Human Cytomegalovirus Seropositivity Is Associated With Impaired Vascular Function

Cairistine Grahame-Clarke, PhD, MRCP; Norman N. Chan, MRCP; Dawn Andrew, MSc; Geoff L. Ridgway, FRCP, FRCPath; D. John Betteridge, PhD, MD; Vincent Emery, PhD; Helen M. Colhoun, MFPHM; Patrick Vallance, MD, FRCP, FMedSci

Background—Herpesvirus infection is a possible risk factor for atherogenesis, and diabetics may be at particular risk. Endothelial dysfunction is an early marker for atherosclerosis, and the present study tests the hypotheses that (1) prior infection with cytomegalovirus (CMV) and herpes simplex virus (HSV) is associated with endothelial dysfunction and (2) this may be more marked in diabetics.

Methods and Results—Serum samples were tested for anti-IgG antibodies to CMV and HSV from 400 subjects (mean age for diabetics and nondiabetics, 37.8±4.3 and 37.9±3.7 [SD]). We also assessed Helicobacter pylori and Chlamydia pneumoniae serology. Coronary atheroma was quantified by means of electron beam computed tomography. Subjects (n=157) underwent venous occlusion plethysmography with acetylcholine, bradykinin, glyceryl trinitrate, norepinephrine, and l-N⁴-monomethyl-l-arginine. Individuals who were seropositive for CMV had reduced responses to bradykinin (P=0.005) and glyceryl trinitrate (P=0.006). The reduced response to bradykinin remained significant (P=0.045) after adjusting for the response to glyceryl trinitrate and was independent of conventional risk factors. Positive serology for the other organisms did not have an independent effect on reactivity. There was a weaker association between CMV and coronary artery calcification (P=0.09). Positive serology for each of the other pathogens did not affect reactivity, but there was a relation between total pathogen burden and impaired vascular reactivity. No significant differences were found between diabetics and nondiabetics.

Conclusions—This study shows that CMV-seropositive individuals have endothelial dysfunction and impaired responses to NO. This association was independent of conventional risk factors and may be associated with increased atherosclerosis burden. (Circulation. 2003;108:678-683.)

Key Words: atherosclerosis ■ risk factors ■ viruses ■ endothelium

There is increasing evidence that chronic infection is associated with atherosclerosis.¹ Although clinical data are not conclusive, specific organisms, including herpesviruses, enhance atherosclerosis in the apolipoprotein E (ApoE)–deficient mouse.²,³ Epidemiological studies have also suggested an interaction between human cytomegalovirus (CMV) infection and diabetes mellitus in promoting vascular disease.⁴ Endothelial function is a mechanism whereby infections might influence atherosclerosis⁵ and is an early marker of the vascular effects of classic risk factors.⁶ Therefore, endothelial dysfunction may be a useful surrogate to determine whether or not there is a relation between individual infectious agents and the type of vascular dysfunction that may predispose to atheroma.

The present study tested the hypothesis that serology for specific herpesviruses, CMV and herpes simplex virus (HSV), is associated with endothelial dysfunction and that this effect is more marked in diabetics. Although the focus was on herpesviruses, we also determined whether positive serology for Helicobacter pylori or Chlamydia pneumoniae or multiple positive serologies affected the responses seen.

