Host Response to Cytomegalovirus Infection as a Determinant of Susceptibility to Coronary Artery Disease
Sex-Based Differences in Inflammation and Type of Immune Response

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Background—Positive and negative associations between cytomegalovirus (CMV) infection and coronary artery disease (CAD) have been reported. We postulated that the susceptibility to CMV-induced CAD might relate to patterns of inflammatory and immune responses to CMV infection and that sex might have an effect on these responses.

Methods and Results—In 151 men and 87 women being evaluated for CAD, blood samples were tested for humoral (Ab+) and cellular (Tc+) responses to CMV and for C-reactive protein (CRP). In men, an elevated CRP level was a significant determinant of CAD even after adjustment for CAD risk factors (OR, 3.1; 95% CI, 1.21 to 7.97). CMV seropositivity was associated with elevated CRP levels on multivariate analysis (P=0.006). In contrast, in women, CMV seropositivity was independently predictive of CAD (OR, 41.8; 95% CI, 4.12 to 423.74). CRP level in women with CAD was >25% higher than those without CAD, but the difference did not reach statistical significance. Importantly, compared with CMV Ab−/Tc− women, CAD prevalence was higher in Ab+/Tc− and Ab+/Tc+ (13% versus 68% and 64%, both P<0.005) but not in Ab−/Tc+ women (25%). There were no differences in age, smoking, diabetes, hypertension, and hypercholesterolemia among women with different types of immune responses to CMV infection.

Conclusions—The mechanisms by which CMV predisposes to CAD in men and women may be different. In men, CMV appears to contribute to CAD risk, insofar as it predisposes to inflammation. In women, other mechanisms, possibly related to the type of immune response generated by the host, appear to be responsible for the proatherogenic effects of CMV. (Circulation. 2000;102:2491-2496.)

Key Words: immunology ■ infection ■ inflammation ■ sex ■ risk factors

Various lines of mechanistic evidence imply a possible role of infection in atherosclerosis. Consonant with these investigations are epidemiological studies demonstrating associations between coronary artery disease (CAD) and several pathogens, including cytomegalovirus (CMV), Chlamydia pneumoniae, Helicobacter pylori, and herpes simplex virus.1–8 Other studies, however, have yielded negative results.9–12

The link between infection and atherosclerosis has traditionally been assessed by identifying infected individuals through the presence or absence of antibodies directed at the pathogen. We thought the issue might be more complex, because previous studies have shown13–15 that the type of immune response (humoral or cellular) importantly determines whether a given infection leads to pathogen-induced disease. The humoral immune response functions mainly to prevent infection by extracellular agents, whereas the cell-mediated immune response is more critical for elimination and control of intracellular pathogens. The relative intensities of the humoral and cellular immune responses generated by an infectious agent depend on multiple factors, including the specific pathogen and the genetic determinants of the individual host.

Data compatible with the importance of the cellular immune response to control intracellular pathogens come from studies of infectious diseases such as AIDS,13,14 chronic hepatitis B,16 and leishmaniasis,17–19 which suggest that a humoral response conveys susceptibility to disease, whereas a cellular response conveys resistance. In addition, our recent studies demonstrated that considerable host variability exists in the inflammatory responses to CMV infection,20 as reflected by elevated C-reactive protein (CRP) levels.

Because of this heterogeneity and the marked influence of sex on susceptibility to CAD and because of the presumed roles of inflammatory and immune responses in CAD, we postulated that overall susceptibility or resistance to CMV-
induced CAD will be determined by sex-related heterogeneity of the inflammatory and immune responses to CMV infection. The purpose of the present investigation, therefore, was to examine the hypotheses that (1) the presence or absence of an inflammatory response to CMV is sex-related and (2) the type of immune response mounted by the host to CMV contributes to susceptibility or resistance to CMV-associated CAD.

Methods

Patient Characteristics
This study was approved by the NHLBI Institutional Research Board. Of the 238 individuals, 151 (63%) were men; 169 (71%) were white. Ages ranged from 30 to 81 years (mean, 57.2 years; median, 57.0 years). Each individual was admitted for evaluation of chest pain or abnormal noninvasive tests and underwent diagnostic coronary angiography. These individuals also formed the basis of another study designed to determine whether the influence of CMV on CAD is modulated by induction of an inflammatory state. For primary analysis, CAD was defined as any angiographic evidence of atherosclerosis, including presence of plaque in any segment of the epicardial coronary arteries. A patient was defined as being free of CAD only if all coronary arteries were angiographically smooth. Approximately 95% of individuals had blood drawn at the time of catheterization, but none had blood drawn >3 years after the diagnostic study. No individual without CAD was admitted to the study unless blood for immunologic testing was drawn within 3 years of coronary angiography. No patient admitted to the study had unstable symptoms, and none had a myocardial infarction within the previous 3 months.

