Cytomegalovirus Infection of Rats Increases the Neointimal Response to Vascular Injury Without Consistent Evidence of Direct Infection of the Vascular Wall

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**Background**—Previous studies suggest that infection may play a role in restenosis and atherogenesis; cytomegalovirus (CMV) is one of the implicated pathogens. To determine a potential causal role of CMV in these disease processes, we assessed whether CMV infection increases the neointimal response to injury of the rat carotid artery.

**Methods and Results**—Carotid injury was performed on 60 rats; immediately thereafter, 30 rats were infected with rat CMV, and the other 30 were mock-infected. Six weeks later, rats were euthanized, and the salivary glands, spleen, and carotid arteries were harvested. CMV infection was associated with significant exacerbation of the neointimal response to injury (neointimal to medial ratio 0.81±0.59 versus 0.31±0.38 in CMV-infected versus control rats; \(P<0.0001\)). This occurred despite absence of infectious virus from vascular tissues and detection of CMV DNA by polymerase chain reaction in the injured artery only at day 3 after infection. Persistent distant infection, associated with systemic cytokine response, was evidenced by isolation of infectious virus from homogenates of both salivary glands and spleen and by higher serum levels of interleukin (IL)-2 and IL-4 (but not interferon-\(\gamma\) and tumor necrosis factor-\(\alpha\)) in infected versus noninfected rats.

**Conclusions**—CMV infection of immunocompetent adult rats increases the neointimal response to vascular injury, suggesting that CMV may play a causal role in atherosclerosis/restenosis. Importantly, this CMV-induced response occurs even without the presence of virus in the vascular wall, suggesting that inflammatory and immune responses to infection of nonvascular tissues may contribute to the vascular response to injury. (*Circulation. 1999;100:1569-1575.)*

**Key Words:** viruses • balloon • restenosis • atherosclerosis • cytokines

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Previous studies have demonstrated an association between several infectious agents and both restenosis and atherogenesis.1–6 One of the pathogens for which such evidence has accrued is cytomegalovirus (CMV): the virus is present in restenosis specimens and in atherosclerotic vessels,1,7 previous CMV infection of patients undergoing coronary angioplasty is associated with the subsequent development of restenosis,1,3 and several seroepidemiological studies have shown an association between seropositivity to CMV and atherosclerosis.3,8,9 In addition, experimental studies demonstrating that infection of vascular wall cells induces changes that are associated with both atherosclerosis and restenosis10–12 provide mechanistic support for CMV-induced vascular disease. However, none of these studies provide direct evidence indicating that the virus has the capacity to contribute causally to these vascular diseases.

One of the processes involved in both restenosis and atherosclerosis is the neointimal response to vascular injury: the resulting neointima contributes importantly to lesion mass and therefore to narrowing of the coronary artery. Therefore, to ascertain whether CMV actually has the capacity to play a true causal role in restenosis, in the present investigation we determined whether CMV infection per se increases injury-induced neointimal formation in the standard rat carotid injury model.

**Methods**

**Virus**

Rat cytomegalovirus (RCMV), Priscott strain, was obtained from American Type Culture Collection (ATCC) and propagated in Rat-2 cells (rat embryonic fibroblast cell line, ATCC). Rat-2 cells were cultured with DMEM supplemented with 5% FBS. The rat-2 cell monolayers at 80% confluence were infected with RCMV at a multiplicity of infection of 0.1. When 100% of the cells exhibited cytopathic effects, 48 to 72 hours later, the medium (which contained virus) was collected and centrifuged to remove cell debris. The supernatant was collected and stored as virus stock at \(\sim 80^\circ\)C until
use. Viral infectivity titrations of the RCMV stock was performed on Rat-2 cells propagated in 96-well microtiter plates. Infectivity was recorded according to the induction of cytopathic effects by serial 10-fold dilutions of the sample and expressed as units of 50% tissue culture infective dose (TCID\(_{50}\)) assay. Stock virus infectivity was 10\(^6\) TCID\(_{50}/0.1\) mL.

Control Injectate
To control for as many variables as possible, the control animals were injected with the same medium as the infected animals, but with the virus removed. Thus, aliquots of the virus-containing supernatants described above were filtered through a 0.1-\(\mu\)m pore size filter device to remove virus. This filtered medium was tested for infectious virus by plating on a monolayer of Rat-2 fibroblasts. No cytopathic effects could be detected despite examination of the cells for 3 weeks after application of the filter medium.

Animals
All animals were studied under protocols approved by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute and in accordance with the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services publication No. NIH 86-23, revised 1985). Adult male Sprague-Dawley rats weighing 400 to 450 g (Zivic-Miller, Zelienople, Pa) were used for the experiments. All procedures were performed under general anesthesia and sterile technique. General anesthesia was administered with ketamine 150 mg/kg and xylazine 15 mg/kg IM and supplemental ketamine/xylazine IP as necessary.