Methods

Subjects were recruited from a cohort of 400 (199 diabetic and 201 nondiabetic subjects; mean age, 37.8±4.3 and 37.9±3.7 [SD] years, respectively); see Colhoun et al⁷ for further details. All subjects were between 30 and 53 years of age, and 50% were female. Of this cohort, 157 (39%) took part in the in vivo endothelial function testing; 44 were not contactable, 15 were ineligible, and 184 refused. There were 88 (56.1%) type 1 diabetic (54 men and 34 women) and 69 (43.9%) nondiabetic subjects (34 men and 35 women). The study cohort did not differ in cardiovascular risk factor profile, diabetes duration, control, or complications from the overall sample; none was receiving nitrate therapy, and only one male diabetic was taking...
Serology
Serum IgG antibodies to CMV, HSV, C pneumoniae, and H pylori were determined through the use of standard ELISA kits: BioElisa CMV-IgG (Biokit SA), ETI-HSVK-G 1/2 kit (DiaSorin Ltd), C pneumoniae (Medac), and H pylori (Meridian Bioscence Inc). All kits use antigen-coated microtiter plates and were performed according to the respective manufacturer’s instructions. The results are quantitative for C pneumoniae, with a cutoff titer of 100 for IgG. Titer ≥400 was considered seropositive. For CMV, HSV, and H pylori, results are qualitative (positive or negative for antibody). In the case of the CMV and HSV assays, the negative cutoff was assigned by averaging the OD450 values for the 2 cutoff calibrators included in the assay. Positive/negative results were assigned when the OD450 was 10% higher or lower than the cutoff in the HSV assay. Samples falling within this range were assigned as equivocal test results. Equivocal tests were retested in duplicate, and those that were again equivocal were regarded as negative (n = 7, for herpesviruses). The HSV test detected HSV1 and HSV2, but previous studies suggest that the vast majority of the positive results would have been due to HSV1 infection.

In Vivo Endothelial Function Studies
Forearm blood flow in response to endothelium-dependent vasodilators ACh (ACh) and bradykinin (BK) and the endothelium-independent vasodilator glyceryl trinitrate (GTN) were measured by venous occlusion plethysmography. Basal NO-dependent vascular tone was assessed with the use of L-N^6-monomethyl-L-arginine (L-NMMA), a NO-synthase inhibitor, with the response to norepinephrine assessed as a control. Forearm blood flow was measured in response to intrabrachial infusion of ACh (Sigma; doses of 25, 50, and 100 mmol/L; each dose for 3 minutes); BK (Clinalfa; doses of 10, 30, and 100 pmol/min; 3 minutes each); GTN (David Bull Laboratories; of 4, 8, and 16 mmol/min; 5 minutes each); norepinephrine (Levophed; Sanofi Winthrop Ltd; 60, 120, and 240 pmol/min; 5 minutes each); and L-NMMA (Clinalfa; 1, 2, and 4 μmol/min; 5 minutes each). Each infusion was separated by a 10-minute saline washout. The order of vasodilators was randomized.

Electron Beam Computed Tomography
All subjects had coronary artery calcification (CAC) score assessed by electron beam computed tomography ~12 months before the in vivo vascular function study, as described.

Cholesterol Measurements
Total cholesterol, HDL cholesterol, and triglyceride measurements on serum samples were performed with the use of standard enzymatic colorimetric methods. LDL cholesterol was calculated by means of the Friedewald formula.

Statistical Analysis
Data were analyzed through the use of STATA 6.0, and a multivariate analysis was performed. This allowed identification of the impact of individual risk factors and for each pathogen separately. Comparison of background characteristics between the sexes and between diabetic and control groups was made by using multiple linear regression. The response to drug was calculated as described previously: (flow in drug arm/flow in control arm during drug infusion) ÷ (flow in drug arm/flow in control arm at baseline).

This was summarized across the 3 doses as the area under the dose-response curve. The inverse of this ratio was used for constrictor. Differences in the area under the curve were tested for statistical significance through the use of multiple linear regression to adjust for age, sex, diabetes, and then other risk factors. Social class was defined through the use of the Registrar General’s Classification of Occupation as being manual (social classes III manual, IV and V) and nonmanual (I, II, and III nonmanual).

Results
Thirty-seven percent of those studied (142 of 381) were CMV-seropositive. These individuals had higher body mass index (1 kg/m^2, P = 0.03) and systolic blood pressure (3 mm Hg, P = 0.03) but lower LDL cholesterol (0.2 mmol/L, P = 0.03) when adjusted for age, sex, and diabetes (Table 1). There was no difference in C-reactive protein (CRP) values, hemoglobin A1C levels (7%), or smoking between seropositive and seronegative subjects.