Determination of Risk Factors for CAD
Risk factors for CAD analyzed included age, sex, cigarette smoking, diabetes, hypercholesterolemia, hypertension, and seropositive CMV status. A patient who had stopped smoking >20 years ago and who was <30 years of age when he or she stopped smoking was considered not to have smoking as a risk factor. A patient was considered to have diabetes if he or she was taking insulin or oral hypoglycemic agents or had previously received such treatment and was currently using dietary modification to control the condition. A patient was considered to have hypercholesterolemia if he or she had a serum cholesterol value >240 mg/dL (6.2 mmol/L) or was receiving cholesterol-lowering treatment. A patient was considered to have hypertension if he or she had received the diagnosis or was being treated with antihypertensive medications and/or dietary modification.

Immune Response to CMV Antigens
Blood samples from each individual were tested for (1) anti-CMV IgG antibodies and (2) the proliferation of T lymphocytes from peripheral blood mononuclear cells (PBMCs) in response to CMV antigens.

Antibody Status to CMV
Serum collected for detection of antibodies was frozen at −80°C. CMV IgG antibodies were determined by ELISA (Cytomeg ella II, BioWhittaker). Antibody results were calculated from standard curves provided by the manufacturer. A positive result was determined prospectively: an ELISA value <0.25 U was considered negative, and a value of ≥0.25 U was considered positive, indicating prior exposure to CMV. Samples were tested in triplicate and in 2 separate experiments.

Isolation of PBMCs
PBMCs were separated from whole blood on lymphocyte separation medium (Organon Teknika Corp) by centrifugation at 1800 rpm for 25 minutes at room temperature. Separated cells were collected and washed twice in PBS (Gibco Laboratories). The number of viable cells was determined by trypan blue exclusion. PBMCs were cryopreserved in aliquots in liquid nitrogen until used.

CMV Antigen Preparations
Human CMV, Towne strain, was obtained from the American Type Culture Collection and grown in human fibroblasts, HEL299 (ATCC CCL-137) for preparation of the viral antigens. Growth media consisted of MEM (Gibco) supplemented with 2% FBS and antibiotics. Virus titer was measured on HEL299 cells. Protocols for CMV antigen preparations have been published. CMV antigens were prepared with (1) heat-inactivated CMV (1 hour at 56°C) obtained from supernatants of CMV-infected fibroblasts; final viral concentration was 10⁵ pfu before inactivation; (2) cell lysates of CMV-infected fibroblasts by repeated freezing and thawing; and (3) 0.08% glutaraldehyde-fixed CMV-infected fibroblast cells. Both cell lysates and fixed cells were prepared from 2×10⁶ cells/mL by infecting a 90% confluent monolayer of human fibroblasts with CMV at a multiplicity of infection of 10. Cells were collected by centrifugation at 50% cytopathic effect. Stocks were divided into aliquots and stored at −70°C. CMV antigen controls were obtained from noninfected fibroblasts (mock-infected cells), prepared exactly as described for CMV-infected cells.

T-Lymphocyte Proliferation
T-lymphocyte proliferative responses were performed in 96-well flat-bottom plates (Costar). PBMCs (100 μL; 3×10⁶/mL) were added to each well. PBMCs were cultured at 37°C with 5% CO₂ in RPMI 1640 (Gibco) containing 5% human AB serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and HEPES buffer, with or without antigen stimulation. Six days after culture, or 3 days for phytohemagglutinin stimulation, each well was pulsed with 1 μCi of [3H]thymidine and harvested 18 hours later, and thymidine incorporation was determined. Samples were assayed in triplicate and expressed as mean cpm. Data are presented as stimulation index (cpm of cultures in the presence of CMV antigens divided by cpm of cultures in the absence of antigens). If a sample responded to two thirds CMV antigen preparations (heat-inactivated supernatants of CMV-infected fibroblasts, CMV-infected cell lysates, or fixed CMV-infected fibroblasts) by a stimulation index >4, the response was considered positive. Positive controls included (1) 3 days of stimulation with phytohemagglutinin (1:200; Gibco); (2) influenza A/Bangkok RX73 (flu; grown in embryonated eggs and used as infectious allantoic fluid at an infectivity of 2×10⁶ tissue culture infectious dose₅₀/well at a final dilution of 1:1000); (3) Candida antigen (Greer Laboratories, Inc, final dilution of 20 mg/mL); and (4) a pool of irradiated (5000 rad) PBMCs from 3 unrelated healthy donors (2×10⁶/mL). Negative controls included (1) supernatants, cell lysates, and fixed cells from mock-infected fibroblasts prepared exactly as described for CMV-infected cells and (2) RPMI media control.

