Infections, Immunity, and Atherosclerosis

Associations of Antibodies to \textit{Chlamydia pneumoniae}, Helicobacter pylori, and Cytomegalovirus With Immune Reactions to Heat-Shock Protein 60 and Carotid or Femoral Atherosclerosis

Manuel Mayr, MD; Stefan Kiechl, MD; Johann Willeit, MD; Georg Wick, MD; Qingbo Xu, MD, PhD

\textbf{Background}—Atherogenesis involves inflammatory processes in which infections are incriminated as possible contributors.

\textbf{Methods and Results}—We evaluated cardiovascular risk factors as well as seropositivity to \textit{Chlamydia pneumoniae}, \textit{Helicobacter pylori}, and cytomegalovirus in a population-based study. A significant association between prevalence and severity of atherosclerosis in carotid and femoral arteries and IgA antibodies to \textit{C pneumoniae} was demonstrated that was not substantially altered after adjustment for established risk factors. For anti-\textit{H pylori} IgG antibodies, significant correlations to vascular disease were restricted to low social status and lesions in carotid arteries. In addition, the study design allowed us to monitor lesion progression over time. In this prospective analysis, \textit{C pneumoniae} seropositivity emerged as a significant risk predictor. Antibody titers against cytomegalovirus were not a marker for prevalence or incidence of atherosclerosis in this population. Further infection parameters added to the predictive value of chlamydial serology in risk assessment: Mean odds ratios for the prevalence of carotid atherosclerosis were 4.2 and 6.3 for seropositive subjects with elevated C-reactive protein levels and clinical evidence for chronic respiratory infection, respectively. For subjects with all 3 infection parameters, the odds ratio of carotid atherosclerosis reached 10.3 (\(P<0.0001\)). Concomitantly, serum antibodies to mycobacterial heat-shock protein 65 (mHSP65) correlated with seropositivity to \textit{C pneumoniae} and \textit{H pylori} but not to cytomegalovirus.

\textbf{Conclusions}—This prospective population-based study provides strong evidence for a potential atherogenic role of persistent bacterial infection, especially \textit{C pneumoniae}, as indicated by serological and clinical data and demonstrates a correlation between immune reactions to mHSP65 and bacterial infections in atherogenesis. (\textit{Circulation}, 2000;102:833-839.)

\textbf{Key Words:} aging ■ infection ■ antibodies ■ atherosclerosis ■ immunology

Over the past decade, awareness of possible associations between atherosclerosis and certain persistent bacterial and viral infections has steadily increased. The most compelling evidence derives from seroepidemiological studies, which focused mainly on 3 pathogens: \textit{Chlamydia pneumoniae}, \textit{Helicobacter pylori}, and cytomegalovirus (CMV). The hypothesis is strongest for \textit{C pneumoniae} on the basis of the association between chlamydial seropositivity and risk of cardiovascular and cerebrovascular disease. Prevalences of IgG antibodies in middle-aged adults exceed 50%, because this common respiratory pathogen causes widespread epidemics and frequent reinfection. These obligate intracellular bacteria are detected frequently within atherosclerotic lesions but rarely in normal vessels. They reside mainly in plaque macrophages but also colonize the vessel wall by infecting endothelial and smooth muscle cells. Animal models support their possible atherogenic effect, and preliminary data from small-scale clinical trials suggest that macrolide antibiotic treatment is beneficial in vascular disease, but this remains to be confirmed by larger studies.

Human heat-shock protein (HSP) production in the arterial wall is essential for cell protection against unfavorable conditions, such as hemodynamic stress, oxidants, and cytokine stimulation. However, HSPs bear the risk of autoimmunity because of their high sequence homology between different species, from prokaryotes to humans. Previous epidemiological studies from our laboratory demonstrated that increased serum antibodies to mycobacterial HSP65 (mHSP65), a bacterial homologue of human HSP60, were associated with the prevalence of carotid atherosclerosis independently of other established risk factors, and this was subsequently confirmed by other laboratories. Furthermore,
elevated anti-mHSP65 antibody titers were sustained during a 5-year follow-up and were strongly associated with advanced atherosclerosis and mortality in prospective analyses. These serum antibodies cross-react with human HSP60 and mediate cytotoxicity on stressed endothelial cells. Therefore, the antimicrobial HSP60/65 immune response may contribute to vascular endothelial injury, which is believed to be a key event in the pathogenesis of atherosclerosis.