Fifty-eight percent of those studied (220 of 379) were HSV-positive. These individuals had higher body mass index (1 kg/m^2, P = 0.03) and systolic blood pressure (4 mm Hg higher, P = 0.004), adjusted for age, sex, and diabetes. Unlike for CMV, those who were seropositive for HSV had significantly higher CRP values (P < 0.001), although this was no longer significant when the sexes were considered separately (Table 1).

For those who took part in the endothelial function studies (n = 157), there were no significant differences in baseline characteristics (including CRP values) between seronegative and seropositive subjects, except in the case of systolic blood pressure for women in the CMV group and men in the HSV group (Table 2).

There was no difference in the seroprevalence for either virus between diabetic and nondiabetic subjects (CMV: odds...
Table 2. Characteristics of Those Undergoing Endothelial Function Studies

<table>
<thead>
<tr>
<th></th>
<th>Men (n=84) (DM, n=51)</th>
<th>Women (n=66) (DM, n=33)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>CMV BMI, kg/m²</td>
<td>24 (2)</td>
<td>25 (3)</td>
</tr>
<tr>
<td>LDL-c, mmol/L</td>
<td>3.2 (0.9)</td>
<td>3.3 (1.1)</td>
</tr>
<tr>
<td>HDL-c, mmol/L</td>
<td>1.7 (0.3)</td>
<td>1.6 (0.4)</td>
</tr>
<tr>
<td>TG, mmol/L</td>
<td>1.2 (0.7)</td>
<td>1.2 (0.7)</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>128 (11)</td>
<td>128 (12)</td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>0.7 (0.8)</td>
<td>0.8 (1.4)</td>
</tr>
<tr>
<td>% Now smoking</td>
<td>57 (10)</td>
<td>51 (7)</td>
</tr>
</tbody>
</table>

Table 3. Seroprevalence of CMV and HSV in Diabetic Versus Nondiabetic Subjects and in Men Versus Women

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NDM (n=88)</td>
<td>DM (n=99)*</td>
</tr>
<tr>
<td>CMV seropositive</td>
<td>% (±SEM)</td>
<td>% (±SEM)</td>
</tr>
<tr>
<td>HSV seropositive</td>
<td>30 (5)</td>
<td>29 (5)</td>
</tr>
</tbody>
</table>

Table 4. Area Under the Curve for Drug Response by Seropositivity Status

<table>
<thead>
<tr>
<th></th>
<th>Seropositive</th>
<th>Seronegative</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMV ACh</td>
<td>3.32</td>
<td>3.16</td>
</tr>
<tr>
<td>BK</td>
<td>4.21</td>
<td>4.99*</td>
</tr>
<tr>
<td>GTN</td>
<td>2.71</td>
<td>2.96*</td>
</tr>
<tr>
<td>NE</td>
<td>2.10</td>
<td>2.12</td>
</tr>
<tr>
<td>L-NMMA</td>
<td>2.05</td>
<td>2.01</td>
</tr>
<tr>
<td>HSV ACh</td>
<td>3.23</td>
<td>3.32</td>
</tr>
<tr>
<td>BK</td>
<td>4.62</td>
<td>4.82</td>
</tr>
<tr>
<td>GTN</td>
<td>2.84</td>
<td>2.89</td>
</tr>
<tr>
<td>NE</td>
<td>2.20</td>
<td>1.97</td>
</tr>
<tr>
<td>L-NMMA</td>
<td>2.07</td>
<td>1.96</td>
</tr>
</tbody>
</table>

*P<0.01.

Coronary Artery Calcification and CMV/HSV

CMV-positive individuals had a 1.5-fold odds ratio of having any CAC compared with the seronegative group (odds ratio = 1.5, 95% CI, 0.9 to 2.3, P = 0.09). A similar nonsignificant relation was seen between HSV-positive serology and CAC (odds ratio = 1.5, 95% CI, 0.8 to 2.7, P = 0.19) and nondiabetic groups (CMV: odds ratio = 1.3, 95% CI, 0.7 to 2.6, P = 0.4; HSV: odds ratio = 1.4, 95% CI, 0.7 to 2.8, P = 0.29).

