American Journal of Pathology, Vol. 147, No. 2, August 1995

Infrequency of Cytomegalovirus Genome in Coronary Arteriopathy of Human Heart Allografts

James M. Gulizia,* Reinhard Kandolf,† Todd J. Kendall,* Sheldon L. Thieszen,* Janet E. Wilson,* Stanley J. Radio,* Maria R. Costanzo,‖ Gayle L. Winters,‡ Leslie L. Miller,§ and Bruce M. McManus*

From the Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, Nebraska; the Institute of Pathology,† University of Tuebingen, Tuebingen, Germany; the Department of Medicine‖ and Pathology,‡ Loyola University of Chicago, Maywood, Illinois, and the Division of Cardiology,§ St. Louis University, St. Louis, Missouri*

In heart transplantation, long-term engraftment success is severely limited by the rapid development of obliterative disease of the coronary arteries. Data from various groups have been suggestive of a pathogenetic role of herpesviruses, particularly human cytomegalovirus, in accelerated allograft coronary artery disease; however, results are not yet conclusive. This study examines the hypothesis that human cytomegalovirus infection of allograft tissues is related pathogenetically and directly to accelerated coronary artery disease. Using in situ DNA hybridization and polymerase chain reaction, we examined particular coronary artery segments from 41 human heart allografts (ranging from 4 days to greater than 4 years after transplantation; mean, 457 days) and 22 donor age- and gender-comparable, coronary site-matched trauma victims for presence of human cytomegalovirus DNA. Human cytomegalovirus genome was detected in 8 of 41 (19.5%) allografts and in 1 of 22 (4.5%) control hearts. This difference in positivity was not statistically significant (P = 0.10). In the human cytomegalovirus-positive hearts, viral genome was localized to perivascular myocardium or coronary artery media or adventitia. Human cytomegalovirus genome was not detected in arterial intima of any allograft or control heart, although human cytomegalovirus genome was readily identified within intima of small pulmonary arteries from lung tissue with human cytomegalovirus pneumonitis. By statistical analyses, the presence of human cytomegalovirus genome was not associated with the nature or digitized extent of transplant arteriopathy, evidence of rejection, allograft recipient or donor serological data suggestive of human cytomegalovirus infection, duration of allograft implantation, or causes of death or retransplantation. Thus, our data indicate a low frequency of detectable human cytomegalovirus genome in accelerated coronary artery disease and do not support a direct role for human cytomegalovirus in vascular wall infection or in the development of accelerated coronary artery disease. (Am J Pathol 1995, 147:461–475)

Use of contemporary immunosuppressive regimens for allograft rejection has led to increased early survival after heart transplantation. Accelerated coronary artery disease (ACAD), however, remains a major limitation to long-term engraftment success. Olivari and colleagues reported an angiographic incidence of ACAD of 10% at 1 year, 25% at 3 years, and 36% at 5 years, with ACAD being responsible for 60% of late deaths in 139 allograft recipients.[1] Through January, 1991, 465 heart or heart-lung retransplantations were recorded in the Registry of the International Society for Heart and Lung Transplantation, and 40% of those procedures were done for chronic rejection/ACAD.[2]

Supported by a Max Planck Research Prize (BMM and RK), National Heart, Lung and Blood Institute 5 RIO HL33778 (BMM), and the Thyssen Foundation (RK).

Accepted for publication April 26, 1995.

Address reprint requests to Dr. Bruce M. McManus, Department of Pathology and Laboratory Medicine, St. Paul's Hospital, University of British Columbia, 1081 Burrard St., Vancouver, BC V6Z 1Y6.

Dr. Gulizia's and Dr. Winter's current address: Department of Pathology, Brigham and Women's Hospital, Boston, Massachussetts.

Dr. Wilson's and Dr. McManus's current address: Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, British Columbia.

Dr. Costanzo's current address: Department of Medicine, Rush Presbyterian-St. Luke's Medical Center, Chicago, Illinois.

Transplant-associated ACAD is distinguishable pathologically from native atherosclerosis; ACAD is typically more concentric, diffuse, and cellular, involving intramural and extramural vessels. In addition, ACAD develops over a much shorter period of time (months) than classical atherosclerosis (years). Degenerative features such as plaque calcification are thus much less frequently seen in arteriopathy. These data, combined with the fact that risk factors for traditional atherosclerosis are less well linked to ACAD,[3] have focused investigative attention on other potential etiological mechanisms in ACAD.

Much effort has been directed towards evaluation of possible etiological links between herpesviruses and classical, native atherosclerosis. Early studies demonstrated induction of occlusive atherosclerotic lesions in normocholesterolemic, pathogen-free chickens by the avian herpesvirus, Marek's disease virus.[4,5] Several groups have since reported antigens or nucleic acids of herpesviruses, including human cytomegalovirus (HCMV), in atherosclerotic vessels by immunological or molecular techniques.[6–13]

Interest in a role for herpesviruses in transplant-associated ACAD was spurred by ubiquity of systemic HCMV colonization of immunocompromised cardiac transplant recipients[14–16] and two, independent, retrospective analyses of a combined total of 403 cardiac transplant recipients in whom a relationship was observed between post-transplant HCMV infection and ACAD.[17,18] More recent molecular studies of human allograft tissue for HCMV are also suggestive.[19–21]

The present study was undertaken to further examine the hypothesis that HCMV is involved pathogenetically, and particularly directly, in ACAD. *In situ* DNA hybridization and polymerase chain reaction (PCR) to detect HCMV immediate early and late genes were employed to compare the presence and distribution of HCMV genome in coronary arteries of human heart allografts to that in donor age- and gender-comparable and coronary site-matched control hearts. Comparison of allograft arteries to those of control hearts provides two types of valuable data. First, the potential role of HCMV in allograft ACAD is evaluable, and second, the prevalence of HCMV nucleic acids in early, classical atherogenesis in native hearts is studied. Furthermore, these molecular data were analyzed in conjunction with histopathological characterization of the coronary arteries and myocardium, as well as serological and historical information, including recipient pre-transplantation diagnoses, donor and recipient HCMV serologies, allograft operative ischemic times, and patient clinical courses post-transplantation.

Materials and Methods

Subjects and Tissues Studied

The study population consisted of 41 allograft recipients from collaborating transplantation centers. The patients were treated with contemporary, multidrug immunosuppression, including combinations of cyclosporine, azathioprine, cyclophosphamide, and corticosteroids. Monoclonal antibody therapy with OKT-3 was also used in some patients in induction protocols and in rescue situations. Controls included hearts of 22 individuals from the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) Study who died of traumatic causes.[22] The groups were donor age and gender comparable and coronary site matched. The mean age of heart donors was 27.7 ± 10.4 (SD) years old, with 34 males and 7 females, and the mean age of control individuals was 24.3 ± 6.6 years old, with 19 males and 3 females. The difference between the mean age of the allograft donors and that of the PDAY controls was not statistically significant ($P = 0.12$). Study subject and allograft characteristics are summarized in Tables 1 and 2.

Intact cardiac allografts, obtained at autopsy or explant, were transported in RPMI medium via overnight express mail to the Cardiovascular Registry, University of Nebraska Medical Center. The entire coronary arterial vasculature and sections of myocardium were triaged by protocol.[23] Coronary arteries of allografts and controls were perfusion-fixed in neutral, phosphate-buffered formalin, and two sequential segments of the proximal, left anterior descending coronary arteries from each heart were studied. Formalin-fixed, paraffin-embedded, positive control specimens from HCMV-viremic patients were demonstrated to be HCMV infected by immunohistochemical staining with a monoclonal antibody specific for the HCMV immediate early (IE) antigen. Positive control tissues were also observed to contain HCMV inclusion bodies based on histopathological examination of hematoxylin and eosin (H&E)-stained slides.

For purposes of this study, ACAD is defined pathologically. Thus, by subjective and quantitative morphometric features the disease was established as present in all allografts to various degrees. In other