Despite the rapidly growing number of studies about associations between infections and vascular disease, consensus on the possible atherogenic effects of infectious agents has not been achieved; pathogenetic mechanisms remain unclear, and the available seroepidemiological studies are subject to certain limitations. First, most previous surveys focused on clinical end points, such as coronary heart disease or stroke. The associations obtained do not necessarily prove the existence of atherogenic effects, because infections may provoke ischemia by prothrombotic and other mechanisms. In particular, extrapolation of results from clinical studies to early stages of atherogenesis, which pathologically resembles chronic inflammatory disease, requires caution. Second, the studies that investigated the association of infections with atherosclerosis were, with few exceptions, cross-sectional, and those with a prospective design were not conducted in a random population. Finally, seropositivity as a marker for infections has the advantage of clinical applicability, but the assessment of infection status based on serology without further clinical or laboratory characterization is subject to diagnostic inaccuracies, especially if seropositivity is common because of the widespread distribution of the incriminated microorganism.

In this prospective survey, we investigated the association of immune reactions to *C pneumoniae, H pylori*, and CMV with carotid and femoral atherosclerosis as quantified by ultrasound in the population-based Bruneck study. In addition, we scrutinized this association by laboratory and clinical markers for persistent infection and by correlating bacterial infections with serum antibodies to mHSP65.

**Methods**

**Subjects**

Human sera were derived from the Bruneck Study, a large population-based study on the epidemiology and etiology of atherosclerosis and arterial disease. The study population is an age- and sex-stratified random sample of all inhabitants of Bruneck 40 to 79 years old. In each of the fifth to eighth decades of age (40 to 49, 50 to 59, 60 to 69, and 70 to 79 years), 125 men and 125 women were selected for inclusion (n = 1000). A total of 93.6% participated, with data assessment completed in 919 subjects. Between 1990 and 1995, a subgroup of 63 individuals died or moved away. In the remaining population, follow-up was 96.5% complete, which left 826 subjects for the present analysis. All participants gave informed consent before entering the study.

**Clinical Examination and Laboratory Methods**

All participants underwent a clinical examination with cardiological and neurological priority and completed standardized questionnaires on current and past exposure to candidate vascular risk factors as described previously. The term “chronic respiratory infections” encompassed chronic bronchitis, emphysema, and asthma, which were defined according to standard criteria. Blood samples were taken from the antecubital vein after subjects had fasted and abstained from smoking for ≥12 hours. Laboratory parameters were examined by standard methods.

**Scanning Protocol and Definition of Ultrasound End Points**

The ultrasound protocol involves scanning of the internal (bulbous and distal segments) and common (proximal and distal segments) carotid arteries on either side with a 10-MHz imaging probe and 5-MHz Doppler. Atherosclerotic lesions were defined by 2 ultrasound criteria: (1) wall surface (protrusion into the lumen or roughness of the arterial boundary) and (2) wall texture (echogenicity). The maximum axial diameter of plaques was assessed in each of the additional 16 vessel segments, and a sensitive and reproducible atherosclerosis score was calculated by addition of all diameters. The accuracy of this procedure had been established previously. Scanning was performed twice, in 1990 and 1995, by the same experienced sonographer, who was unaware of the subjects’ clinical and laboratory characteristics. Incident (early) atherosclerosis was defined by the occurrence of new plaques in previously normal segments (1990 to 1995). Advanced atherogenesis shown to originate primarily from atherosclerosis was assumed whenever the progression criterion (increase in plaque diameter exceeding twice the measurement error) was met and a narrowing of the lumen >40% was achieved. The cutoff of 40% was adopted from previous epidemiological analyses in this cohort. In 1995, the ultrasound examination was extended to the femoral arteries (40 mm proximal and 10 mm distal to the bifurcation into the superficial and deep branches). The intima-media thickness was measured in 1995 at the far wall of the femoral arteries and common carotid arteries with the ultrasound beam directed through the axis of the vessel. It was defined as the distance between the lumen-intima interface and the leading edge of the media-adventitia interface.