Response to Bradykinin

The vasodilator response to BK was lower in those who were CMV-positive (P = 0.005, adjusting for age, sex, and diabetes; Table 4 and Figure 1), despite there being no difference in the odds ratio of being seropositive for CMV (odds ratio = 1.5, 95% CI, 0.5 to 1.3, P = 0.35; HSV: odds ratio = 0.67, 95% CI, 0.4 to 1.0, P = 0.06). Women had a 2-fold odds ratio of being seropositive for CMV (odds ratio = 1.97, 95% CI, 1.3 to 3.0, P = 0.002) or HSV (odds ratio = 2.06, 95% CI, 1.3 to 3.1, P = 0.0008) (Table 3). There were no significant sex differences for any of the results presented below.

CMV, HSV, and Social Class

There is a relation between atherosclerosis and social class that may confound any relation found here. The majority of the group were from the nonmanual classes (82%). CMV seropositivity was similar in nonmanual (37%) and manual (40%) workers (odds ratio = 0.8, 95% CI, 0.5 to 1.3, P = 0.35; HSV: odds ratio = 0.67, 95% CI, 0.4 to 1.0, P = 0.06). Women had a 2-fold odds ratio of being seropositive for CMV (odds ratio = 1.97, 95% CI, 1.3 to 3.0, P = 0.002) or HSV (odds ratio = 2.06, 95% CI, 1.3 to 3.1, P = 0.0008) (Table 3). There were no significant sex differences for any of the results presented below.

Figure 1. Vascular response to BK stratified by CMV serostatus (values are mean ± SEM).
TABLE 5. Area Under the Curve for Drug Response by Seropositivity Status in Nondiabetic and Diabetic Subjects

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>NDM</th>
<th>DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMV Positive</td>
<td>4.23</td>
<td>4.23</td>
</tr>
<tr>
<td>CMV Negative</td>
<td>5.56</td>
<td>4.59</td>
</tr>
<tr>
<td>HSV Positive</td>
<td>4.94</td>
<td>4.40</td>
</tr>
<tr>
<td>HSV Negative</td>
<td>5.27</td>
<td>4.49</td>
</tr>
<tr>
<td>BK</td>
<td>NDM</td>
<td>3.02</td>
</tr>
<tr>
<td>HSV</td>
<td>NDM</td>
<td>3.36</td>
</tr>
<tr>
<td>GTN</td>
<td>NDM</td>
<td>3.13</td>
</tr>
<tr>
<td>DM</td>
<td>3.33</td>
<td>2.57</td>
</tr>
</tbody>
</table>

NDM indicates nondiabetic subjects; DM, diabetic subjects.

*P<0.05.
†P<0.01.

in basal flow (P=0.7). This relation was independent of systolic blood pressure, lipids, smoking, social class, or serum CRP levels (P=0.005). Excluding the individual who was receiving an HMG-coenzyme A inhibitor made no difference in the result. Although the difference in vascular responses with CMV seemed greater in nondiabetics, there was no significant interaction between CMV seropositivity and diabetes (P=0.3 for the diabetes-CMV interaction, Table 5). Being HSV-seropositive made no difference in the response to BK (P=0.3, Table 4).

Responses to GTN and Other Agents

The response to GTN was lower in those who were CMV-seropositive (P=0.006, adjusted for age, sex, and diabetes; Table 4 and Figure 2). This relation remained significant after adjusting for systolic blood pressure, lipids, and smoking (P=0.001) and for social class (P=0.003). There was no significant interaction between CMV seropositivity and diabetes (P=0.5 for diabetes-CMV interaction; Table 5). There was no difference between the GTN responses with HSV status (P=0.6).

We examined the association of CMV seropositivity while adjusting for the GTN response. The difference in the BK response in CMV-positive compared with CMV-negative subjects was decreased by a third but remained significant (P=0.045).

There was no significant difference between the response to ACh, norepinephrine, or L-NMMA in those who were seropositive for either virus compared with those who were seronegative (Table 4).

