine the indications for endomyocardial biopsy. Of the 48 biopsy samples with positive PCR results, 22 (46 percent) were obtained because of clinically suspected rejection (in 15 patients) or as a follow-up procedure after an episode of rejection (in 7 patients). The other 26 biopsies with positive PCR results (54 percent) were obtained during routine surveillance for evidence of rejection.

The results of previous PCR testing were available for the patients from whom 33 of the 48 PCR-positive samples were obtained (69 percent). Thirty of these samples were from patients who had previously had negative results on PCR, as compared with just three from patients who had previously had positive results. In six patients, virus was detected in the first biopsy after transplantation; therefore, no previous PCR results were available. No previous PCR testing had been performed in the remaining nine patients with positive results, all of which were obtained soon after transplantation.

The results of subsequent PCR testing were available for the patients with 42 of the 48 PCR-positive samples (88 percent). In 37 cases, the subsequent test was negative, and in 5 cases, the subsequent test was positive. One patient who tested positive for Epstein–

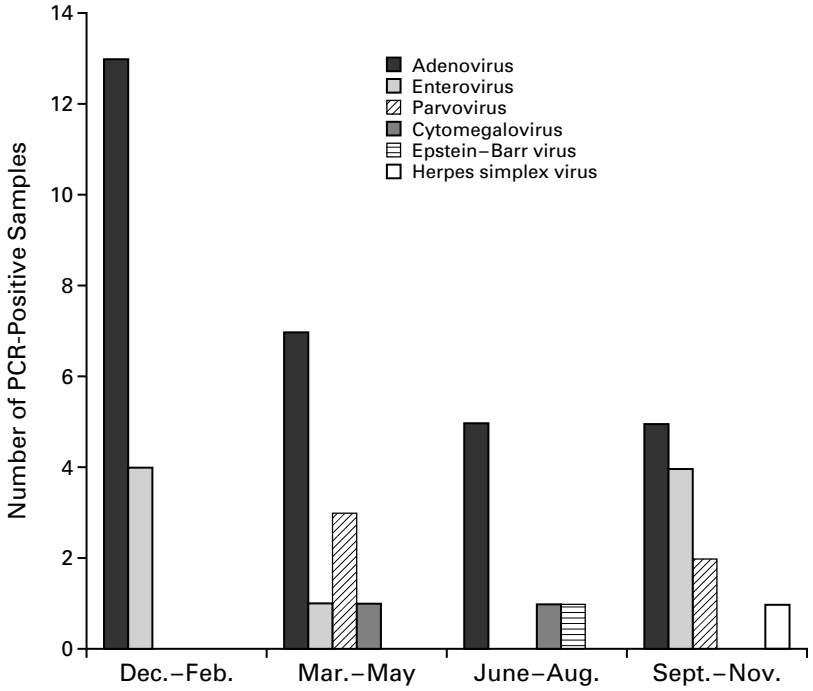

Figure 2. Seasonal Variation in the Amplification of Viral Genomes from the Biopsy Samples Obtained from Pediatric Cardiac-Transplant Recipients.

Barr virus underwent retransplantation because of coronary vasculopathy. A biopsy sample obtained after retransplantation was also positive for Epstein–Barr virus. Of the remaining six patients, two died shortly after having a positive result on PCR, and a lack of vascular access precluded repeated biopsy in the other four patients. Persistence of adenovirus was noted (three patients had positive results on PCR on a total of 10 occasions), as well as parvovirus (one patient had positive results on PCR twice during a one-month period).

Correlation between Positive PCR Results and Clinical Conditions

There was no evidence of rejection (i.e., rejection was assessed as grade 0 or 1), infection, or vasculitis in the vast majority of biopsy specimens we evaluated according to the ISHLT criteria (518 of the 553 samples [94 percent] were negative). However, of the 35 samples with histopathological evidence of rejection (assessed as grade 3A or higher), 27 were associated with positive results on PCR (77 percent); the remainder of the patients with such samples never had positive PCR results, and in these cases, the rejection occurred soon after transplantation (in six patients) or was the result of noncompliance with immunosuppressive regimens (in two patients).

In 16 of the 34 patients with positive results on PCR (47 percent), an endomyocardial-biopsy specimen was simultaneously graded 3A (indicating moderately high grade rejection). In six other instances, a biopsy specimen classified as grade 0 (no rejection) was found in association with chronic graft dysfunction (in three patients), new arrhythmias (supraventricular tachycardia in one patient and atrial flutter in another) with heart failure, and death from coronary arteriopathy two days later (in one patient). In contrast, a simultaneously obtained endomyocardial-biopsy specimen was graded 3A in only 8 of the 115 patients with negative results on PCR (7 percent) — a significantly smaller proportion than among the patients with positive results on PCR (odds ratio for rejection associated with detection of virus, 11.9; 95 percent confidence interval, 4.4 to 31.8; $P<0.001$).