Left Carotid Artery Balloon Injury and RCMV Inoculation
Standard left carotid balloon injury was performed on 60 rats as described by Clowes et al.\(^9\) Each rat was anesthetized with intramuscular injection of ketamine and xylazine. The distal left common carotid artery was exposed at the bifurcation of the internal and external carotid arteries through a midline incision in the neck. A 5-mm arteriotomy was made in the external carotid artery, and the catheter was introduced in a retrograde fashion to the arch of the aorta. The balloon was inflated with 1.5 mL of normal saline to generate slight resistance and passed 3 times through the common carotid. The external carotid was then tied off, and the wound was closed with 2–0 silk. Immediately after injury, 30 of the balloon-injured rats received an intraperitoneal injection of 1 mL RCMV (10\(^6\) TCID\(_{50}/mL), and 30 received a 1-mL injection of the filtered virus-free medium.

Neointimal/Medial Ratio Determination
The rats of both the infected and noninfected groups were euthanized 6 weeks after balloon injury. Preparation of the balloon-injured left carotid arteries was performed as previously described.\(^14\) Briefly, the rats were perfused with 10% formalin for ~5 minutes, after which the left carotid arteries were isolated and excised. Then the arteries were cut into 3 to 5 segments, embedded in paraffin, and Movat stained. The slides were inspected visually to select the segment with greatest luminal narrowing. The slides were coded so that the individual carrying out the measurements was unaware of treatment assignment. The areas of the media and the neointima were assessed for normality by the Shapiro-Wilk test, and nonparametric analyses were performed for statistical significance with the Mann-Whitney test. The statistical significance of serum cytokine changes was assessed by 2-tailed Student’s t test.

Virus Recovery
To determine whether infectious virus was present in the injured carotid artery, separate groups of rats underwent carotid injury and RCMV infection exactly as the primary group of rats had; 2 to 3 animals were euthanized at 3 days, 3 weeks, and ~6 weeks after infection. The salivary glands, spleen, and carotid arteries (both left and right) were harvested. The carotid artery was homogenized by standard homogenizer. The serial dilutions of the tissue homogenate were then used to inoculate a monolayer of permissive indicator cells (Rat-2 cells).

The presence of infectious virus was determined by whether or not cytopathic effects (due to replicating virus) were observed in the monolayer of permissive cells. The salivary glands and spleen of each animal were also analyzed in this way to determine whether these tissues, known to be the site of CMV persistence in mice,\(^13,16\) harbored infectious virus under the conditions used in the present investigation.

Polymerase Chain Reaction to Detect CMV DNA Sequences in Salivary Gland and Carotid Artery
For detection of viral DNA in these organs, DNA was isolated by standard procedures from frozen tissues. Polymerase chain reaction (PCR) was performed with specific primers for the RCMV exon 4 region.\(^17\) Primer set 1 was 5'-CTGTAATGGCATAAAGCMC' and 5'-CTCTCCTGCCATGTTAGGATGAAGA-3'; primer set 2 (nested) was 5'-CAAATGATACATGAGAATGT-3' and 5'-CACTTTATCAATCTGCATGCAGAATGT-3'. A DNA PCR kit (Perkin-Elmer Cetus) was used according to the manufacturer’s recommended procedures under the following conditions: denaturation, 94\(^\circ\)C for 1 minute; primer annealing, 65\(^\circ\)C for 1 minute; and extension, 72\(^\circ\)C for 1 minute in a thermocycler for 2 rounds (35 cycles each). Amplified products were transferred to a nylon membrane and detected by hybridization. To confirm the integrity of the DNA samples, PCR was performed on extracted DNA with commercially available primers specific for the rat β-actin gene (Clontech). The expected (429-bp) genomic fragment was detected in all samples (data not shown).

Assay for Serum levels of Cytokines
Serum samples were collected 6 weeks after carotid artery balloon injury before the rats were killed. All samples were divided into aliquots and stored at ~80\(^\circ\)C before study. Serum cytokine levels of interleukin (IL)-2, IL-4, IL-10, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ were determined by standard solid-phase sandwich ELISA kit (Biosource International, Inc). Assays were performed in duplicate in 96-well microplates according to the manufacturer’s protocol directions. Serum samples were diluted 1:1 with standard diluent buffer. The values were determined by use of an automated microplate reader and were fitted to a standard curve ranging from 23.4 to 1500 pg/mL (IL-2, IL-4), 39.0 to 2500 pg/mL (IL-10), 2.3 to 150 pg/mL (TNF-α), and 21.8 to 1400 pg/mL (IFN-γ).