Effects of Multiple Positive Serology—“Pathogen Burden”

The number of subjects seropositive for 1, 2, 3, or 4 pathogens was 142, 100, 37, and 6, respectively (88 were negative for all 4). Positive serology for C. pneumoniae was not associated with altered vascular responses or CAC. Although positive serology for H. pylori was associated with reduced GTN (P=0.03) and BK (P=0.034) responses, this was not independent of other risk factors. Responses to BK and GTN were slightly lower in individuals who were seropositive for both HSV and CMV compared with individuals positive only for CMV (P=0.06 and 0.01, respectively). Similarly, when individuals were stratified according to positive serology for 1, 2, 3, or 4 organisms, there was a significant relation between “pathogen burden” and reduced responses to both BK (P=0.01, test for trend) and GTN (P=0.04, test for trend). These effects remained significant on adjustment for other risk factors, but the only single organism for which the effect remained significant, independent of other organisms and other risk factors was CMV (P=0.01). The effect of “pathogen burden” was independent of CRP levels. Interestingly, “pathogen burden” was also related to higher CAC so that individuals who were positive for all 4 organisms had a 7-fold odds of CAC (P=0.034), although this effect was not independent of other risk factors (P=0.2 on adjustment).

Discussion

This study shows that relatively young asymptomatic individuals seropositive for CMV have abnormal vascular reactivity consistent with endothelial dysfunction. This was independent of systemic inflammation as assessed by CRP, and there was no specific interaction with diabetes. The results also suggest that previous CMV infection might be associated with increased atherosclerotic burden, as assessed by coronary artery calcification. In contrast, there was no statistically significant independent relation between HSV, H. pylori, or C. pneumoniae seropositivity and vascular dysfunction. These findings extend work in the ApoE-deficient mouse, in which murine gammaherpesvirus-68 and murine cytomegalovirus1 enhance atheroma but HSV-1 does not10 and is consistent with the clinical finding that CMV but not HSV-1 seropositivity is associated with advanced atherosclerosis.11

Reduced responses to endothelium-dependent agonists are often taken as indicative of endothelial dysfunction and increased cardiovascular risk.8 In the present study, although responses to BK were reduced in seropositive individuals, those to ACh were unaffected. Because both BK and ACh cause vasodilation in part by stimulating endothelial NO
synthesis, this differential effect of CMV on BK/ACh responses may seem surprising. However, differential impairment has been found in previous studies in the presence of classic risk factors, and a reduced BK response may be a particularly good marker of inflammation-induced vascular dysfunction. BK responses are also diminished in certain groups at risk of cardiovascular disease in which ACh responses are maintained, and BK responses but not ACh responses are diminished in the coronary vessels of the ApoE-knockout mouse. The mechanisms underlying such agonist-specific defects are unclear, but neither BK nor ACh work exclusively through NO in humans or mouse, and inflammation/CMV infection could be affecting one effector mechanism more than another. Alternatively, compensatory mechanisms may normalize the response to ACh but not to BK. Indeed, in endothelial NOS-knockout mice, responses to ACh and BK are preserved in resistance vessels and may be differentially mediated by EDHF or cytochrome P450 metabolites. Whatever the mechanism, the present study shows a marked association between CMV seropositivity and BK-induced vasodilation. The finding that responses to L-NMMA are unchanged suggests that basal NO-mediated dilation is unaffected by CMV infection. Again, this type of divergence between effects on agonist-induced and basal NO-mediated dilation has been observed in the presence of classic cardiovascular risk factors.

The GTN response was also diminished in those seropositive for CMV. This might be due to reduced vascular smooth muscle responsiveness to NO, increased destruction of NO by superoxide, a specific effect on GTN metabolism, or by some nonspecific effect on vasodilation. Increasingly, studies of vascular responses in the presence of cardiovascular risk factors have shown some impairment in GTN responses, suggesting that not all the defects described as “endothelial” are confined to this cell layer. Overproduction of superoxide has been implicated in vascular dysfunction of this type and would provide an attractive mechanism to link certain types of infection to increased atherogenesis.