One or more adverse cardiac events occurred in 29 of the 34 patients with positive PCR results (85 percent) within three months before or after the positive PCR analysis. These events included biopsy-verified rejection in 26 patients, new or chronic ventricular dysfunction in 7, and graft loss because of death in 1 and because of the need for retransplantation in 1. In 11 patients, a simultaneously obtained biopsy specimen was assessed as grade 0, but biopsy-verified rejection occurred during the three months before the positive PCR result was obtained (in 6 patients) or thereafter (in 5 patients). Echocardiographic criteria did not consistently predict rejection or adverse cardiac events. In contrast, an adverse cardiac event occurred in 39 of 115 patients (34 percent) within three months before or after a negative PCR result — a significantly smaller proportion than among the patients with positive PCR results (odds ratio for an adverse cardiac event with a positive PCR result, 11.3; 95 percent confidence interval, 4.1 to 31.5; $P<0.001$). These events consisted of concomitant biopsy-verified rejection (in 8 patients) or rejection during the three months before the negative result was obtained (in 26 patients) or thereafter (in 5 patients). Neither ventricular dysfunction nor graft loss occurred in any patient with negative PCR results.

In order to address the possibility that the nature of the patient pool might bias the results because it included a disproportionate number of patients who required biopsies for surveillance for rejection, a contingency-table analysis of PCR results (chi-square test) was performed according to the indication for biopsy. This analysis was restricted to the first PCR-tested biopsy specimen for each patient. Of the 149 patients studied, rejection was suspected in 39 at the time of their first biopsy; 8 of these patients were positive for virus on PCR, whereas of the remaining 110 patients, who underwent a biopsy as part of routine surveillance, only 6 had positive results for virus (odds ratio, 4.47; 95 percent confidence interval, 1.44 to 13.87; $P<0.01$).

Outcomes

There was a statistically significant difference between the rate of graft survival among the patients with positive results on PCR and the rate among those with only negative results (Fig. 3). Graft loss occurred in 9 of the 34 patients with positive PCR results (26 percent) 2 to 43.4 months after the first positive PCR result was obtained (median, 28.9 months). In six of these nine patients (67 percent), coronary vasculopathy developed in the transplant. One of these patients died suddenly, and five were placed on the waiting list for retransplantation; two of the five died while awaiting retransplantation, and three underwent retransplantation. Pathological examination of the three explanted hearts revealed severe (grade 3 or 4) coronary vasculopathy. In one patient, parvovirus had been detected before the development of coronary vasculopathy; the patient subsequently died of aplastic anemia. The other two patients are doing well after retransplantation.

Chronic graft failure developed in two of the three patients with graft loss in whom coronary vasculopathy did not develop. One of these patients underwent retransplantation and is doing well; the other patient died. The third patient with graft loss but without coronary vasculopathy died of acute rejection. Thus, six of the nine patients with graft loss died, as did one patient with positive PCR results for Epstein–Barr virus who had lymphoproliferative disease after transplantation.

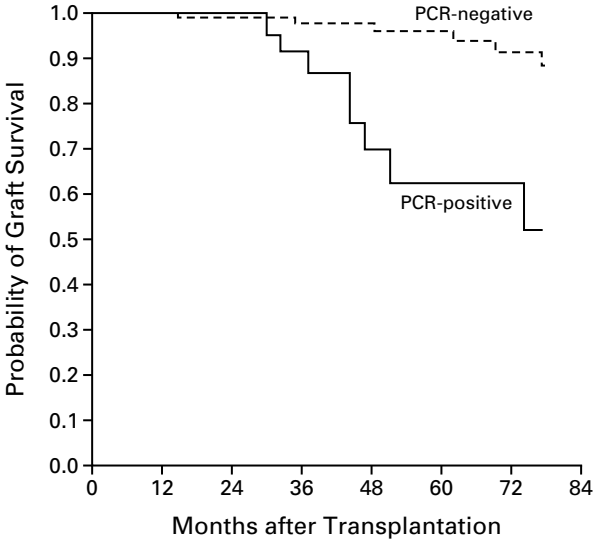

Figure 3. Kaplan–Meier Analysis of Actuarial Graft Survival in Children with Positive PCR Results for Any Viral Genome at Any Time on Serial Biopsies (PCR-Positive), as Compared with Those Who Had Consistently Negative PCR Results (PCR-Negative).