Table 1. *Patient and Allograft Characteristics*

Patient No.	Patient age/ sex	Pre-Tx Dx	Patient pre-Tx HCMV titer	Donor age/ sex	Donor HCMV titer	Ischemic time (minutes)	HCMV titer rise	Duration post-Tx (dys)	Cause of death or retransplantation
1	15/F	CMY	1:10	15/M	1:80	200	+	20	Acute rejection
2	20/F	CMY	1:10	15/M	0	85	+	208	Allograft failure
3	58/M	CMY	1:160	30/M	1:40	200	–	55	Aspergillosis, heart; HCMV pneumonitis
4	50/M	CMY	1:40	19/M	0	225	–	238	Acute rejection
5	21/F	CMY	1:320	21/F	1:160	135	+	311	Acute rejection
6	39/F	IHD	1:320	25/M	1:20	60	–	153	Allograft failure
7	43/M	CMY	0	35/M	1:40	95	+	36	Acute rejection
8	35/M	CMY	1:10	17/M	1:10	80	+	639	Fungal pneumonitis
9	38/M	IHD	1:10	17/M	1:10	135	–	13	Acute rejection
10	53/M	IHD	1:160	21/M	1:80	140	–	617	Pulmonary embolism
11	55/M	IHD	1:640	21/M	NT	92	–	45	Acute rejection; ACAD
12	41/M	IHD	1:40	16/F	1:10	95	–	402	Myocardial toxoplasmosis
13	46/M	CMY	1:160	31/M	0	180	–	294	ACAD
14	37/M	IHD	1:320	37/M	1:160	110	+	638	Hepatic failure; pneumonia
15	46/M	CMY	0	24/M	1:20	65	–	85	Fungal pneumonitis; ACAD
16	27/F	CMY	1:20	27/M	1:10	56	+	1432	Allograft failure
17	28/M	IHD	1:10	25/M	1:40	210	+	426	ACAD
18	60/F	CMY	1:1281	44/F	1:20	145	–	46	PTLD
19	56/M	CMY	1:10	29/M	1:320	128	–	55	PTLD
20	53/M	CMY	0	27/M	NT	NA	NT	718	PTLD
21	66/M	CMY	1:320	36/M	1:10	150	–	153	PTLD
22	51/M	IHD	1:20	13/M	1:20	175	–	635	PTLD
23	16/M	CMY	1:1281	47/M	1:20	85		611	Allograft failure; ACAD
24	61/M	IHD	0	17/M	0	180	NT	180	Pneumonia; pulmonary fibrosis
25	54/F	CMY	1:40	46/F	0	216	+	33	ACAD
26	44/F	CMY	1:10	33/F	1:227	105	–	4	Acute rejection; ACAD
27	64/M	CMY	1:160	32/M	1:10	180	+	323	ACAD
28	65/M	IHD	NT	35/M	NT	316	NT	147	ACAD
29	43/M	IHD	0	51/M	0	90	NT	1349	Arrhythmia/sudden death
30	28/M	CMY	1:233	45/F	NT	183	NT	411	ACAD
31	60/M	IHD	1:80	31/M	0	154	–	63	Guillain-Barré; ACAD
32	57/M	IHD	0	33/M	0	98	NT	1145	Arrhythmia/sudden death; ACAD
33	57/M	IHD	1:1281	22/M	1:320	250	–	1190	Chronic rejection; ACAD
34	54/M	IHD	0	47/M	0	94	NT	1610	Arrhythmia/sudden death; ACAD
35	57/M	IHD	0	35/M	0	148	NT	1510	Arrhythmia/sudden death
36	54/F	CMY	0	18/F	0	180	+	63	Pneumonia, sepsis
37	66/M	IHD	1:80	17/M	0	275	+	199	Infection
38	50/M	CMY	1:160	18/M	0	165	–	300	ACAD
39	45/M	CMY	1:20	20/M	0	148	–	210	Chronic rejection
40	49/F	CMY	0	22/M	0	150	–	450	*P. carinii* pneumonia
41	66/M	CMY	1:40	22/M	1:20	130	+	1712	ACAD

Tx, transplantation; Dx, diagnosis; ACAD, allograft coronary artery disease with microscopic evidence of myocardial ischemic injury; CMY, cardiomyopathy; IHD, ischemic heart disease; NA, data not available; NT, not tested; PTLD, post-transplantation lymphoproliferative disorder.
HCMV antibody titers of <1:10 are recorded as zero; postoperative HCMV titer rise of more than fourfold is recorded as positive. The post-transplantation HCMV titer of patient 23 remained elevated at 1:1281 throughout course of study.

words, the pathological evidence of alloarteritic (endothelialitis) and intimitis processes was present in grafts from the very shortest period of time post-transplant to those with the longest. The process of ACAD then, is a long-term process, appearing early post-transplant, and reflected in various degrees of intimal thickening. It may not be detectable in clinical studies, angiographic or ultrasonic, but still be present pathologically. This principle of universal presence is founded on previous studies demonstrating the ubiquity of the alloimmune phenomena in graft arteries and the characteristic intimal changes in virtually every human heart allograft implanted.

Serological Diagnosis of Cytomegalovirus Infection

All organ donors and recipients immediately before transplantation had serum samples obtained for the determination of anti-cytomegalovirus antibody titers. These titers were also obtained weekly for the first 3 weeks post-transplant, quarterly, and when clinically indicated. Immunoglobulin (Ig)G and IgM cytomegalovirus-specific antibody levels were measured by the cytomegalovirus IgM test (Gull Laboratories, Salt Lake City, UT), an indirect fluorescent antibody method. A serum dilution of 1:10 or greater

Table 2. *Presence of HCMV Genome and Histopathological and Histometric Features of Allografts*

Patient No.	HCMV ISH result	HCMV PCR result	Evidence of rejection at autopsy	Ranges of % luminal narrowing of coronary arteries	
				Mean	Maximum
1	–	NT	3–4+	0–25	0–25
2	–	–	Tr–1+	0–25	91–100
3	–	NT	0	26–50	51–75
4	–	–	0–Tr	5–30	26–50
5	+	+	1–2+	10–35	26–50
6	+	+	0	0–25	0–25
7	–	–	1+	22–46	51–75
8	–	–	0	6–31	26–50
9	–	–	1–2+	6–31	26–50
10	–	–	0	51–75	51–75
11	–	–	2+	8–33	26–50
12	–	–	0	0–25	26–50
13	–	–	Tr	51–75	91–100
14	–	–	Tr–1+	32–56	51–75
15	–	–	Tr	32–56	51–75
16	–	–	0	0–25	0–25
17	–	NT	1–2+	64–82	76–90
18	–	NT	0	0–25	26–50
19	–	NT	0	18–42	51–75
20	–	NT	0–Tr	12–38	26–50
21	+	–	0	12–38	26–50
22	+	–	1+	51–72	76–90
23	–	+	1–2+	26–50	76–90
24	NT	–	0	0–25	76–90
25	NT	+	0	13–38	25–50
26	NT	–	3–4+	26–50	76–90
27	NT	–	0	51–75	76–90
28	NT	–	1–2+	6–31	76–90
29	NT	+	1–2+	18–42	51–75
30	NT	–	0	12–37	51–75
31	NT	–	0	8–33	51–75
32	NT	–	0	39–63	91–100
33	NT	–	0	46–71	91–100
34	NT	–	0	50–70	76–90
35	NT	–	0	8–33	26–50
36	NT	+	0	5–30	26–50
37	NT	–	0	0–25	0–25
38	NT	–	1+	30–54	76–90
39	NT	–	0–Tr	0–25	26–50
40	NT	–	0	0–25	51–75
41	NT	–	0	38–59	91–100

ISH, *in situ* hybridization; NT, not tested; Tr, trace.

Coronary arteries (*CA*) evaluated were left main, left anterior descending, first diagonal branch of left anterior descending, first marginal branch of left CA, proximal and distal left CA, and right main, right marginal, and posterior descending CA. Mean ranges are mean upper and lower limits of cross-sectional luminal narrowing (that is, reciprocally, intimal area) considering all evaluated CA. Maximum ranges reflect maximal luminal narrowing at any site in the coronary tree.

indicated the presence of cytomegalovirus antibodies. A current cytomegalovirus infection was positive if IgM antibodies were present at a serum dilution of 1:10 or greater. An antibody titer of fourfold increase or seroconversion of IgG antibodies was indicative of active cytomegalovirus disease.

Preparation of Labeled DNA Probes for In Situ *Hybridization*

A probe specific for the HCMV (AD169 strain) major IE gene was derived from the 10-kb HCMV *Eco*RI J fragment of the pCM5018 cosmid clone.[24] The 6.6-kb HCMV *Bam*HI R fragment of the pCM3 plasmid was used to generate a probe specific for the HCMV gene encoding mRNAs for the 65-kd and 75-kd late phosphoprotein antigens.[24,25] Both HCMV gene regions are devoid of sequence homologies to human DNA.[24] Recombinant coxsackievirus B3 (CVB3) cDNA clone pCB3-M1, containing the full-length transcript of the viral genome,[26] was used to generate *Kpn*I or *Bam*HI fragments (6.2 kb and 1 kb, respectively), together representing 95.4% of the viral genome. Agarose gel-purified probe DNA was radiolabeled by nick-translation using (α-[^{35}S]thio)dATP and (α-[^{35}S]-thio)dCTP (1200 Ci/mmol, NEN Research Products, Boston, MA) as described.[27]

In Situ *Hybridization*

Paraffin-embedded tissue sections on silanated glass slides were deparaffinized as described.[27,28] Before hybridization, samples were processed as follows: 20 minutes at room temperature in 0.2 N HCl; 30 minutes at 70°C in 2X standard-saline citrate (SSC); 15 minutes at 37°C in 20 mmol/L Tris-HCl (pH 7.4), 2 mmol/L $CaCl_2$, and proteinase K (1 μg/ml); 15 minutes at 65°C in 95% formamide with 0.1% SSC; and finally washed in 0.1X SSC for 5 minutes at 4°C.[28] Hybridization mixture contained either the ^{35}S-labeled, *Eco*RI J fragment probe (1.1×10^7 cpm/ml, 21 ng/ml), specific for the HCMV IE gene region, the *Bam*HI R fragment (1.1×10^7 cpm/ml, 23 ng/ml), specific for the HCMV late antigen gene region, or the negative control, CVB3 cDNA probe[28] (1.1×10^7 cpm/ml, 16 ng/ml) in 50% formamide, 10 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA, 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.05% bovine serum albumin, 10% dextran sulfate, 10 mmol/L dithiothreitol, 200 μg/ml denatured, sonicated salmon sperm DNA, 0.1% sodium dodecyl sulfate, 600 mmol/L NaCl, and 100 μg/ml rabbit liver tRNA. Hybridization was done at 25°C for 48 hours. Slides were then washed in 50% formamide, 10 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA, and 600 mmol/L NaCl for 18 hours at 35°C, followed by a 1-hour wash in 2X SSC at 55°C, two rinses in 2X SSC at room temperature, and dehydration in graded ethanol solutions containing 300 mmol/L ammonium acetate. After 6 weeks of exposure, slides were autoradiographed as described,[27] developed with Kodak D19, and lightly counterstained with H&E.