C-Reactive Protein
Serum CRP was measured by fluorescence polarization immunoasay technology (TDxFLEx analyzer, Abbott Laboratories). CRP levels of 95% and 98% of healthy individuals (n=202) were ≤0.5 mg/dL and ≤1.0 mg/dL, respectively. The between-run coefficients of variation (CVs) of this assay (n=31) were 4.3% and 2.2% at mean levels of 1.10 mg/dL and 2.94 mg/dL, respectively.

Statistical Analysis
Tests were 2-sided. Categorical data were analyzed by Fisher’s exact test. The dichotomous variable indicating presence or absence of CAD was modeled as a function of other factors or variables by multiple logistic regression. The odds ratio was used as a measure of risk of CAD in patients with a given risk factor compared with those without that factor. Covariates considered were age, sex, cigarette smoking, diabetes, hypercholesterolemia, hypertension, and seropositive CMV status. All covariates were individually examined as predictors of CRP and CAD by simple correlation analyses. They were further analyzed as a group for their predictive value for CAD by multiple logistic regression. Multiple linear regression was used.
to analyze their predictive value for CRP. These analyses were performed with SAS procedures (SAS software system for PC Windows).24

**Results**

Of the 238 subjects, 158 (66%) had CAD ranging from presence of plaque to significant stenoses. As previously found,20 factors significantly associated with CAD were age, male sex, smoking, diabetes, hypercholesterolemia, elevated CRP (>0.5 mg/dL), and CMV seropositivity in univariate analysis. On multivariate analysis, after adjustment for these factors, age (OR, 2.3; P < 0.0001), male sex (OR, 6.0; P = 0.0005), and hypercholesterolemia (OR, 3.5; P = 0.0007) were retained as significant risk factors, whereas diabetes was of borderline significance (OR, 3.0; P = 0.0068).20

**Sex Differences in Associations Among CMV Infection, CRP Levels, and CAD**

**Men**

Of 151 men, 113 (75%) had CAD; mean age was 57.2 years (median age, 57.0 years). Anti-CMV IgG antibodies were detected in 62% of CAD patients, compared with 61% of those without CAD (Figure 1). Mean CRP value was higher in men with CAD than in those without (0.86±0.05 versus 0.59±0.07 mg/dL, P = 0.01; Figure 2). Of men with CAD, 76% had CRP levels >0.5 mg/dL, versus 50% of those without CAD (P = 0.004). When adjusted for traditional CAD risk factors (age, smoking, diabetes, hypercholesterolemia, and hypertension) and CMV seropositivity by multivariate analysis, elevated CRP level was a significant independent predictor of CAD (odds ratio, 3.1; 95% CI, 1.21 to 7.97; P = 0.02). Although CMV seropositivity was not significantly associated with CAD in men, CMV seropositivity was associated with elevated CRP levels (P = 0.03 on univariate analysis) and was an independent determinant of CRP on multivariate analysis after adjustment for CAD and CAD risk factors (β=0.27; 95% CI, 0.08 to 0.46, P = 0.006).

**Women**

Of 87 women, 45 (52%) had CAD. Their mean age was 57.0 years (median age, 57.0 years). The frequency of traditional CAD risk factors (except smoking, P < 0.05) was similar in men and women (Table 1). Of 45 women with CAD, 40 (89%) had anti-CMV IgG antibodies, whereas only 20 of 42 (48%) of those without CAD were CMV-seropositive (P = 0.001; Figure 1). After adjustment for traditional CAD risk factors, the presence of anti-CMV antibodies was a highly significant predictor of CAD (odds ratio, 41.8; 95% CI, 4.12 to 423.74; P = 0.0016). In contrast to the findings in men, although mean CRP in women with CAD was 25% higher than CRP in women without CAD, the difference did not reach statistical significance (0.95±0.06 versus 0.75±0.05 mg/dL, P = 0.1; Figure 2).

**Influence of the Type of Immune Responses to CMV on CAD Prevalence**

We next determined whether the type of immune response to CMV influenced the prevalence of CAD. Four types of immune response patterns were found. The most common was a humoral response (Ab+Tc−); the least common was a cellular response (Ab−Tc+). The types of immune responses to CMV infection generated by the total cohort and by the women and men are shown in Table 2.