Some clinical studies have shown an association between herpesvirus serology and atherosclerosis, although larger studies have refuted this. However, animal studies have shown that certain herpesviruses enhance atherosclerosis and may cause endothelial dysfunction. It is possible that the large epidemiological studies are negative because they rely on a single serology measurement taken late in the disease and cannot take into account when infection occurred or how often reactivation has taken place. Alternatively, it may be that the clinical end points are determined by other factors that influence the atherosclerotic plaque. Whatever the explanation, it cannot detract from this association between CMV seropositivity and vascular dysfunction. The specificity of this finding is confirmed by the lack of such an association with HSV seropositivity. Indeed, many individuals were seropositive for both HSV and CMV, but in all analyses, only CMV seropositivity was an independent predictor of vascular dysfunction. We also found that “pathogen burden” was associated with impaired vascular responses, but again, an independent relation was found only for CMV. Therefore, although the present study concurs with the overall findings of Prasad et al., in which total pathogen burden was associated with endothelial dysfunction, the results and those from animal studies suggest that pathogens differ in their ability to produce detrimental vascular effects. It is also interesting to note that although greater “pathogen burden” was associated with more CAC, this relation was not independent of other risk factors. It will clearly be important to disentangle confounding from causal association in some of the reported relations between positive serology and vascular dysfunction. It is worth noting that although we have reported previously that CRP levels are related to CAC in this population, the effects of CMV seropositivity and “pathogen burden” were independent of CRP levels. This is consistent with our observations in healthy volunteers in whom acute systemic inflammation produced by vaccination impairs BK responses but does not cause a rise in CRP and suggests that understanding the type of local or systemic inflammatory response evoked would be of interest.

**Limitations of the Study**

A high level of false-negative or false-positives results for serology might affect the power of the study. However, it would only influence the association if the results were nonrandomly assigned to those with or without endothelial dysfunction. A false-negative result is unlikely, given the sensitivity of the assay. There may have been some individuals acutely infected with virus who would express an IgM rather than an IgG response, but, given the incidence of infection rates, such numbers are likely to be small or nonexistent. The chance of false-positive results is heightened with repeated freeze/thawing in the specimens. Specimens had only been previously thawed twice before at most. Furthermore, the number of equivocal tests was very low (7 in total), suggesting that neither a false-positive nor a false-negative test result was common. The young age range of the subjects could have accounted for the relatively low seroprevalence for herpesviruses and the low calcium scores. However, this would have weakened rather than strengthened any associations.

The lack of an interaction with diabetes may seem counterintuitive, but we have found in a murine model that γ-herpesvirus infection enhances atheroma induced by a high cholesterol diet but does not enhance atheroma induced by a diabetogenic diet (unpublished data). This observation merits further study, as does the finding that overall, diabetics had a lower “pathogen burden” as assessed by serology. A final limitation is that the relation between coronary calcification and CMV seropositivity did not reach statistical significance, and the relation between “pathogen burden” and CAC was not independent of other risk factors. Although the CAC data were collected 1 year before the vascular study, the rank order of CAC and absolute scores should not change significantly over this period (reference 23 and personal communication with author).

**Gender Difference in Antibody Levels**

Women had a higher seroprevalence of antibodies to both HSV and CMV than men and therefore may have been expected to demonstrate more atheroma. However, the im-
munological defense against herpesviruses involves both antibody- and cell-mediated immune responses, and the presence of IgG antibodies gives no indication of how quickly the virus has been cleared or how much inflammation it provoked. Sex differences in the immunological response to CMV infection have been found, and it is possible that this, or interaction with other classic risk factors, determines the overall effect of infection on disease.

Summary
We detected a relation between CMV and not HSV, H pylori, or C pneumoniae infection and impaired vascular responses to BK and GTN in young asymptomatic subjects. Similarly, in the ApoE-knockout mouse, CMV but not HSV-1 accelerated atherogenesis. Together these findings implicate infection with CMV in the pathogenesis of long-term vascular dysfunction of a type that might predispose to atheroma formation and increased cardiovascular risk. The results suggest that not all infectious agents individually alter vascular function but that total “pathogen burden” may exert complex effects through interactions or association with other risk factors.

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References
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