Preparation of Tissues for PCR

For analysis by PCR, three tissue sections (5 μm each) in series to those used for *in situ* hybridization were placed in a sterile, 0.5-ml microfuge tube. Extreme care was taken to avoid product carryover contamination by soaking microtome blades in 0.1 N HCl between cutting of each case. Sections were deparaffinized by a modified published procedure.[29] Cut sections were deparaffinized by incubation in 450 μl of xylene at 60°C for 15 minutes. Specimens were then pelleted by centrifugation, and the xylene supernatant was removed. Xylene extraction was then repeated twice. After the final xylene incubation, tissue fragments were washed three times in absolute ethanol and dried thoroughly in a 55°C heat block. Tissues were then resuspended in 200 μl of digestion buffer containing 200 μg/ml proteinase K (Boehringer Mannheim, Mannheim, Germany), 50 mmol/L Tris-HCl, 1 mmol/L EDTA, and 0.5% Tween 20 (Boehringer Mannheim). Digestion was carried out for 12 hours at 55°C with continuous shaking, followed by heat inactivation of proteinase K for 15 minutes at 95°C. More extensive purification by phenol/chloroform extraction and ethanol precipitation did not improve PCR amplification yields.

Primers Used in PCR

A positive control, oligonucleotide primer pair, specific for the constitutive human β-globin gene,[30] served to examine the integrity of sample DNA for PCR. The β-globin gene primer pair, consisting of GH20 (5′-GAAGAGCCAAGGACAGGTAC-3′) and PC04 (5′-CAACTTCATCCACGTTCACC-3′), generated an amplified fragment of 268 bp. PCR detection of HCMV genome was attempted in only those samples from which β-globin could be amplified. HCMV genome was amplified with an oligonucleotide primer pair specific for exon 4 of the HCMV IE gene. The upstream primer sequence was 5′-GGTCACTAGTGACGCTTGTATGATGACCA-3′, corresponding to sense strand sequence 2113–2141 of the HCMV IE gene.[31] The downstream primer sequence was 5′-TTCTCAGCCACAATTACTGAGGACAGA-3′, complementary to sense strand sequence 2488–2514 of the HCMV IE gene. The HCMV IE gene primer pair generated an amplified fragment of 402 bp.

PCR

Reaction conditions and cycling profile for the β-globin primer set were standard[30] and included 10 μl of patient sample DNA, 50 pmol of each primer, 0.2 mmol/L of each dNTP (dATP, dTTP, dGTP, and dCTP), 1.5 mmol/L $MgCl_2$, 2.5 U of *Taq* DNA polymerase (Promega, Madison, WI), and buffer components (described by the manufacturer) in a final volume of 30 μl. Reaction mixtures were overlaid with 100 μl of mineral oil to prevent evaporation. The cycling profile for the β-globin primer set consisted of 5 minutes of denaturing at 94°C, followed by 40 cycles of 94°C, 55°C, and 72°C for 1 minute each in a Perkin-Elmer Cetus (Norwalk, CT) DNA thermal cycler. Completion of the 40 cycles was followed by 2 minutes at the annealing temperature (55°C) and 10 minutes at the extension temperature (72°C) to ensure full product synthesis.

Reaction conditions and cycling parameters for the HCMV IE gene primer pair were extensively charac-

terized and optimized for highest sensitivity. The optimal reaction conditions and cycling profile for the HCMV IE gene primer set included 50 pmol of each primer, 0.2 mmol/L of each dNTP, 2 mmol/L $MgCl_2$, 100 μg/ml bovine serum albumin, 2.5 U of *Taq* DNA polymerase, and buffer components (described by the manufacturer). The cycling profile consisted of 5 minutes of denaturing at 94°C, followed by 40 cycles of 94°C, 60°C, and 72°C for 1 minute each in the DNA thermal cycler. Completion of the 40 cycles was followed by 2 minutes at the annealing temperature (60°C) and 10 minutes at the extension temperature (72°C) to ensure full product synthesis.

Sensitivity of the HCMV IE gene primer set and the fully optimized reaction conditions were assessed by serial dilution analysis of purified HCMV genome. Unpurified HCMV genome was isolated and purified from an infected fibroblast culture by the methods of Josephs and colleagues.[32] Purified HCMV DNA was then diluted to various known concentrations (ranging from 1 pg to 4 ng) and used as template DNA in sensitivity determination PCR assays.

Analysis of PCR-Amplified Products

PCR-amplified products were resolved on 2% agarose gels, stained with ethidium bromide, and photographed under ultraviolet light. For Southern blotting, separated DNA fragments were blotted onto Zeta Probe nylon membrane (Bio-Rad Laboratories, München, Germany) as described.[33] The oligonucleotide, PC03 (5′-ACACAACTGTGTTCACTAGC-3′),[29] served as a probe to identify internal sequences of the PCR-amplified β-globin gene fragment by Southern blot. The oligonucleotide probe used to confirm the identity of the PCR-amplified HCMV IE gene fragment by Southern blot consisted of the internal sense strand sequence 2329–2358 (5′-TCTGAGAGTCTGCTCTCCTAGTGTGGATGA-′3). Ten picomoles of oligonucleotide probes were end-labeled with 125 μCi of [γ-^{32}P]ATP (6000 Ci/mmol) (NEN Research Products) and bacteriophage T4 polynucleotide kinase (Boehringer Mannheim), essentially as described.[33] Probes were end-labeled to a determined radiospecific activity and were used in the hybridization solution at a final concentration of 1×10^6 cpm/ml. Prehybridization, hybridization, and wash steps were carried out as specified by the manufacturer of the nylon membrane. Blots were autoradiographed on Hyperfilm-MP (Amersham, Solna, Sweden) at −70°C in the presence of an intensifying screen.

Data Analyses

In situ-hybridized microscopic slides and H&E-stained serial sections were analyzed in blinded fashion by two investigators. Serial sections of the entire coronary tree of each allograft were numerically scored for arteriopathic disease, particularly the extent of artery luminal narrowing. Sections of allograft myocardium were analyzed independently by two investigators for evidence of ischemic injury and rejection (manifested by myocardial or perivascular inflammatory infiltrate, interstitial edema, or myocyte damage or necrosis) that was graded numerically from 0 to 4+. Although the principles of the rejection grading criteria developed for biopsies by the International Society for Heart and Lung Transplantation[34] could be considered, they were not meant for application to transmural sections of myocardium. Thus, for the present study a score of 0 reflects no infiltrates throughout the myocardium; 1+, minimal, focal perivascular or interstitial cell mononuclear cell infiltrates; 2+, rare, aggressive foci of mononuclear infiltrates; 3+, multifocal, aggressive mononuclear cell infiltrates with readily visible myocyte damage and edema; and 4+, diffuse, cellular infiltrates with myocyte damage or necrosis and possible vasculitis. The molecular and histopathological findings were analyzed in conjunction with the following clinical and serological data: donor and recipient HCMV serologies, allograft operative ischemic times, durations of allograft implantation, and causes of death or retransplantation.

Statistical comparison between mean ages of allograft donors and PDAY control subjects was carried out by the paired and unpaired Student's *t*-test. Pearson's product-moment and Spearman's rank-order correlation algorithms were employed to evaluate possible relationships between various sets of allograft donor or recipient data, with correlation coefficients (corr. coef.) indicated where applicable. Statistical comparison between prevalence of HCMV DNA in allograft hearts *versus* that in control hearts and comparison between results of *in situ* hybridization and PCR were made by the Fisher's exact test. For all data analyses, the probability of an α error was set at $P < 0.05$.

Results

In Situ *Hybridization*

Of the 23 allograft hearts tested by *in situ* hybridization, 4 (17.4%) were positive for HCMV DNA (Table 2). HCMV genome was focally present in perivascular

myocardium of two allografts, in the perivascular myocardium and coronary artery adventitia of one allograft (Figure 1), and in the coronary artery media of another allograft. Of the 22 control hearts, 1 (4.5%) contained HCMV genome within histopathologically normal, perivascular myocardium (not shown). HCMV DNA was not detected in the arterial intima of any allograft or control heart. HCMV DNA was, however, detected in positive control tissues of HCMV-viremic immunodeficient patients (Figure 2). When HCMV genome was detected, it was not associated with an inflammatory infiltrate in the immunodeficient patient. By *in situ* hybridization, the difference between prevalence of HCMV DNA in allografts *versus* control hearts did not reach statistical significance ($P = 0.19$). The negative control probe did not label any of the tissues studied (Figures 1 and 2).

