Inflammatory Response After Influenza Vaccination in Men With and Without Carotid Artery Disease

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Objective—Inflammatory markers are associated with vascular disease; however, variation in the acute phase response (APR) has not been evaluated. We evaluated whether the APR magnitude in men with severe carotid artery disease (CAAD) (>80% stenosis) differed from that of men without stenosis (<15% stenosis).

Methods and Results—White males with (n=43) and without (n=61) severe CAAD receiving clinical influenza vaccinations were recruited. Their baseline and 24-hour after vaccination blood samples were assayed for C-reactive protein (CRP), IL-6, and serum amyloid A (SAA). In vivo APR to vaccination was measurable and varied among subjects. Adjusted for age, smoking, oral hypoglycemics, aspirin, and stain use, the relative 24-hour changes in levels of ln(CRP), ln(IL-6), and ln(SAA) were higher in men with CAAD than in men without, but only the SAA response was significant (P=0.02); the relative SAA response was 1.6 (95% confidence interval, 1.1 to 2.5) times higher in men with than without CAAD. The APR for all markers appeared to be independent of baseline levels.

Conclusions—Influenza vaccination results in a mild, but measurable, APR in men with and without CAAD. SAA APR variability may be a predictor of severe vascular disease that is independent of basal SAA level. (Arterioscler Thromb Vasc Biol. 2006;26:2738-2744.)

Key Words: acute phase response • inflammation • carotid artery disease

Inflammatory mechanisms play a key role in the initiation and progression of cardiovascular disease. Increased levels of inflammatory markers such as fibrinogen, C-reactive protein (CRP), serum amyloid A (SAA), and IL-6 are associated with an increased risk of cardiovascular disease. These proteins are also key components of the acute phase response (APR) to injury or infection. After an insult, the acute phase response is characterized by a rapid increase in levels of inflammatory cytokines and acute phase proteins that regulate immune response, mediate inflammation, transport products generated during the inflammatory process, or participate in tissue repair and remodeling. The APR is largely regulated by the nuclear factor kappa beta (NFKB) pathway, which is activated systemically by cytokines.

To date, most studies of inflammation and cardiovascular disease risk have focused on chronic, basal levels of inflammatory markers, but the total burden of inflammation, both chronic and the acute response, may also be etiologically relevant for the development of cardiovascular disease. We recently detected genetic variation in the NFKBIA locus that predicted carotid artery disease status, suggesting a role of APR in disease etiology (Carlson et al, submitted, Human Genetics). Additionally, it has been reported that common acute infections are associated with an increased risk of myocardial infarction and stroke, presumably when the APR has been activated. A high degree of inter-individual variation in the acute phase response has been described by Verschuur et al, who assessed changes in fibrinogen, CRP, and IL-6 in 25 healthy volunteers after yellow fever vaccination. This variation suggests possible genetic and other determinants of APR, which may predispose to vascular disease.

We are unaware of previous studies that test whether APR predicts or varies with vascular disease status. To address this issue, we investigated the APR in white men with severe carotid artery disease (CAAD) and similarly aged men without vascular disease. Using influenza vaccination as a standard immune stimulus, we measured the association between CAAD disease status and change in plasma levels of inflammatory markers (CRP, IL-6, SAA) after vaccination.

Study Population
White males aged 38 to 88 years who planned to receive clinical influenza vaccinations during October to December of 2005 were
recruited from the Seattle-based Carotid Lesion Epidemiology and Risk (CLEAR) study at the Puget Sound Veterans Affairs Health Care System (PSVAHCS) and the University of Washington Medical Center (UWMC). A subset of participants in the ongoing CLEAR study were contacted by letter and phone and invited to enroll in this sub-study. Enrollment target size was 100 subjects. No subject received vaccination solely to enroll in this sub-study. Because of the planned small sample size, enrollment was limited to the CLEAR study’s most prevalent racial group and sex to minimize these sources of variation. Exclusion criteria included current acute illness (assessed by subjects’ symptoms or CRP ≥16 mg/dL at baseline and declining at 4 hours after vaccination) and current use of systemic steroids (assessed by VA pharmacy records and self-report).

A total of 43 men with stenosis were recruited from the CLEAR study population. They had CAAD, >80% internal carotid artery stenosis unilaterally or bilaterally on ultrasound, or previous carotid endarterectomy at enrollment into CLEAR. Before CLEAR recruitment, they presented clinically and were ascertained through diagnosis codes and clinical referral. The 61 men recruited from CLEAR without severe stenosis had no previous vascular disease by patient report or medical record review and <15% internal carotid artery stenosis, bilaterally, on carotid duplex ultrasound. Similar to men with stenosis, they were recruited from the PSVAHCS and UWMC patient population, but did not have a medical history of vascular disease or vascular disease codes present in their medical records.