**Specific Tests for Infections and Measurement of Anti-mHSP65 Antibodies**

Serum antibodies against *C pneumoniae, H pylori*, and CMV were determined by 3 commercial test systems as part of the 1995 evaluation: SeroCP-IgA (Savyon Diagnostics Ltd), Helicobacter-IgG (Medac), and CMV-IgG ELISA (Medac). The analyses were performed and calculated according to the manufacturer’s instructions. Antibodies against mHSP65 were determined by ELISAs according to an established protocol. In brief, microtiter plates were coated with 1 μg/mL PBS with recombinant mHSP65 overnight at 4°C and incubated with 100 μL human serum diluted 1 in 10 to 1 in 5160 for 1 hour. A serum dilution was considered positive for antibodies to mHSP65 if the optical density at 410 nm exceeded 0.400.

**Statistical Analyses**

The association between immune reaction to bacterial/viral pathogens and anti-mHSP65 antibodies was investigated by comparing antibody levels across categories of increasing titers of antibodies to *C pneumoniae, H pylori*, and CMV. Logistic regression models were built to analyze strength and type of association between antibody titers and prevalent or incident atherosclerosis (incident nonstenotic atherosclerosis or incident stenosis). The test procedure was based on maximum-likelihood estimators, and the goodness of fit of each model was assessed by the test of Hosmer and Lemeshow. Effect modification was tested by the inclusion of interaction terms. Base models were adjusted for age and sex only (plus baseline atherosclerosis score in the prospective analysis). Multivariate equations were controlled for potential effects of age, sex, smoking, alcohol intake, diabetes, impaired glucose tolerance, lipoprotein (a), apolipoprotein B, ferritin, hypertension, fibrinogen, and social status. The forced entry of all these variables yielded results virtually identical to those of a forward stepwise selection procedure (data not presented). In the main analysis (Tables 1 to 3), log-transformed antibody titers were treated as a continuous variable, and odds ratios were calculated for a 1-titer increase in given serological parameters. This procedure, contrary to most previous evaluations in this field, avoided arbitrary categorizations of subjects according to their antibody titer. Because
there is no consensus on how to define seropositivity, categorizations may give rise to spurious relations, especially if guided by a post hoc decision. In a second step, antibody titers were modeled as a set of indicator variables, and odds ratios of atherosclerosis were computed for each category to investigate the scale of the association (Figure 1). Finally, the relation between atherosclerosis and immune reaction to *C pneumoniae* was reanalyzed with respect to clinical (manifest chronic respiratory diseases) and/or laboratory (high C-reactive protein [CRP]) evidence of persistent infection status (Figure 2). For ease of presentation, we used a categorization of IgA antibody titers to *C pneumoniae* (negative $<16$, positive $\geq 16$) in this analysis.

Logistic regression models were supplemented by linear regression analyses using intima-media thickness as a continuous outcome variable. Multivariate regression models were built as described above.

**Results**

**Association of Antibody Titers With Carotid and Femoral Atherosclerosis**

Seroprevalence was assessed by commercial ELISA tests measuring IgA antibodies to *C pneumoniae* and IgG antibodies to *H pylori* and CMV. Chlamydial IgA antibodies are expected to be a more reliable marker for chronicity of chlamydial infections than seroprevalences of IgG antibodies. Antibody titers to *C pneumoniae* were strongly associ-
ated with male sex and age ($P<0.001$); a moderate association was observed with smoking ($P<0.1$). Anti–$H$ pylori antibodies were more frequent in subjects of low social status and patients with ulcer disease ($P<0.001$ and $P<0.1$, respectively). Serum antibodies to CMV were significantly higher in women ($P<0.001$) and increased with age and low social status ($P<0.05$).