PCR

Sections of paraffin-embedded coronary arteries of 36 allograft hearts and of 11 control hearts were stud-

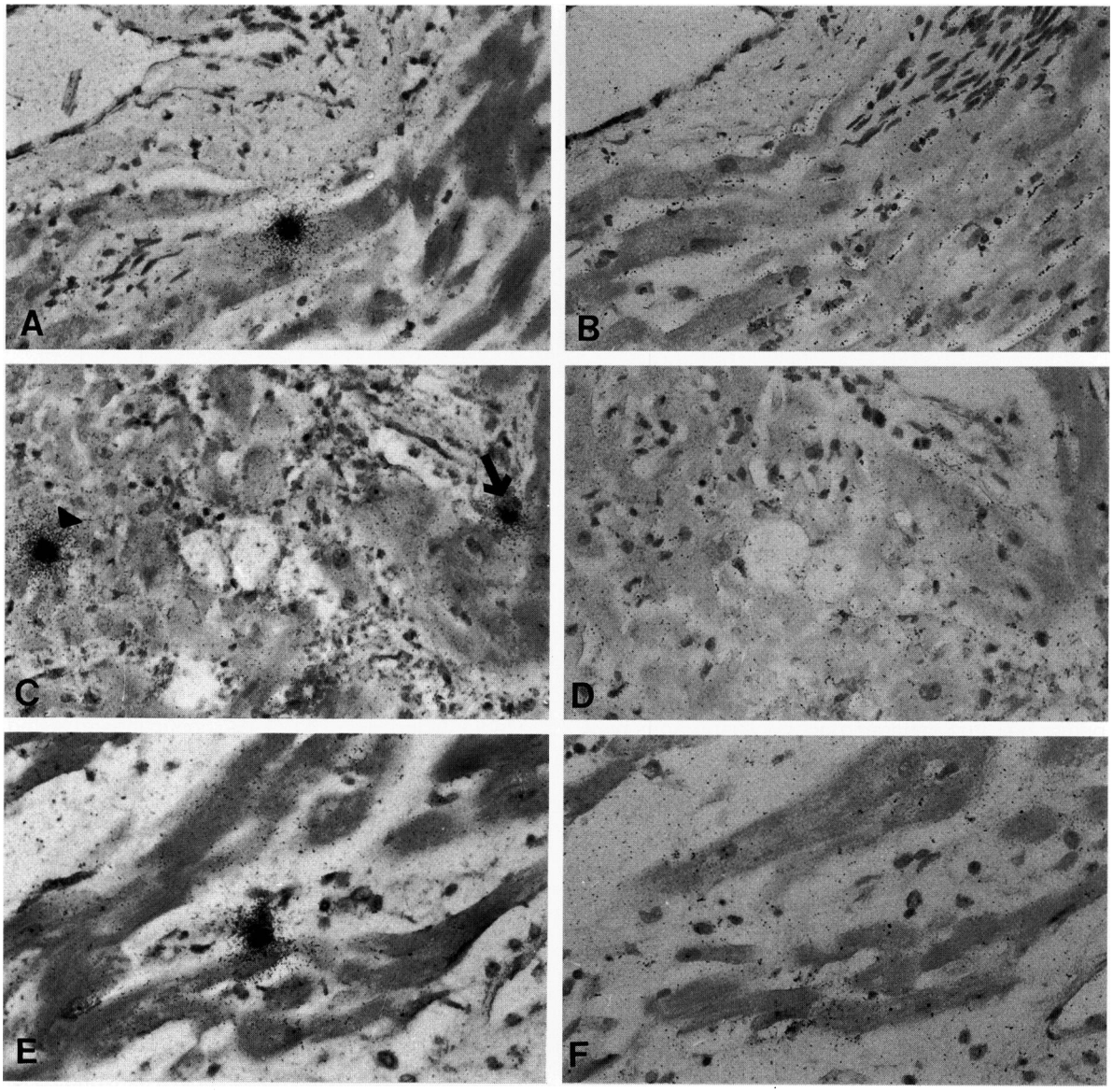

Figure 1. *Sections of cardiac allografts labeled by in situ hybridization.* **A:** *Patient 5. Labeling with the HCMV IE gene probe is apparent within a cardiac cell adjacent to an unlabeled, normal myocardial vessel.* **B:** *Serial section showing no labeling with the negative control probe.* **C:** *Patient 9. Labeling with the HCMV-LA gene probe is apparent within the myocardial interstitium (arrow) and possibly within a cardiac myocyte (arrowhead).* **D:** *Serial section labeled with the negative control probe.* **E:** *Patient 23. Labeling with the HCMV IE gene probe is seen within the edematous myocardial interstitium.* **F:** *Serial section labeled with the negative control probe. All panels were counterstained with H&E.* **A** *and* **B**, ×*400;* **C** *and* **D**, ×*330;* **E** *and* **F**, ×*500.*

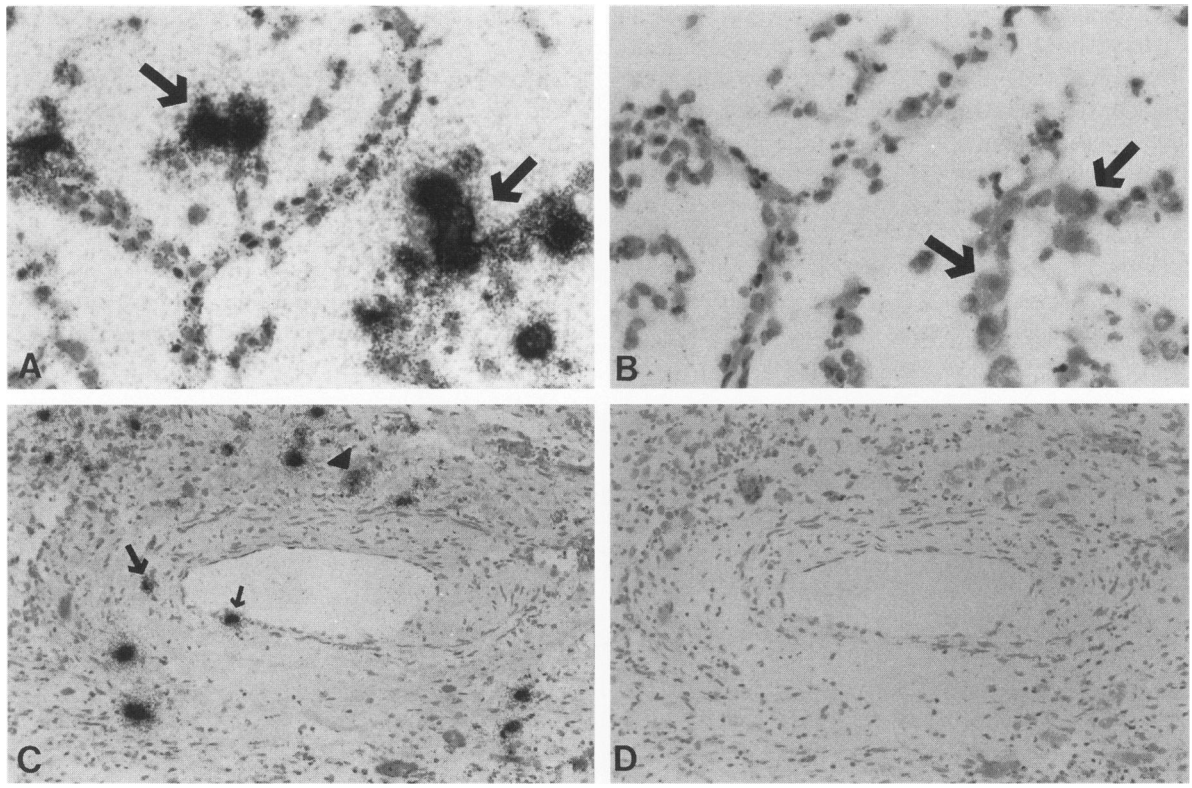

Figure 2. In situ-*hybridized positive control lung tissue from a HCMV-viremic immunodeficient individual.* **A:** *Prominent labeling is seen by* in situ *hybridization with the radiolabeled probe specific for the HCMV IE gene. Labeling is localized to intra-alveolar cells (arrows) characterized by viral cytopathic effect, well seen in* **B** *(arrows), which is the serial section showing no labeling when hybridized with the negative control probe.* **C:** *Small pulmonary artery with labeling by* in situ *hybridization with the HCMV late antigen probe. Labeling is localized to cytomegalic cells along the endothelial lining (small arrow), the vessel outer media (larger arrow) and adventitia (arrowhead), as well as in the surrounding lung parenchyma.* **D:** *Serial section hybridized with the negative control probe. Sections were lightly counterstained with H&E.* **A** *and* **B**, *original magnification ×400;* **C** *and* **D**, *×200.*

ied by PCR. The allograft specimens studied by PCR included 17 of the allograft hearts that were also examined by *in situ* hybridization. To confirm the suitability of DNA from prepared specimens, all samples were tested initially for presence of the constitutive human β-globin gene, employing an oligonucleotide primer pair that generated an amplified fragment of 268 bp (Figure 3). The PCR-amplified DNA fragment was confirmed to represent a portion of the β-globin gene by Southern blotting with an oligonucleotide probe specific for an internal region of the human β-globin gene fragment (not shown). Prepared DNA samples from 35 of 36 (97.2%) allograft specimens were positive for the β-globin gene.

β-globin-positive samples were further tested for HCMV DNA with HCMV IE gene-specific oligonucleotide primer pair generating a PCR-amplified DNA fragment of 402 bp (Figure 3). PCR conditions and cycling parameters for detection of HCMV IE DNA were extensively characterized and optimized for highest sensitivity. Serial dilutions of purified HCMV DNA were used to determine a PCR sensitivity of 2 pg of viral DNA (not shown), which corresponds to approximately 4×10^3 molecules of HCMV DNA.[35] Southern blotting with an oligonucleotide probe specific for an internal sequence of the HCMV IE gene confirmed that the amplified fragment was derived from the HCMV IE gene (not shown). HCMV DNA was detected by PCR in 6 of 35 (17.1%) allograft hearts (Table 2) and in 1 of 11 (9.1%) control hearts tested. This difference, however, was not statistically significant ($P = 0.46$).