In the larger CLEAR cohort, subjects without CAAD were age-distribution matched with the clinically recognized vascular disease onset age of men with CAAD. However, this age matching was not retained in the flu shot sub-study population (n = 104), which is a convenience sample of the CLEAR study population. Because the age distribution varied by stenosis status in the sub-study, we adjusted for it in all of our analyses. It is possible that the participants in our sub-study were healthier, more mobile, and more likely to seek preventive healthcare than the nonparticipating members of the CLEAR study. Controls were more likely to enroll in this sub-study than cases. Further CLEAR study recruitment details are reported elsewhere.13

Subjects appeared for a brief clinic visit during which a short questionnaire was completed and a blood sample was collected before standard 2005 inactivated influenza vaccination injections were administered. No subjects had nasal vaccination. Subjects returned for an additional brief visit at both 4 and 24 hours after vaccination, during which additional blood samples and questionnaires were collected. Smoking status and current use of aspirin and nonsteroidal anti-inflammatory drugs (NSAIDS) were self-reported and verified during each visit. Race, age, and medication use were obtained from PSVAHCS records and self-report. Body mass index was measured by study personnel, missing data were obtained from subject surveys. The present study was approved by the University of Washington (UW) and the PSVAHCS human subjects review boards. All subjects gave written informed consent.

**Laboratory Methods**

Inflammation and lipid measures were performed by the North-west Lipid Metabolism and Diabetes Research Laboratories. Highly sensitive Luminescent bead assays were used for the measurement of SAA and IL-6. Analyses were performed on the Bio-Rad Bioplex platform using commercially available reagents from Linco Research Inc. Bio-Plex assays are designed in a capture sandwich immunoassay format. LINCO-specified intra-assay and inter-assay variability were 13.4% and 20.0%, respectively, for SAA and both <8.5% for IL-6. Samples from all time points for each subject were assayed on the same plate to further reduce measurement variability.

The immunochemical measurement of CRP was performed by a highly sensitive method using Dade Behring reagent on a Behring II Nephelometer. Polystyrene particles coated with monoclonal antibodies specific to CRP are agglutinated when mixed with CRP in the plasma samples. The intensity of the scattered light in the nephelometer is compared against a standard curve performed by serially diluting a known CRP concentration. Intra-assay and inter-assay variability are below 2.5% and 4.9%, respectively.

Baseline fasting lipid measurements were performed on whole plasma. Standard enzymatic methods were used to determine levels of total cholesterol, triglyceride, very low-density lipoprotein, and high-density lipoprotein (HDL) on an Abbott Spectrum analyzer.14–16 Low-density lipoprotein (LDL) levels were calculated by the Friedewald equation.17

**Statistical Analysis**

Inflammatory marker levels were neutral log (ln)-transformed for analysis. Baseline levels for men with and without CAAD were compared using 2-sided t tests with P < 0.05 as the threshold for statistical significance, as were 24-hour levels. For each inflammatory marker, a relative change variable was calculated as the ln (24-hour level/baseline level), which is equivalent to ln(level at 24 hours) − ln(level at baseline). This variable defined the “response.” In all linear regression models, the “response” or change in marker levels between the 2 time points, ln(level at 24 hours/baseline level) was the outcome.

In univariate analyses in the controls, we investigated the association between response and the following potential confounders: age, body mass index, current smoking, HDL cholesterol levels, chronic co-morbidities and use of aspirin, anti-inflammation medication, and statins. Multivariable linear regression models with robust standard errors were used to test for differences in the response variables between men with and without CAAD.

Using Bland-Altman plots,18 in which the mean of 2 measurements is plotted on the x-axis and the difference between the 2 measurements is plotted on the y-axis, we investigated whether variation in the 24-hour level was dependent on the magnitude of the mean level. We further tested the independence of baseline SAA and the SAA response in the prediction of APR using logistic regression models. Specifically, we tested whether the addition of the response variable to models with and without a baseline level variable improved prediction of CAAD status. We also report the increase in risk of severe CAAD associated with higher SAA baseline level and with a higher SAA response (both in terms of a 1-ln increase). Data were analyzed using STATA (v.8.2) statistical software (StataCorp, College Station, Tex; 2005).

**Results**

Traditional cardiovascular risk factors such as advanced age and current cigarette smoking were significantly higher in men with CAAD than in men without (Table 1). Men with CAAD were also more likely than men without CAAD to be currently using statins (84 versus 26%) and aspirin (61 versus 33%), which may suppress the APR.

CRP levels of subjects with baseline CRP ≥16 mg/dL (range, 17.4 to 41.7) were lower at 4 hours, suggesting a pre-vaccination insult that began resolving at 4 hours; thus supporting exclusion of those subjects because of acute illness. Two other subjects with CRP >10 mg/dL (15.7 and 10.9) had a 4-hour change in CRP that fell on the regression line with the remaining subjects and, thus, were not excluded. As reported in the literature, insulin has an anti-inflammatory effect,19,20 which we also observed for the 2 men with CAAD and 1 man without CAAD using insulin in our study. They had a significantly reduced APR and were also currently using statins and aspirin. Rather than estimate an adjustment for insulin use based on only 3 data points, we excluded the 3 men from our analyses. Excluded subjects are not shown in Table 1 and all exclusions were blinded to case status. CRP and SAA responses were expected to be maximal at 24 hours and
had changed little by 4 hours. IL-6 levels were also higher at 24 hours than at 4 hours, thus, the baseline to 24-hour response was considered for all 3 markers.