Statistical Analysis
Data were analyzed with JMP release 3.2.1 (SAS Institute, Inc) and expressed as mean±SD when appropriate. The N/M data were assessed for normality by the Shapiro-Wilk W test, and nonparametric analyses were performed for statistical significance with the Mann-Whitney test.

Effects of CMV on the Vascular Response to Injury
Rats were euthanized 6 weeks after left carotid balloon injury. To quantify the increase in neointimal area, the N/M ratio was determined by digital planimetry as described.\(^14\) Figure 1 demonstrates the differences in neointimal development between an uninfected rat (A) and an infected rat (B). These examples were selected because the N/M ratios were repre-
Figure 1. A and B, Representative histological sections demonstrating neointimal response to injury in an uninfected rat (A) and an infected rat (B). Vessels selected for display had N/M ratios that were close to mean values for their respective groups. Sections were processed with Movat stain (magnification ×200). C and D, Effect of RCMV infection on vascular response to injury. Mean value of N/M ratio (C) and percent luminal stenosis (D) of CMV-infected and CMV-noninfected animals assessed 6 weeks after balloon injury are shown (n=30 animals per group; bars represent mean±SD).

Virus Recovery
In RCMV-infected animals, infectious virus could not be recovered from injured or noninjured carotid arteries at any time point. However, infectious virus was consistently recovered from both salivary glands and spleen at all time points.
Detection of CMV DNA
Although infectious virus was not present in the injured carotid artery, the virus might still be present and exert cellular effects through expression of its immediate early gene products in the absence of viral replication. To determine whether RCMV was present in the vessel wall, PCR amplification to detect RCMV DNA sequences in the carotid artery and in the salivary gland was performed.

RCMV DNA sequences were found in salivary glands of all RCMV-infected animals at all time points (in Figure 2, lanes 15 and 16 depict salivary glands sampled at 6 weeks as representative). However, although RCMV DNA sequences were present in injured carotid arteries of each animal studied 3 days after infection, RCMV DNA sequences were found in only 1 of 11 rats studied from 1 to 6 weeks after infection (Figure 2, lane 14). RCMV DNA sequences were not found in noninjured carotid arteries of either RCMV-infected or mock-infected groups at all time points.

Cytokine Response to CMV Infection
Six weeks after RCMV infection, serum IL-2 and IL-4 levels were significantly greater in the infected than in the mock-infected group (65.9 ± 7.9 versus 29.5 ± 4.3 pg/mL, P < 0.01, and 44.2 ± 7.9 versus 26.9 ± 2.8 pg/mL, P < 0.05, respectively). There were no statistically significant differences between the infected and the mock-infected groups in the levels of IFN-γ, IL-10, and TNF-α (Figure 3).

Discussion
Previously, seroepidemiological studies have demonstrated an association between CMV infection and both atherosclerosis and restenosis. In addition, considerable evidence has accrued providing mechanistic support for a role of this virus in vascular disease, including infection-induced cellular changes that could predispose to increased neointimal accumulation, procoagulant effects, and activation of NF-κB, a transcription factor that increases the expression of many genes, including those responsible for inflammatory and immune responses.

Such seroepidemiological associations and the demonstration of potentially proatherosclerotic mechanistic effects support a possible role of this virus in atherosclerosis and restenosis. So too do the findings of Span et al. who demonstrated that CMV infection of rats produced changes in the aorta similar to those seen early in the atherosclerotic process. These changes included minimal endothelial cell damage, an increased number of leukocytes adhering to the aortic intima, and accumulation of leukocytes and lipid in the subendothelium. However, no neointimal accumulation was reported in either the control or infected rats, even when infected rats were fed high-cholesterol diets.

Although compatible with a causal role in atherogenesis, none of the above studies prove that the virus has the capacity to contribute causally to an increase in lesion mass. The present investigation establishes, for the first time, that CMV has the genetic program to evoke, on infection, cellular responses that lead to an increase in the neointimal accumulation resulting from vascular injury, a response that is an essential component of both atherogenic and restenotic processes. The investigation therefore provides direct evidence of potential causality.

To determine whether CMV increases the neointimal response to injury by infecting the vessel wall and inducing local effects, we harvested multiple organs at various time points during the experiment and assayed for the presence of infectious virus and for the presence of viral DNA through PCR amplification. Infectious virus was present in the salivary glands and spleen, a finding consistent with the results observed by other investigators in murine and rat models of CMV infection. Most importantly, however, infectious virus was not detected in the injured carotid artery.

CMV can infect a tissue and express only its immediate early gene products (ie, an abortive infection) and thereby exert effects on the host cell in the absence of viral replication. We therefore tested for the presence of CMV DNA sequences by PCR amplification. Although we did detect such sequences in injured carotid arteries 3 days after infection, evidence of virus was only rarely found in the injured arteries after that time. It therefore appears that presence of the virus within the injured vessel is not necessary for CMV infection to exacerbate the healing response of the
vessel to injury. Because CMV DNA was detected in the vessel wall very early after infection, we cannot rule out the possibility that this early viral presence led to a brief abortive infection that contributed to the exaggerated neointimal response, even though virus was no longer present in the vessel wall after 3 days.