Concordance between In Situ *Hybridization and PCR*

Seventeen of the allografts were available for study by both *in situ* hybridization and PCR. In fourteen of these samples (82.4%), there was agreement between the two methods, and there was no statistical difference between the results of *in situ* hybridization and PCR ($P = 0.12$). In each of the eleven control hearts that were tested by both *in situ* hybridization and PCR, there was agreement between the two methods. Finally, the overall prevalence of HCMV ge-

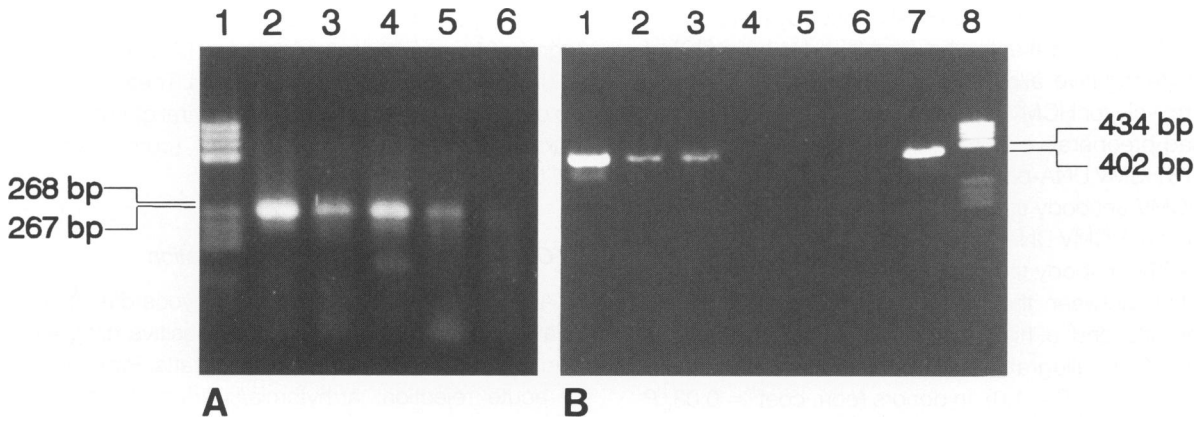

Figure 3. *Illustration of PCR-amplified DNA fragments separated in ethidium bromide-stained agarose gels.* **A:** *PCR-amplified β-globin DNA fragment (268 bp).* **Lane 1**, *molecular weight standard (pBR322 DNA digested with HaeIII);* **lanes 2** *to* **5**, *patient tissue samples containing DNA suitable for PCR;* **lane 6**, *patient without amplifiable DNA.* **B:** *PCR-amplified HCMV IE gene DNA fragment (402 bp).* **Lanes 1** *to* **3**, *patients positive for HCMV IE DNA;* **lanes 4** *to* **6**, *patients negative for HCMV IE DNA;* **lane 7**, *positive control tissue from a HCMV-viremic, immunodeficient individual;* **lane 8**, *molecular weight standard.*

nome, determined by either method, in the allografts (8 of 41, 19.5%) did not differ significantly from that in control hearts (1 of 22, 4.5%; $P = 0.10$).

Relationship between Presence of HCMV Genome and Serological and Clinical Features

Severity of Arteriopathic Disease

H&E-stained sections of the entire coronary artery tree of each allograft heart were examined microscopically for degree of arteriopathic luminal narrowing (Table 2), based on the following ranges of narrowing: 0 to 25%, 26 to 50%, 51 to 75%, 76 to 90%, and 91 to 100%. Reported mean ranges are calculated as mean minimum and mean maximum ranges based on evaluation of the entire coronary tree. Reported maximum ranges reflect sites of maximal narrowing at any given point in the coronary tree. There was no association between nature or extent of transplant arteriopathy and presence of HCMV genome. Two of eight (25%) HCMV-positive allografts (patients 22 and 23) had coronary artery luminal narrowing of greater than 76% at any site of the coronary tree. One of those patients (23) died with histological evidence of previous myocardial infarction, whereas the other patient (22) died of post-transplantation lymphoproliferative disease and without microscopic evidence of myocardial ischemic injury. Patients 22 and 23 had survived 635 and 611 days post-transplant, respectively. Of the 33 allograft hearts that were negative for HCMV genome, 12 (36%) had foci of coronary luminal narrowing of at least 76%, and 10 (83%) of the latter HCMV-negative allografts were found to have microscopic evidence of myocardial ischemic injury.

Evidence of Rejection

Inflammatory infiltrates in ventricular allograft myocardium were numerically graded from 0 to 4+ in increasing severity as noted earlier (Table 2). Four (50%) of the eight HCMV-positive allograft hearts had an inflammatory infiltrate of any magnitude. Fifteen (45%) of the 33 HCMV-negative allografts had an inflammatory infiltrate of any degree, and 8 (24%) of the allografts had an infiltrate of at least 1+ in grade. There was no correlation between positivity for viral DNA and evidence of rejection (corr. coef. = 0.22, $P = 0.16$).

Donor and Recipient HCMV Serology

Before transplantation, allograft recipients and donors were screened for serum antibodies to HCMV (Table 1). Of the 40 tested allograft recipients 30 (75%) had preoperative antibody titers of ≥1:10 (range, 1:10 to 1:1280). Of these, 6 (20%) were found to harbor HCMV DNA in their allografts. Of the 37 tested allograft donors, 22 (59.5%) had HCMV antibody titers of ≥1:10 (range, 1:10 to 1:320). The hearts of 5 (22.7%) of these donors were ultimately found positive for HCMV DNA. Of the 8 allograft cases found to harbor HCMV DNA, 5 (62.5%) had both preoperative recipient and donor HCMV antibody titers of ≥1:10. Of the 29 HCMV DNA-negative cases with available serological data, 15 (51.7%) had HCMV antibody titers of ≥1:10 in both the preoperative recipient and donor. Of the 20 total cases with known preoperative recipient and donor antibody titers of ≥1:10, 5 (25%) were found to have HCMV DNA-positive allografts. Of the 8 transplant patients who were found to have HCMV DNA-positive allografts, 6

(75%) had preoperative HCMV antibody titers of ≥1:10. Of the 33 patients who were found to have HCMV DNA-negative allografts, 32 were screened preoperatively for HCMV antibody titers; of these, 24 (75%) had preoperative titers of ≥1:10. Donors of 5 (62%) of 8 HCMV DNA-positive allografts were found to have HCMV antibody titers of ≥1:10. Of the 29 tested donors of HCMV DNA-negative allografts, 17 (59%) had HCMV antibody titers of ≥1:10. There was no correlation between the presence of HCMV DNA in allografts and a history of HCMV antibody titers of ≥1:10 in allograft recipients preoperatively (corr. coef. = 0.0, $P = 1.0$), in donors (corr. coef. = 0.03, $P = 0.85$), or in both recipients preoperatively and donors (corr. coef. = 0.02, $P = 0.93$).

Allograft recipients were monitored serologically for evidence of active HCMV infection, with new or reactivated infections suspected in the event of appearance of postoperative HCMV antibodies or fourfold or greater titer increases. Post-transplantation HCMV antibody titers were available on seven of eight patients with HCMV DNA-positive allografts. Four (57%) of these seven patients were found to have increased HCMV antibody titers. Three of these four patients generated at least a fourfold rise in HCMV antibody titers during their clinical courses, whereas one patient (23) maintained high postoperative HCMV titers equivalent to his preoperative titer of 1:1281.

Postoperative HCMV antibody titers were available on 26 of 33 patients with HCMV DNA-negative allografts. Of these patients, 10 (38%) generated at least a fourfold rise in HCMV antibody titers during their clinical course post-transplantation. There was no correlation between incidence of fourfold HCMV antibody titer rises and the presence of HCMV DNA in allograft tissues (corr. coef. = 0.04, $P = 0.84$). Serological data on the control trauma victims were not available.

Allograft Operative Ischemic Times

Operative ischemic times were available in all but 1 of the 41 allograft hearts. There was no correlation between the mean operative ischemic time (148 ± 60 minutes; range, 56 to 316 minutes) and the mean duration of allograft implantation (457 ± 493 days; range, 4 to 1712 days, corr. coef. = 0.23, $P = 0.17$). There also appeared to be no correlation between operative ischemic times and coronary luminal narrowing or causes of death or retransplantation.

Duration of Allograft Implantation

The mean durations of allograft implantation in the HCMV-positive allografts (414 ± 444 days; range, 33 to 1349 days) and those of the HCMV-negative allografts (467 ± 510 days; range, 4 to 1712 days; Table 1) did not differ significantly ($P > 0.05$). There was also no correlation between presence of viral genome and durations of allograft implantation (corr. coef. = −0.04, $P = 0.79$).

Causes of Death or Retransplantation

ACAD with evident histological myocardial ischemia was present in 25% of HCMV-positive allografts and in 42% of HCMV-negative allografts. Prevalence of acute rejection, arrhythmia/sudden death, and noncardiac deaths were similar in the HCMV group *versus* the non-HCMV group. Interestingly, the HCMV group appeared to have a higher percentage (25%) of idiopathic allograft failure, relative to the non-HCMV group (6%).