Most importantly, we found that the type of immune response to CMV influences CAD prevalence in women, whereas no relationship between the type of immune response and CAD prevalence was found in men (Figure 3).
CAD prevalence was 5 times higher in Ab+/Tc− (P=0.0005) and in Ab+/Tc+ women (P=0.003) than in Ab−/Tc− women (those with no immunologic evidence of prior CMV infection). CAD prevalence in women with a cellular response (Ab−/Tc+) was not different from the Ab−/Tc− group but was significantly lower than that of the Ab+/Tc+ group (P=0.016). There were no differences in age, smoking, diabetes, hypercholesterolemia, or hypertension among the women with different types of immune response to CMV infection (all P>0.1).

**Discussion**

In a previous investigation of the same cohort, we demonstrated that one mechanism by which CMV infection contributes to CAD appears to be through the inflammatory response it evokes, as reflected by elevated CRP levels. We also found that the inflammatory response to CMV infection varied considerably among individuals infected with CMV. In the present investigation, we further explored the variability of host responses to CMV infection and how these might influence susceptibility and resistance to CMV-associated CAD. We focused specifically on sex differences and differences in the type of immune responses; cellular or humoral.

The conclusion presented in our previous paper, that CMV is a risk factor for CAD only insofar as it contributes to an inflammatory response, derived from an analysis of the total cohort. In the present study, however, we found a sex-based heterogeneity in mechanisms and found that the data suggesting a possible link between CMV and CAD via induction of an inflammatory response were driven by the data in men. Women, who constituted a minority of the study cohort (37%), displayed different results, which were masked when data from the population as a whole were assessed.

In the present study, an elevated CRP level was a significant determinant of CAD in men (adjusted odds ratio, 3.1; 95% CI, 1.21 to 7.97; P=0.02). Whereas CMV infection was not associated with CAD, it was independently associated with elevated CRP levels (P=0.006). In contrast to the data in men, CMV infection in women was not significantly associ-
ated with elevated CRP levels. This finding is compatible with the first hypothesis examined in the present investigation, that the presence or absence of an inflammatory response to CMV is sex-related. Most interestingly, in women, CMV seropositivity was independently predictive of CAD, a relationship that was highly significant (odds ratio, 41.8; 95% CI, 4.12 to 423.74; \( P = 0.002 \)). However, the association between elevated CRP levels and CAD was not as strong in women as it was in men. It therefore seems that men, more consistently than women, mount an inflammatory response to CMV infection and that this response appears to predispose to CAD (although the data do not prove a causal relationship between CMV and CAD via a CMV-induced inflammatory response). In women, conversely, the dominant mechanism relating CMV to CAD appears not to relate to inflammation, at least as assessed by CRP levels, but rather to act by some other, as yet undefined mechanisms.

In an attempt to obtain further insights as to how differences in pathogen-host interaction might contribute to host susceptibility versus resistance to CMV-related CAD, we examined our second hypothesis, which proposed that the type of immune response to CMV infection contributes to susceptibility or resistance to CAD. This was stimulated by our recent finding in a group of healthy blood donors that CMV infection evokes diverse immune responses (J.Z. et al, unpublished data, 1998). Some individuals had neither a humoral (antibody) nor T-cell response to CMV antigens (Ab−/Tc− subgroup); these individuals either were never exposed to CMV or were successful in clearing the virus and at the time of testing had no immunologic evidence of prior infection. Others, all of whom demonstrated immunologic evidence of prior infection, had either a humoral phenotype (Ab+/Tc− subgroup), a cellular response (Ab−/Tc+ subgroup), or a combined response (Ab+/Tc+ subgroup). A similar variety of immune responses was found in the present population (Table 2).

We found that although there was no influence of immune response patterns on disease susceptibility in men, susceptibility to CMV-related CAD was limited to women with a humoral immune response to CMV infection. Thus, compared with the Ab−/Tc− women, CAD prevalence was higher in the Ab+/Tc− and in the Ab+/Tc+ women (13% versus 68% and 64%, both \( P \leq 0.005 \)) but not in Ab−/Tc+ women (25%). These differences could not be explained by subgroup-related differences in age, smoking, diabetes, hypercholesterolemia, and hypertension (all \( P > 0.1 \)).