Significance of the Detection of Virus

Viral genome was detected in 34 of 149 cardiac-transplant recipients who underwent serial biopsies (23 percent), and graft loss occurred in 26 percent. The crude odds ratio for graft loss among patients with positive results on PCR, as compared with those with negative results, was 6.5 (95 percent confidence interval, 2.1 to 19.9; Bonferroni-corrected $P=0.006$). Among patients without positive results on PCR, the five-year graft-survival rate was 96.1 percent, whereas the rate among patients with positive results on PCR was 64.7 percent (Bonferroni-corrected $P=0.003$) (Fig. 3).

A greater number of episodes of rejection (associated with positive PCR results) was associated with a higher risk of graft loss. Each episode of rejection increased the risk of graft loss by 57 percent (95 percent confidence interval, 26 to 95 percent; Bonferroni-corrected $P=0.009$). In a multivariate logistic-regression analysis, the association between positive PCR results and graft loss was greatly diminished by adjustment for the number of episodes of rejection, suggesting that the association between positive PCR results and graft loss was largely mediated by an increase in the number of episodes of rejection. The mean number of episodes of rejection among patients with positive PCR results was 2.1 times that among patients with negative PCR results ($P<0.001$).

Association of the Type of Virus with Outcome

In patients with biopsy samples that were positive for adenovirus, five-year graft survival was 62.3 percent ($P=0.002$ for the comparison between adenovirus-positive patients and adenovirus-negative patients). The odds ratio for graft loss in adenovirus-positive patients, as compared with patients who had persistently negative PCR results for adenovirus, was 4.7 (95 percent confidence interval, 1.3 to 17.1; $P=0.02$). Because of the small numbers of patients with positive results for other viruses, we could not make a reliable determination of the relative risk of graft loss for patients with those viruses.

DISCUSSION

Our study demonstrates a close temporal association between the detection of viral genome and adverse cardiac events over the short term and identifies the detection of viral genome as an independent predictor of graft loss over the intermediate-to-long term. The association between cytomegalovirus infection and allograft rejection, vasculopathy, and death is well known.[26-30] However, previous studies were retrospective, relying on serologic test results, and did not involve analysis of myocardial samples. Some studies have shown an association between the presence of adenovirus, enterovirus, or cytomegalovirus genome in the myocardium and concomitant rejection.[2]

In this prospective, blinded study, adenovirus was shown to be the most common agent associated with adverse cardiac events, including graft loss, in both the short and the long term. The next most common viral agents were enterovirus, cytomegalovirus, and parvovirus. Adenovirus and enterovirus are the most common pathogens associated with myocarditis as well,[4-7,9,10,16,17] and adenovirus has been associated with graft loss and bronchiolitis obliterans after lung transplantation.[31] The association of a positive PCR assay for virus with concomitant biopsy-verified rejection is consistent with an episode resembling viral myocarditis: like myocarditis, a positive PCR result frequently occurred in the absence of evidence of inflammation.[16,17,32-34] In this study, a concomitant biopsy with no evidence of rejection (grade 0) was occasionally seen in association with an adverse cardiac event. Previous or subsequent biopsy samples typically tested negative for virus on PCR but frequently revealed signs of rejection. This combination of findings suggests a delayed immune response with a late expression of inflammatory mediators. Although our data suggest that viral infection causes rejection, another possibility is "reverse causality": the transplanted heart in the early phases of rejection could be more prone to viral infection as a secondary phenomenon. However, this appears unlikely, since several of the patients we studied had a positive result on PCR even when they had a biopsy assessed as grade 0, which is not consistent with the presence of rejection. In these patients, signs and symptoms of rejection later became apparent, within three months after the positive PCR result was obtained.

The virus-mediated triggering or acceleration of coronary arteriopathy after heart transplantation is not yet understood. Cytomegalovirus has been implicated in the pathogenesis of atherosclerosis.[35,36] Our study implicates adenovirus and enterovirus in the development of post-transplantation coronary vasculopathy, but the mechanism is unclear. It is possible that a persistent subclinical inflammatory response is responsible for this effect as well. Since pulsed steroid therapy is commonly used as a first-line response to rejection, it is plausible that the reduction in infiltrate is not representative of the immune response.

The understanding that viral infections result in late rejection and other long-term sequelae should lead to a reconsideration of the treatment options for late rejection. The use of viral vaccines before transplantation could be considered for study, and the use of intravenous immune globulin either prophylactically or during the course of a rejection episode is another possible option. Finally, the development of pharmaceutical antiviral agents to treat infected patients would also be worthwhile.

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