Discussion

Improvements in postoperative care and immunosuppression have markedly enhanced early survival of heart transplant recipients. In contrast, long-term survival remains severely limited by rapidly progressive allograft coronary artery disease (ACAD) as well as other causes. The pathogenesis of the arteriopathic lesion is still unresolved, and this uncertainty underlies the additional examination we have pursued of the hypothesis that cytomegalovirus infection of arterial walls is directly at play.

Our concordant results of *in situ* DNA hybridization and PCR analyses of coronary arterial segments of human heart allografts and hearts of donor age-, gender-, and coronary site-matched trauma victims argue against a direct, pathogenetic role of HCMV in ACAD. The difference between the prevalence of HCMV genome in allografts and that in control hearts failed to reach statistical significance. In HCMV genome-positive allograft or control hearts, viral DNA was focally present in perivascular myocardium and/or in coronary artery adventitia or media and could not be detected in arterial intima. Viral DNA was, however, detected in the intimal layer of pulmonary vessels in positive control, HCMV pneumonitis tissues. Examination of the entire coronary arterial tree, to determine character of arteriopathic injury and degree of luminal narrowing throughout, failed to show a relationship between the presence of viral genome and arteriopathic injury and repair. There was also no more histopathological evidence of allograft rejection in the HCMV DNA group than in the group without allograft HCMV genome. The presence of allograft HCMV DNA could not be predicted based on

HCMV antibody titers suggestive of previous infection of allograft recipients or donors with HCMV. Moreover, postoperative appearance of HCMV antibodies or fourfold antibody titer increases were also not associated with detection of HCMV DNA in allografts. HCMV DNA in allograft hearts also had no bearing on or relationship to the duration of allograft implantation.

Much effort has been directed toward evaluation of possible etiological links between herpesviruses and classical, native atherosclerosis. Early studies demonstrated induction of occlusive atherosclerotic lesions in normocholesterolemic, pathogen-free chickens by the avian herpesvirus, Marek's disease virus.[4,5] Several groups have since reported antigens or nucleic acids of herpesviruses, including HCMV, in atherosclerotic vessels, by immunological or molecular techniques.[6–13] Although Benditt and colleagues[6] reported the presence of herpes simplex viral mRNA (by *in situ* hybridization) in association with human atherosclerotic injury, they were unable to detect HCMV genome in the same tissues. In one study, Hendrix and colleagues implicated HCMV infection in the pathogenesis of atherosclerosis.[12] Using PCR, they detected viral DNA in 90% of severely atherosclerotic abdominal aortas and femoral arteries and in 53% of minimally diseased, control abdominal aortas. In a subsequent study, however, the same group examined several major elastic arteries in nine HCMV-seropositive subjects for HCMV DNA.[13] In that report, HCMV DNA was apparently detected by PCR in 95% of atherosclerotic vessels and in 90% of normal vessels. These latter data argue against a direct relationship between HCMV infection and native atherogenesis but suggest high prevalence of HCMV genome in these tissues.

HCMV is a major pathogen in immunosuppressed hosts, such as organ transplant recipients[14–16,36–38] or AIDS patients.[39–42] Although HCMV-infected cells are readily found to be widespread in these individuals, including lung, liver, salivary and adrenal glands, gastrointestinal tract, pancreas, kidneys, and blood, the virus is rarely detectable in hearts. Interest in a role for herpesviruses in transplant-associated ACAD was spurred by ubiquity of systemic HCMV colonization (virus presence without pathological consequence) of immunocompromised heart transplant recipients[15] and two, independent, retrospective analyses of a combined total of 403 cardiac transplant recipients in which a relationship was observed between post-transplant HCMV infection and ACAD.[17,18] Others have since suggested a link between serological evidence of HCMV exposure and allograft atherosclerosis.[43] Our data agree with those of Pahl and colleagues,[44] who studied 21 pediatric allograft recipients surviving the perioperative period and found no correlation between the incidence of post-transplant, serological HCMV infection and development of ACAD. Similarly, in multivariate analysis of 323 transplant recipients, Sharples and colleagues[45] failed to find correlation between HCMV infection and ACAD. Cooper and colleagues[37] reported clinically significant, noncardiac HCMV infections in 39% of 57 heart allograft recipients studied. There appeared to be higher incidence of early acute rejection and angiographic evidence of graft atherosclerosis in the first postoperative year in their HCMV group, when compared with their group in whom HCMV disease did not develop. The association between noncardiac HCMV infection and graft atherosclerosis, however, did not reach statistical significance, and direct viral infection of allografts was not determined in that study. It is also unclear whether HCMV infection promotes rejection or actually results from increased immunosuppressive treatment thereof. Similar to our findings of no correlation between the presence of myocardial cellular infiltrates and serological data suggestive of HCMV infection, another group reported no evidence of increased cellular infiltration in allograft endomyocardial biopsies in patients with serological evidence of HCMV infection.[46]

More recent molecular studies in search of HCMV in human heart allograft tissue have been suggestive but not compelling. Hruban, Wu, and colleagues compared the prevalence of HCMV nucleic acids in coronary arteries of 9 allograft hearts with ACAD to that in coronary arteries of 10 allografts without ACAD.[19,20] By *in situ* hybridization with a DNA probe and a riboprobe, HCMV nucleic acids were detected in 3 of 10 (30%) hearts without ACAD and in 6 of 9 (67%) hearts with ACAD. This difference, however, was not statistically significant. The strongest hybridization with the DNA probe (from the L-segment repeats of the HCMV Towne strain) was in spindle cells, presumed to be intimal smooth muscle cells, whereas the most abundant hybridization with the riboprobe (specific for the HCMV Towne strain IE gene) was in cells thought to represent lymphocytes. No cytomegalic inclusion bodies were seen. Neither of these studies included native, atherosclerotic coronary artery controls. Arbustini and colleagues[21] examined 45 endomyocardial biopsy specimens from 44 heart transplant recipients with primary or recurrent HCMV infections for the presence of a viral cytopathic effect (histologically), viral antigens (immunohistochemically), or viral genome by *in situ* hybridization and PCR. They concluded that 6 of 45 (13%) specimens were positive (myocardial, endothelial or PCR positive) for HCMV. Jäkel and colleagues[47] evaluated 164

endomyocardial biopsy specimens from 29 allograft recipients for HCMV DNA using *in situ* hybridization. HCMV DNA was detected in 31% of 29 patients or in 8.5% of 164 total specimens. Both of these studies, however, did not include seronegative transplant recipient controls or non-allograft, native biopsy specimens. Also, allograft coronary arteries were not examined for evidence of HCMV infection.

In recent work of relevance to our observations, Nadas and colleagues[48] sought HCMV genome and proteins in renal allograft blood vessels. None of 24 patients' biopsy samples revealed the presence of HCMV despite the fact that nephrectomies (13) and biopsies (11) revealed transplant arteriopathy.[49]

Several lines of evidence argue against an infective process and in favor of immune mechanisms in the pathogenesis of heart allograft arteriopathy. The disease process is diffuse in nature but confined to the graft, including the donor aorta and endocardium; recipients' native arteries are unaltered post-transplantation.[50] Several authors report an association between human leukocyte antigen (HLA) incompatibility, the frequency and severity of rejection episodes, and development of allograft arteriopathy.[44,51–54] Much effort is directed toward identification and characterization of various endothelial cell surface molecules that are involved in adhesion of leukocytes to the coronary endovascular surface. Two promising candidates are intercellular adhesion molecule-1 (ICAM-1, CD54) and vascular cell adhesion molecule-1 (VCAM-1).[55–57] Whether up-regulation of adhesion molecules precedes, or results from, rejection remains unknown. HCMV infection and subsequent interleukin-1 induction may lead to enhanced expression of an endothelial cell adhesion molecule (possibly ELAM-1) and neutrophil recruitment.[58] Up-regulation of endothelial cell VCAM-1 expression has also been noted to occur in temporal association with HCMV infection, which preceded an episode of rejection in a heart transplant patient.[59] Collins recently reviewed the possible link between atherosclerosis and an inducible, nuclear transcription factor complex, NF-κB, which is essential for expression of a large number of genes during an immune response.[60] NF-κB elements are also found in the important cell surface molecules, ICAM-1, VCAM-1, and MHC class I.[61] NF-κB is also activated after HCMV infection[62] and may play a role in the cascade of immunological events that occurs in initiation and propagation of allograft arteriopathic injury.

Animal models of cardiac transplantation have been developed without introduction of infectious agents. We are unaware of any reports of natural cytomegalovirus infection in dogs or rabbits. One group studied HCMV chorioretinitis induced by intraocular virus inoculation,[63] but, to our knowledge, hearts of cytomegalovirus-infected rabbits have not been studied. Rat cytomegalovirus (RCMV) has been found to exist in a latent state in immunocompetent rats after asymptomatic primary infection.[64,65] Span and colleagues[66] studied aortas of rats from 1 to 16 weeks after infection with RCMV. Although no damage to the endothelium was observed, RCMV infection apparently led to increased adherence of leukocytes to and lipid accumulation in endothelial cells. Lemström and colleagues[67] studied the influence of early (day 1 post-transplantation) or late (day 60) RCMV infection on aortic allograft arteriosclerosis in the rat. The authors concluded that RCMV infection at the time of transplantation led to enhanced allograft adventitial inflammatory infiltrate and increased proliferation rate of intimal smooth muscle cells when compared with allografts in uninfected rats. Infection of the rats 2 months after transplantation did not affect the development of arteriosclerotic injury. Moreover, RCMV infection had no effect on recipients of syngeneic aortas. Together, these data suggest that RCMV infection alone is insufficient to promote intimal changes in this model but that RCMV, if present in high enough titers, may somehow enhance the acute allogeneic immune response and subsequent allograft intimal injury.