Anti–$C$ pneumoniae IgA antibodies were associated with the prevalence of atherosclerosis in carotid and femoral arteries ($P=0.011$ and $P=0.035$, Table 1). Results remained significant after adjustment for multiple risk factors, including age, sex, hypertension, smoking, apolipoprotein B, lipoprotein (a), diabetes, impaired glucose tolerance, fibrinogen, and ferritin levels, alcohol consumption, and social status. Anti–$H$ pylori IgG antibodies correlated significantly with carotid atherosclerosis when the statistical analysis was restricted to low social status ($P=0.027$). No correlation between immune responses to $H$ pylori and atherosclerosis in femoral arteries could be obtained. Although antibodies to $C$ pneumoniae and $H$ pylori predicted the risk of carotid atherosclerosis (Figure 1), elevated antibody titers to CMV were not associated with atherosclerotic lesions in carotid or femoral arteries. The power of our statistical analysis was $0.9$ to detect a difference of 1 titer between outcome categories at a level of $\alpha=0.05$.

Intima-media thickness is a well-established surrogate and precursor of definite atherosclerosis. When this outcome variable was substituted for the atherosclerosis categories, $C$ pneumoniae remained a significant risk factor (Table 2). Significance for anti–$H$ pylori IgG antibodies was again limited to subjects of low social status and lesions in carotid arteries. Finally, IgG titers to CMV were definitely unrelated to vessel wall thickness. In this analysis, the power of our study would allow detection of correlates as low as $r=0.09$ ($power=0.8, \alpha=0.05$).

The study design permitted us to study changes in carotid atherosclerosis during a 5-year follow-up and to define distinct stages in the atherogenesis process. Immune reactions

<table>
<thead>
<tr>
<th>Antibody and Model</th>
<th>Common Carotid Artery IMT</th>
<th>Femoral Artery IMT</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{IgA to C pneumoniae}$</td>
<td>Reg Coeff</td>
<td>95% CI</td>
</tr>
<tr>
<td>Adjusted for age/sex</td>
<td>0.010</td>
<td>0.001–0.019</td>
</tr>
<tr>
<td>Multivariate†</td>
<td>0.008</td>
<td>0.004–0.012</td>
</tr>
<tr>
<td>$\text{IgG to H pylori}$</td>
<td>Reg Coeff</td>
<td>95% CI</td>
</tr>
<tr>
<td>Adjusted for age/sex</td>
<td>0.011</td>
<td>0.001–0.021</td>
</tr>
<tr>
<td>Multivariate†</td>
<td>0.009</td>
<td>0.001–0.017</td>
</tr>
<tr>
<td>$\text{IgG to CMV}$</td>
<td>Reg Coeff</td>
<td>95% CI</td>
</tr>
<tr>
<td>Adjusted for age/sex</td>
<td>0.001</td>
<td>−0.008–0.010</td>
</tr>
<tr>
<td>Multivariate†</td>
<td>0.001</td>
<td>−0.008–0.010</td>
</tr>
</tbody>
</table>

*Analysis restricted to low social status (see text).
†Adjustment (see Table 1).
to *C pneumoniae* were associated with early and advanced stages of atherosclerosis, whereas seroprevalences of antibodies to *H pylori* and CMV were not (Table 3).

Notably, none of these findings were substantially altered when the analysis was restricted to nonsmokers. When subjects seropositive to *C pneumoniae* had additional serological evidence for infections with either *H pylori* or CMV or both, predictive significance for atherosclerosis did not change.

**Respiratory Infections, CRP Levels, and Anti-mHSP65 Antibodies**

The presence of IgA antibodies to *C pneumoniae* is supposed to be indicative for ongoing chronic infection, but substantial fluctuations in antibody responses during chlamydial disease may weaken statistical associations. Hence, evaluation of infection activity should rely on further clinical and laboratory parameters. In our study, high CRP levels (Figure 2B) were positively correlated with carotid atherosclerosis and substantially improved the predictive accuracy (Figure 2B) were positively correlated with carotid atherosclerosis, whereas seroprevalences of antibodies to *H pylori* and CMV were not (Table 3).