Although CMV was found in the injured vessel only briefly, replicating virus persisted in the salivary glands and spleen throughout the course of the experiment. It therefore appears likely that infection of nonvascular tissues plays an important contributory role in the increased neointimal response to injury. In this regard, Lemstrom and colleagues demonstrated in immunocompetent and immunosuppressed rats that RCMV infection increased smooth muscle cell proliferation and neointimal thickness of aortic allografts, even though there was no evidence of active viral infection in the graft tissue.

Given these findings, we propose that additional effects, other than those caused by local infection of the injured vessel, contribute to the CMV-induced increased neointimal response to injury observed in the present study. Such effects could be mediated by responses to viral infection of more permissive tissues (such as the salivary glands or spleen), leading to the local production of factors that are humorally transmitted to sites of vessel injury, where they then influence the vascular response to injury. Alternatively, the effect could be mediated by the immune response to viral infection through humorally transmitted cytokines and/or inflammatory cells.

That this may be the case is suggested by the studies we performed on circulating cytokine levels. Cytokine profiles in response to infection were obtained at the time of euthanization (6 weeks after infection and injury). Significantly higher circulating levels of IL-2 and IL-4 were found in the infected versus noninfected animals; there were no differences in circulating levels of IL-10, IFN-γ, and TNF-α. It is unclear at this time whether these particular differences induced by infection were in any way causally related to the increase in neointimal response to vessel injury. The changes do demonstrate, however, that in the rat model of CMV infection, changes in circulating cytokine levels are present in a model in which virus is essentially absent from the vessel wall. These findings are therefore compatible with the concept that systemic influences on the vessel wall may contribute to the vascular effects of virus.

It should be emphasized that we have no information at this time whether infection-induced cytokine differences change over time; in particular, whether the levels of IFN-γ or TNF-α are increased during the earlier phases of infection. However, elevated levels of IFN-γ and TNF-α are considered to be markers of a Th-1–type immune response, and IL-4 is considered to reflect a Th-2–type response. In this regard, it has been demonstrated that a Th-1–type response is essential to clear the host of intracellular pathogens such as CMV. The findings of increased IL-4 levels with normal IFN-γ levels

Figure 3. Cytokine response to RCMV infection. Serum samples were collected 6 weeks after carotid artery balloon injury before rats were euthanized. IL-2, IL-4, IL-10, TNF-α, and IFN-γ were determined by standard ELISA kit in duplicate in 96-well microplates. Six weeks after RCMV infection, serum IL-2 and IL-4 levels were significantly greater in infected than in mock-infected group. There were no statistically significant differences between infected and mock-infected groups in levels of IFN-γ, IL-10, and TNF-α.
therefore suggest that in our model, infection led, at least at 6 weeks, to a predominant Th-2-type response, a finding that may contribute to viral persistence (as evidenced by infectious virus being present in the spleen and salivary glands). Such an immune response, by contributing to the failure of the host to eliminate the virus, could thereby facilitate any vasculopathic effects of the virus.

Persoons et al,\(^9\)\(^\text{9}\) using the same rat carotid injury model, found that CMV infection did not alter the neointimal response to injury. One possible explanation for this difference is the differences in the timing of infection in relation to injury. In our investigation, rats were infected immediately after injury. We obtained similar results, with CMV infection significantly increasing the neointimal response to injury, in a preliminary study with infection 24 hours after injury (data not shown). Persoons et al, however, infected the rats 14 and 18 days after injury. Considerable neointima accumulates by 2 weeks after injury of the rat carotid artery, and it is likely that any influence the virus might have had on augmenting neointimal development would, at this late time, be minimized. An alternative explanation for the disparate results is that Persoons et al used a different strain of RCVM from the one we used. It is possible that vascular effects of RCVM infection are strain-specific. We also wish to point out that the differences we observed were measured at 6 weeks after injury, after any “catch-up” that might have occurred would have taken place. Thus, the CMV-induced increase in neointimal response to vascular injury was not a transient response.

In conclusion, the finding that CMV infection augments the vascular response to injury in an animal model provides evidence compatible with the concept that CMV does play a causal role in the development of atherosclerosis and of restenosis and that such an effect is not necessarily entirely mediated by direct infection of the vessel wall. On the basis of these results, we believe that this model not only provides a means for further expanding our understanding of the molecular mechanisms whereby CMV may contribute to atherogenic and restenosis processes but also may be used to assess the benefit of anti-CMV–based therapy to attenuate these disease processes.

References


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