In our study, allograft recipients and donors were serologically screened for elevated HCMV antibody titers, suggestive of previous infection, and recipients were routinely monitored postoperatively for changes in antibody titers. The frequent observation of lack of viral DNA in hearts of seropositive patients may be explained by antibodies induced by previous HCMV infection with latent viral DNA present in noncardiac tissues, such as salivary glands. As reviewed by Löning and colleagues,[68] there are disadvantages of clinical monitoring for HCMV infection by determination of serological immunoglobulin (IgM and IgG) responses. The primary humoral response (IgM) to HCMV takes place 3 to 4 days after symptoms. Diagnosis of HCMV infection, however, is usually made several weeks after the immunoglobulin isotype switch to IgG, so that close monitoring of serum antibody titer changes may be of limited value. Also, antigenic heterogeneity among different strains of HCMV[69,70] and marked individual variation in the host's immune response[71] may cause misleading results. Serological screening for antibodies to HCMV is routine, although determination of HCMV antigenemia may have more diagnostic utility, particularly in active infection (primary or recurrent).[72,73]

Although our results do not support a direct role of HCMV infection in this disease, they do not rule out the

possibility that HCMV may be involved indirectly or that virus-related factors may play an adjunctive role in lesion development. One such example may be virus-mediated dysregulation of cellular lipid metabolism and accumulation[74] in concert with aggressive alloimmune attack and associated release of potent inflammatory cytokines. We have recently discussed other potential mechanisms by which HCMV infection may propagate ACAD.[75] Additional epidemiological, pathological, and molecular work is needed in transplantation to determine whether or how HCMV infection may be involved pathogenetically in accelerated vascular disease.

Acknowledgments

We are indebted to Ms. Helga Rieseman for excellent technical assistance and to Dr. Kashinath Patil for statistical analysis and advice. The support of Dr. Dale Renlund is also appreciated. A Max Planck Research Prize Fellowship was awarded to Dr. Gulizia for the period during which this work was conducted.

References

1. Olivari MT, Kubo SH, Braunlin EA, Bolman RM, Ring WS: Five-year experience with triple-drug immunosuppressive therapy in cardiac transplantation. Circulation 1990, 82:IV276–280
2. Kriett JM, Kaye MP: The Registry of the International Society for Heart Transplantation: eighth official report. J Heart Lung Transplant 1991, 10:491–498
3. Winters GL, Kendall TJ, Radio SJ, Wilson JE, Costanzo-Nordin MR, Switzer BL, Remmenga JA, McManus BM: Posttransplant obesity and hyperlipidemia: major predictors of severity of coronary arteriopathy in failed human heart allografts. J Heart Transplant 1990, 9:364–371
4. Fabricant CG, Fabricant J, Litrenta MM, Minick CR: Virus-induced atherosclerosis. J Exp Med 1978, 148: 335–340
5. Minick CR, Fabricant CG, Fabricant J, Litrenta MM: Atheroarteriosclerosis induced by infection with a herpesvirus. Am J Pathol 1979, 96:673–706
6. Benditt EP, Barrett T, McDougal JK: Viruses in the etiology of atherosclerosis. Proc Natl Acad Sci USA 1983, 80:6386–6389
7. Melnick JL, Petrie BL, Dreesman GR, Burek J, McCollum CH, Debakey ME: Cytomegalovirus antigen within human arterial smooth muscle cells. Lancet 1983, 2:644–647
8. Gyorkey F, Melnick JL, Guinn GA, Gyorkey P, DeBakey ME: Herpes viridae in the endothelial and smooth muscle cells of the proximal aorta of atherosclerosis patients. Exp Mol Pathol 1984, 40:328–339
9. Petrie BL, Melnick JL, Adam E, Burek J, McCollum CH, DeBakey ME: Nucleic acid sequence in cytomegalovirus in cells cultured from human arterial tissue. J Infect Dis 1987, 155:158–159
10. Yamashiroya HM, Ghosh L, Yang R, Robertson AL: Herpesviridae in the coronary arteries and aortae of young trauma victims. Am J Pathol 1988, 130:71–79
11. Hendrix MGR, Dormans PHJ, Kitslaar P, Bosman F, Bruggeman CA: The presence of cytomegalovirus nucleic acids in arterial walls of atherosclerotic and nonatherosclerotic patients. Am J Pathol 1989, 134: 1151–1157
12. Hendrix MGR, Salimens MMM, van Boven CPA, Bruggeman CA: High prevalence of latently present cytomegalovirus in arterial walls of patients suffering from grade III atherosclerosis. Am J Pathol 1990, 136: 23–28
13. Hendrix MGR, Daeman M, Bruggeman CA: Cytomegalovirus nucleic acid distribution within the human vascular tree. Am J Pathol 1991, 138:563–567
14. Dummer JS, Hardy A, Poorsattar A, Ho M: Early infections in kidney, heart, and liver transplant recipients on cyclosporine. Transplantation 1983, 36:259–267
15. Dummer JS, White LT, Ho M, Griffith BP, Hardesty RL, Bahnson HT: Morbidity of cytomegalovirus infection in recipients of heart or heart-lung transplants who received cyclosporine. J Infect Dis 1985, 152:1182–1191
16. Pollard RB: Cytomegalovirus infections in renal, heart, heart-lung and liver transplantation. Pediatr Infect Dis J 1988, 7:S97–102
17. Grattan MT, Moreno-Cabral CE, Stames VA, Oyer PE, Stinson EB, Shumway NE: Cytomegalovirus infection is associated with cardiac allograft rejection and atherosclerosis. JAMA 1989, 261:3561–3566
18. McDonald K, Rector TS, Braunlin EA, Kubo SH, Olivari MT: Association of coronary artery disease in cardiac transplant recipients with cytomegalovirus. Am J Cardiol 1989, 64:359–340
19. Hruban RH, Wu TC, Beschomer WE, Cameron DE, Ambinder RF, Baumgartner WA, Reitz BA, Hutchins GM: Cytomegalovirus nucleic acids in allografted hearts. Hum Pathol 1990, 21:981–983
20. Wu TC, Hruban RH, Ambinder RF, Pizzomo M, Cameron DE, Baumgartner WA, Reitz BA, Hayward GS, Hutchins GM: Demonstration of cytomegalovirus nucleic acids in the coronary arteries of transplanted hearts. Am J Pathol 1992, 140:739–747
21. Arbustini E, Grasso M, Diegoli M, Percivalle E, Grossi P, Bramerio M, Campana C, Goggi C, Gavazzi A, Vigano M: Histopathologic and molecular profile of human cytomegalovirus infections in patients with heart transplants. Am J Clin Pathol 1992, 98:205–213
22. Pathobiological Determinants of Atherosclerosis in Youth (PDAY) Study Group: Natural history of aortic and coronary atherosclerotic lesions in youth: findings from the PDAY study. Arterioscler Thromb 1993, 13: 1291–1298