Consensus about the role of infections in atherosclerosis has not been achieved despite several seroepidemiological studies. Available data derive almost exclusively from cross-sectional and retrospective analyses using such clinical end points as coronary heart disease or stroke as outcome variables. To the best of our knowledge, the present study is the first prospective survey about atherosclerosis and immune reactions to *C pneumoniae*, *H pylori*, and CMV conducted in a large random population. Among the 3 microorganisms, significant with anti-mHSP65 antibodies (*P* = 0.03 and *P* = 0.001). Conversely, levels of anti-CMV antibody titers were not associated with either atherosclerosis or anti-mHSP65 antibodies (*P* = 0.17). Subjects seronegative to CMV had detectable anti-mHSP65 antibodies, as did all individuals in this population. But in contrast to *C pneumoniae* and *H pylori*, seropositivity to CMV did not alter these titers significantly (Table 4). Altogether, our findings suggest that immune reactions to mHSP65 are linked with bacterial but not viral infections.

**Discussion**

Consensus about the role of infections in atherosclerosis has not been achieved despite several seroepidemiological studies. Available data derive almost exclusively from cross-sectional and retrospective analyses using such clinical end points as coronary heart disease or stroke as outcome variables.

<table>
<thead>
<tr>
<th>Antibody and Model</th>
<th>Incident Nonstenotic Atherosclerosis</th>
<th>Incident Stenosis &gt;40%*</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgA to <em>C pneumoniae</em></td>
<td>OR 1.12</td>
<td>95% CI 1.02–1.24</td>
</tr>
<tr>
<td>Multivariate‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG to <em>H pylori†</em></td>
<td>OR 1.10</td>
<td>95% CI 1.00–1.22</td>
</tr>
<tr>
<td>Multivariate‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG to CMV multivariate‡</td>
<td>OR 1.01</td>
<td>95% CI 0.88–1.17</td>
</tr>
<tr>
<td>Multivariate‡</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*‡* Adjustment (see Table 1) plus baseline atherosclerosis score (AS).

*OR, 95% CI, and *P* value were derived from logistic regression analyses.

* Analysis restricted to low social status (see text).

*‡* Adjustment (see Table 1) plus baseline atherosclerosis score (AS).

**TABLE 3. Association of Serum Antibodies to Infectious Agents With Incident Carotid Atherosclerosis and Stenosis (1990 to 1995)**

---

**TABLE 4. Association of Seropositivity to *C pneumoniae*, *H pylori*, and CMV With Immune Reactions to mHSP65**

<table>
<thead>
<tr>
<th>Antibody and Model</th>
<th>Mean Anti-mHSP65 Antibody Titer±SEM</th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>IgA to <em>C pneumoniae</em></td>
<td>&lt;16 (n=245) 240±13</td>
<td>. . .</td>
</tr>
<tr>
<td>≥16 (n=581) 294±12</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>IgG to <em>H pylori</em> Negative (n=118) 213±21</td>
<td>. . .</td>
<td></td>
</tr>
<tr>
<td>≥8 U/mL (n=708) 289±10</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>IgG to CMV Negative (n=226) 269±15</td>
<td>. . .</td>
<td></td>
</tr>
<tr>
<td>≥5 U/mL (n=600) 281±12</td>
<td>0.533</td>
<td></td>
</tr>
</tbody>
</table>

* *P* values were derived from a paired Student’s *t* test.
C pneumoniae was the most reliably associated with atherosclerosis. The general consistency of the data in all analysis, ie, different stages of atherosclerosis (precursor to stenosis), and several vascular territories as well as in the cross-sectional and prospective analysis, supports the existence of a real association. Seropositivity for H pylori yielded some association with carotid atherosclerosis, but data were less consistent. The inconclusive results may be partly explained by the strong association of H pylori infection with confounding factors, such as low social class. In addition, the genetic polymorphism of various strains concerning virulence factors like the cytotoxin-associated gene A seems to be decisive for their pathogenic contribution.