These results indicate that multiple mechanisms exist whereby CMV infection and perhaps infection by other pathogens contribute to CAD. They also indicate that the relative contribution of these mechanisms to atherogenesis is sex-determined and is influenced by whether or not the host mounts an inflammatory response to CMV infection as well as by the nature of the immune response. The data suggest that CMV, at least in men, may contribute to CAD, insofar as it induces an inflammatory response (although it must be emphasized that insofar as an inflammatory response contributes to CAD, CMV can be considered only one possible factor). In women, however, CMV infection is an independent predictor of CAD risk and is not associated with elevated CRP levels.

There are at least 2 possible explanations, not mutually exclusive, to account for the findings in women that susceptibility to CMV-associated CAD occurs in the humoral response subgroups, whereas resistance is observed in the cellular responders. First, it is possible that a cellular response to CMV, an intracellular pathogen, conveys greater control of viral activity than a humoral response. This explanation implies that the cellular response is primary in determining outcome. If this were the sole explanation, however, it might have been expected that greater control of viral activity would be accompanied by lower CRP levels and that women with a combined humoral and cellular response would have a lower prevalence of CAD than women with a humoral response who lacked a cellular response. This was not observed.

The alternative explanation focuses on the humoral immune response as the major player. Thus, it is possible that the humoral response to CMV is a reflection of antibody-induced autoimmune disease. In this regard, there is now growing evidence that autoimmune responses may play a role in atherosclerosis.\(^ {25, 26} \) Even more relevant to our concept that the antibody response to CMV infection may predispose to atherosclerosis through autoimmune mechanisms are the many examples of immunopathology triggered by the host’s immune response to viral infection. Perhaps the best-studied potential mechanism for infection-induced immunopathology is that of molecular mimicry, which is based on the invading pathogen having peptides highly homologous to host peptides. The immune response targeted to the infectious pathogen would, through molecular mimicry, effectively result in the development of autoantibodies or autoaggressive T cells to host peptides.

In addition to the information relating to potential mechanisms by which CMV and presumably other pathogens contribute to atherogenesis, our results also help to explain the conflicting epidemiological evidence relating to the possible role of infectious agents in atherosclerosis. Although some studies have found an association between CMV and atherosclerosis or restenosis based on analysis of CMV seropositivity, other studies have questioned such a relationship. This controversy may be due to the paucity of women in these studies in whom a direct association between CMV seropositivity and CAD is observed and to the failure to concomitantly analyze an index of inflammation, such as CRP elevations. Our study also demonstrates the importance of the type of immune response mounted by the host to the infectious agent in determining whether or not the infection predisposes to vascular disease.

Several caveats must be considered relating to our conclusions. First, the study design of this investigation is cross-sectional in nature. Such a design cannot establish causality. It can only establish an association. Hence, any conclusion derived from such a study must be considered preliminary and hypothesis-generating rather than hypothesis-proving. Second, as implied in our discussion about different populations responding differently to a specific infection, it is possible that our conclusions may be limited to the particular population of men and women we studied. Third, our non-
CAD control group consisted of individuals who, on clinical evaluation, had some suggestion of CAD. These individuals may not be representative of other individuals without CAD who lack clinical features triggering the decision to perform coronary angiography. In addition, it is unclear why the level of CRP appears to be high in our control group. Fourth, our analysis on the type of immune response is based on single assessments of selected immunologic responses; therefore, our proposed hypotheses will have to be evaluated further in the future with more frequent immunologic assessments in larger numbers of men and women. Finally, the number of women in each immune response category is small; the results therefore need to be confirmed by larger studies. Nonetheless, we believe it likely that our conclusions will prove to be valid, considering that (1) our hypotheses were formulated prospectively, (2) the results are consistent with those demonstrating the important role of host response in determining susceptibility or resistance to various diseases induced by many other pathogens, and (3) our results help to explain otherwise disparate results in the literature relating to CMV and CAD.

In summary, the results of this investigation provide further evidence that CMV contributes to atherosclerosis. The data suggest, however, that the dominant mechanisms by which CMV predisposes to CAD in men and women are different. In men, if CMV contributes to CAD, it would appear to do so insofar as it predisposes to inflammation, whereas in women, CMV is an independent risk factor for CAD. These observations raise the possibility of novel therapeutic strategies for the prevention or treatment of atherosclerosis. Thus, it might be possible to alter disease outcome favorably through the use of vaccines or cytokine-based strategies designed to change an immune response directed against a causally relevant pathogen from one that conveys disease susceptibility to one that enhances resistance. This concept would be especially attractive if similar associations were also demonstrated between CAD and infection with other pathogens, such as *C. pneumoniae* and *H. pylori*, and with the immune response to these pathogens.

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References

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