23. Lin H, Kendall TJ, Wilson JE, Thieszen SL, Radio SJ, Winters GL, Costanzo MR, Miller LW, McManus BM: Lack of evidence for greater distal severity of accelerated intimal proliferation in transplant arteriopathy of human heart allografts: histomorphometric documentation. J Heart Lung Transplant, 1994, 13:824–833
24. Rüger R, Bornkamm GW, Fleckenstein B: Human cytomegalovirus DNA sequences with homologies to the cellular genome. J Gen Virol 1984, 65:1351–1364
25. Rüger R, Klages S, Walla B, Albrecht J, Fleckenstein B, Tomlinson P, Barrell B: Primary structure and transcription of the genes coding for the two virion phosphoproteins pp65 and pp71 of human cytomegalovirus. J Virol 1987, 61:446–453
26. Kandolf R, Hofschneider PH: Molecular cloning of the genome of a cardiotropic coxsackie B3 virus: full-length reverse-transcribed recombinant cDNA generates infectious virus in mammalian cells. Proc Natl Acad Sci USA 1985, 82:4818–4822
27. Kandolf R, Ameis D, Kirschner P, Canu A, Hofschneider PH: *In situ* detection of enteroviral genomes in myocardial cells by nucleic acid hybridization: an approach to the diagnosis of viral heart disease. Proc Natl Acad Sci USA 1987, 84:6272–6276
28. Klingel K, Hohenadl C, Canu A, Albrecht M, Seemann M, Mall G, Kandolf R: Ongoing enterovirus-induced myocarditis is associated with persistent heart muscle infection: quantitative analysis of virus replication, tissue damage, and inflammation. Proc Natl Acad Sci USA 1992, 89:314–318
29. Greer CE, Peterson SL, Kiviat NB, Manos AM: PCR amplification from paraffin embedded tissues. Am J Clin Pathol 1991, 95:117–124
30. Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Amheim N: Enzymatic amplification of β-globin sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 1985, 230: 1350–1354
31. Akrigg A, Wilkinson GWG, Oram JD: The structure of the major immediate early gene of human cytomegalovirus strain AD 169. Virus Res 1985, 2:107–121
32. Josephs SF, Salahuddin SZ, Ablashi DV, Schachter F, Wong-Staal F, Gallo RC: Genomic analysis of the human B-lymphotrophic virus (HBLV). Science 1986, 234:601–603
33. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual, ed 2. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press, 1989
34. Billingham ME, Cary NRB, Hammond ME, Kemnitz J, Marboe C, McCallister HA, Snovar DC, Winters GL, Zerbe A: A working formulation for standardization of nomenclature in the diagnosis of heart and lung rejection: heart rejection study group. J Heart Transplant 1990, 9:587–593
35. Chou S, Merigan TC: Rapid detection and quantitation of human cytomegalovirus in urine through DNA hybridization. N Engl J Med 1983, 308:921–925
36. Myerson D, Hackman RC, Nelson JA, Ward DC, McDougall JK: Widespread presence of histologically occult cytomegalovirus. Hum Pathol 1984, 15:430–439
37. Cooper DK, Novitzky D, Schlegel V, Muchmore JS, Cucchiara A, Zuhdi N: Successful management of symptomatic cytomegalovirus disease with gancyclovir after heart transplantation. J Heart Lung Transplant 1991, 10:656–662
38. Spector SA, Rua JA, Spector DH, McMillan R: Detection of human cytomegalovirus in clinical specimens by DNA-DNA hybridization. J Infect Dis 1984, 150: 121–126
39. Drew WL, Miner RC, Ziegler JL, Gullett JH, Abrams DI, Conant MA, Huang ES, Groundwater JR, Volberding P, Mintz L: Cytomegalovirus and Kaposi's sarcoma in young homosexual men. Lancet 1982, 2:125–127
40. Reichert CM, O'Leary TJ, Levens DL, Simrell CR, Macher AM: Autopsy pathology in the acquired immune deficiency syndrome. Am J Pathol 1983, 112:357–382
41. Niedt GW, Schinella RA: Acquired immunodeficiency syndrome: clinicopathologic study of 56 autopsies. Arch Pathol Lab Med 1985, 109:727–734
42. Keh WC, Gerber MA: *In situ* hybridization for cytomegalovirus DNA in AIDS patients. Am J Pathol 1988, 131:490–496
43. Loebe M, Schüler S, Zais O, Warnecke H, Fleck E, Hetzer R: Role of cytomegalovirus infection in the development of coronary artery disease in the transplanted heart. J Heart Transplant 1990, 9:707–711
44. Pahl E, Fricker FJ, Armitage J, Griffith BP, Taylor S, Uretsky BF, Beerman LB, Zuberbuhler JR: Coronary arteriosclerosis in pediatric heart transplant survivors: limitation of long-term survival. J Pediatr 1990, 116: 177–183
45. Sharples LD, Caine N, Mullins P, Scott JP, Solis E, English TAH, Large SR, Schofield PM, Wallwork J: Risk factor analysis for the major hazards following heart transplantation-rejection, infection, and coronary occlusive disease. Transplantation 1991, 52:244–252
46. Stovin PGI, Wreghitt TG, English TAH, Wallwork J: Lack of association between cytomegalovirus infection of heart and rejection-like inflammation. J Clin Pathol 1989, 42:81–83
47. Jäkel KT, Löning T, Arndt R, Rödiger W: Rejection, herpesvirus infection, and Ki-67 expression in endomyocardial biopsy specimens from heart transplant recipients. Pathol Res Pract 1992, 188:27–36
48. Nadas T, Smith J, Laszik Z, Warner JL, Johnson LD, Silva FG: Absence of association between cytomegalovirus infection and obliterative transplant arteriopathy in renal allograft rejection. Mod Pathol 1994, 7:289–293
49. Skowronski EW, Mendoza A, Smith SC Jr, Jaski BE: Detection of cytomegalovirus in paraffin-embedded postmortem coronary artery specimens of heart transplant recipients by the polymerase chain reaction: implications of cytomegalovirus association with graft atherosclerosis. J Heart Lung Transplant 1993, 12: 717–723

50. Billingham ME: Histopathology of graft coronary disease. J Heart Lung Transplant 1992, 11:S38–44
51. Uretsky BF, Murali S, Reddy PS, Rabin B, Lee A, Griffith BP, Hardesty RL, Trento A, Bahnson HT: Development of coronary artery disease in cardiac transplant patients receiving immunosuppressive therapy with cyclosporin and prednisone. Circulation 1987, 76:827–834
52. Keogh A, Simons L, Spratt P, Esmore D, Chang V, Hickie J, Baron D: Hyperlipidemia after heart transplantation. J Heart Transplant 1988, 7:171–175
53. Olivari MT, Homans DC, Wilson RF, Kubo SH, Ring WS: Coronary artery disease in cardiac transplant patients receiving triple drug immunosuppressive therapy. Circulation 1989, 80(Suppl III):111–115
54. Costanzo-Nordin MR: Cardiac allograft vasculopathy: relationship with acute cellular rejection and histocompatibility. J Heart Lung Transplant 1992, 11:S90–103
55. Osborn L, Hession C, Tizard R, Vassallo C, Luhowskyj S, Chi-Rosso G, Lobb R: Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. Cell 1989, 59:1203–1211
56. Elices MJ, Osborn L, Takada Y, Crouse C, Luhowskyj S, Hemler ME, Lobb RR: VCAM-1 on activated endothelium interacts with the leucocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. Cell 1990, 60:577–584
57. Carlos T, Kovach N, Schwartz B, Rosa M, Newman B, Wayner E, Benjamin C, Osborn L, Lobb R, Harlan J: Human monocytes bind to two cytokine-induced adhesive ligands on cultured human endothelial cells: endothelial-leucocyte adhesion molecule-1 and vascular cell adhesion molecule-1. Blood 1991, 77:2266–2271
58. Span AHM, Mullers W, Miltenburg AMM, Bruggeman CA: Cytomegalovirus induced PMN adherence in relation to an ELAM-1 antigen present on infected endothelial cell monolayers. Immunology 1991, 72:355–360
59. Allen MD, McDonald TO, Carlos T, Himes V, Fishbein D, Aziz S, Gordon D: Endothelial adhesion molecules in heart transplantation. J Heart Lung Transplant 1992, 11:S8–13
60. Collins T: Endothelial nuclear factor-κB and the initiation of the atherosclerotic lesion. Lab Invest 1993, 68: 499–508
61. Bours V, Franzoso G, Brown K, Park S, Azarenko V, Tomita-Yamaguchi M, Kelly K, Siebenlist U: Lymphocyte activation and the family of NFκB transcription factor complexes. Curr Top Microbiol Immunol 1992, 182:411–420
62. Sambucetti LC, Cherrington JM, Wilkinson GWG, Mocarski ES: NF-κB activation of the cytomegalovirus enhancer is mediated by a viral transactivator and by T cell stimulation. EMBO J 1989, 8:4251–4258
63. Dunkel EC, Freitas DD, Schleer DI, Siegel ML, Zhu Q, Whitley RJ, Schaffer PA, Pavan-Langston D: A rabbit model for human cytomegalovirus-induced chorioretinal disease. J Infect Dis 1993, 168:336–344
64. Bruggeman CA, Meijer H, Dormans PHJ, Debie WMH, Grauls G, van Boven CPA: Isolation of a cytomegalovirus-like agent from wild rats. Arch Virol 1982, 73:231–241
65. Bruggeman CA, Meijer H, Bosman B, van Boven CPA: Biology of rat cytomegalovirus infection. Intervirology 1985, 24:1–9
66. Span AHM, Grauls G, Bosman F, van Boven CPA, Bruggeman CA: Cytomegalovirus infection induces vascular injury in the rat. Atherosclerosis 1992, 93: 41–52
67. Lemström KB, Bruning JH, Bruggeman CA, Lautenschlager IT, Häyry PJ: Cytomegalovirus infection enhances smooth muscle cell proliferation and intimal thickening of rat aortic allografts. J Clin Invest 1993, 92:549–558
68. Löning T, Milde K, Foss HD: *In situ* hybridization for the detection of cytomegalovirus (CMV) infection. Virchow Arch (Pathol Anat) 1986, 409:777–790
69. Faix RG: Cytomegalovirus antigenic heterogeneity can cause false-negative results in indirect hemagglutination and complement fixation antibody assays. J Clin Microbiol 1985, 22:768–771
70. Volpi A, Britt WJ: Serological heterogeneity of CMV isolates with a monoclonal antibody. J Infect Dis 1985, 152:648–649
71. Dolan J, Briggs JD, Clements GB: Antibodies to cytomegalovirus in renal allograft recipients: correlation with isolation of virus. J Clin Pathol 1989, 42:1070–1077
72. Drew WL: Diagnosis of cytomegalovirus infection. Rev Infect Dis 1988, 10:S468–476
73. Koskinen PK, Nieminen MS, Mattila SP, Häyry SP, Lautenschlager IT: The correlation between symptomatic CMV infection and CMV antigenemia in heart allograft recipients. Transplantation 1993, 55:547–551
74. Hajjar DP, Nicholson AC, Hajjar KA, Sando GN, Summers BD: Decreased messenger RNA translation in herpesvirus-infected arterial cells: effects on cholesterol ester hydrolase. Proc Natl Acad Sci USA 1989, 86:3366–3370
75. Kendall TJ, Wilson JE, Radio SJ, Kandolf R, Gulizia JM, Winters GL, Costanzo-Nordin MR, Malcom GT, Thieszen SL, Miller LW, McManus BM: Cytomegalovirus and other herpesviruses: do they have a role in the development of accelerated arterial disease in human heart allografts? J Heart Lung Transplant 1992, 11:S14–20