Finally, our study could not furnish any evidence for a contribution of CMV to atherogenesis in the general community. At this point, it should be noted that only carotid and femoral arteries were examined in this study, and extrapolation of our results to other vascular territories, eg, coronary arteries, requires caution.

The present study adds further dimensions to the relation between infections and atherogenesis in that the predictive significance of chlamydial IgA antibodies for carotid atherosclerosis substantially increased (OR = 10.3) when elevated CRP levels and clinical evidence for chronic respiratory infections, as well as the fact that chronic respiratory infections amplified atherosclerosis risk in the absence of chlamydial antibodies, were taken into account. The finding that laboratory and clinical markers for persistent infection added significantly to the predictive value of chlamydial serology in risk assessment underscores the importance of chlamydial infections in atherogenesis and demonstrates the limited accuracy of chlamydial antibodies as a single criterion of infection. Discrepancies in the results of the few previous studies in this field may be explained by this methodological shortcoming. The observation that chronic respiratory infections, as defined by clinical criteria, predict the prevalence of carotid atherosclerosis even without seropositivity to C pneumoniae implies that the total infectious load might be even more important than a single infectious agent and that elevated CRP levels in patients with cardiovascular disease might be partly due to infection.

How infections are involved in vascular disease remains to be elucidated. Increased blood viscosity, hypercoagulability, and alterations of the serum lipid profile are postulated mechanisms. As in the case of C pneumoniae or CMV, vessel wall colonization may contribute to local inflammation by cytokine induction or antigen stimulation. In contrast, H pylori infections might stimulate atherogenesis via indirect effects, such as systemic inflammation or (auto) immune reactions, because these bacteria are rapidly eliminated in the circulation before they reach the vessel wall. Immune reactions to HSP60 are a possible link between the various incriminated microorganisms and atherogenesis. Abundant bacterial HSP60 may evoke an anti–self-immune response in susceptible individuals because of its high sequence homology with the human homologue. Indeed, circulating HSP60 antibodies are absent in specific pathogen–free animals, suggesting that induction of HSP antibodies depends primarily on infections. Likewise, the age-dependent increase of anti-HSP60 titers in humans might be due to lifetime exposure to environmental microorganisms. The prospective Bruneck study yielded evidence for a sustained elevation of anti-mHSP65 antibody titers in subjects with atherosclerosis. These serum antibodies cross-react with bacterial and human HSP60 and mediate vascular cytotoxicity on stressed endothelial cells. Cells of the arterial wall overexpress HSP60 in response to various harmful stimuli, including hemodynamic stress, infections, oxidants, and cytokines, to protect themselves against these unfavorable conditions. Interestingly, persistent chlamydial infections are associated with an abundance of HSP60. The frequent colocalization of bacterial and human HSP60 within atherosclerotic lesions provides a possible explanation of how C pneumoniae might contribute to the pathophysiology of atheroma. In addition to molecular mimicry, HSP60 activates vascular adhesion molecule expression, induces the production of proinflammatory cytokines, and regulates the expression of matrix metalloproteinases in macrophages. In the present study, we demonstrated for the first time that anti-mHSP65 correlates significantly with antibodies against C pneumoniae and H pylori in the general community, suggesting that immune reactions to HSP60 in atherogenesis are at least partially due to bacterial infections.

Acknowledgments

This work was supported by grants P-12123-MED (to Dr Wick) and P-13099-BIO (to Dr Xu) from the Austrian Science Fund and P7919 (to Dr Xu) from the Austrian National Bank. We are grateful to the companies Medac (Hamburg) and Savyon Diagnostics Ltd (Israel) for providing their commercial ELISA kits and to A. Jenewein for technical assistance.

References


Infections, Immunity, and Atherosclerosis: Associations of Antibodies to Chlamydia pneumoniae, Helicobacter pylori, and Cytomegalovirus With Immune Reactions to Heat-Shock Protein 60 and Carotid or Femoral Atherosclerosis
Manuel Mayr, Stefan Kiechl, Johann Willeit, Georg Wick and Qingbo Xu

Circulation. 2000;102:833-839
doi: 10.1161/01.CIR.102.8.833
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/102/8/833

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/