Baseline levels of CRP and SAA were slightly higher in men with CAAD than in men without, but the differences were not statistically significant (Figure 1). IL-6 followed a similar pattern (not shown). Similarly, 24-hour postvaccination levels of IL-6, CRP, and SAA were also higher in men with versus without CAAD, but only SAA was significantly higher, \( P < 0.021 \). The postvaccination levels of all markers were significantly higher than baseline levels for both men with CAAD (\( P = 0.009 \) for each marker) and for men without CAAD (\( P = 0.014 \) for each marker). In terms of percent difference (rather than ln-transformed level), the percent difference (SD) in SAA after vaccination was 222.7% (1120.9) in men with CAAD and 33.8% (101.4) in men without CAAD. For CRP and IL-6, respectively, men with CAAD had percent difference (standard deviation [SD]) values of 227.0% (995.9) and 40.1% (113.4) compared with 63.1% (125.5) and 14.0% (34.5) in men without CAAD.

To investigate potential confounders, we examined the association between the potential confounders and APR response in men without CAAD in univariate models. Of the characteristics listed in Table 1, only age was significantly associated with APR; age predicted SAA response (\( P = 0.001 \)) and CRP response (\( P = 0.029 \)) in men without CAAD, with increased age being associated with a slightly reduced APR. Other variables that appeared to have a strong effect on the APR in men without CAAD, regardless of their statistical significance, were included in our model only if they were also associated with CAAD status. Smoking, oral hypoglycemics, statin, and aspirin use were carried forward in the analyses because of reported associations with inflammation. Unless otherwise stated, adjustment was made for age (con-

### Table 1. Selected Characteristics of Men With and Without Severe Carotid Artery Disease at Baseline

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No CAAD (n=61)</th>
<th>CAAD (n=43)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age, y (SD)</td>
<td>66.5 (8.0)</td>
<td>72.9 (8.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mean BMI, kg/m² (SD)</td>
<td>28.5 (3.8)</td>
<td>28.4 (5.1)</td>
<td>0.943</td>
</tr>
<tr>
<td>Mean HDL cholesterol, mg/dL (SD)</td>
<td>49.2 (11.2)</td>
<td>42.3 (9.5)</td>
<td>0.002</td>
</tr>
<tr>
<td>Mean LDL, mg/dL (SD)</td>
<td>119.9 (29.4)</td>
<td>87.9 (27.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mean triglycerides, mg/dL (SD)</td>
<td>147.1 (96.3)</td>
<td>156.0 (96.6)</td>
<td>0.655</td>
</tr>
<tr>
<td>Current cigarette smoking, n (%)</td>
<td>2 (3.3)</td>
<td>12 (27.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Current medication use, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-hypertension</td>
<td>29 (47.5)</td>
<td>40 (93.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Any aspirin</td>
<td>20 (32.8)</td>
<td>26 (60.5)</td>
<td>0.005</td>
</tr>
<tr>
<td>Aspirin (300+ mg/day only)</td>
<td>5 (8.2)</td>
<td>10 (23.3)</td>
<td>0.031</td>
</tr>
<tr>
<td>Oral hypoglycemics</td>
<td>5 (8.2)</td>
<td>14 (32.6)</td>
<td>0.002</td>
</tr>
<tr>
<td>Statins</td>
<td>16 (26.2)</td>
<td>36 (83.7)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*For \( \chi^2 \) and \( t \) tests comparing men with and without CAAD.

Boxes represent the inter-quartile (25%-75%) range. The center line in the box indicates the median.

**Figure 1.** Boxplots of ln(CRP) and ln(SAA) levels at baseline and 24-hours post-vaccination.
Table 2. Linear Regression Results for the Relative Difference in APR Associated With CAAD Status

<table>
<thead>
<tr>
<th>Model Adjustment</th>
<th>$e^{\text{CAAD}}$</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ln(SAA)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age*</td>
<td>1.34</td>
<td>1.02–1.75</td>
<td>0.039</td>
</tr>
<tr>
<td>Age,* current smoking, aspirin use, and oral hypoglycemic use</td>
<td>1.62</td>
<td>1.03–2.55</td>
<td>0.039</td>
</tr>
<tr>
<td>Age,* current smoking, aspirin, oral hypoglycemic, and statin use</td>
<td>1.63</td>
<td>1.08–2.48</td>
<td>0.022</td>
</tr>
<tr>
<td>Ln(CRP)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>1.19</td>
<td>0.90–1.58</td>
<td>0.236</td>
</tr>
<tr>
<td>Age,* current smoking, aspirin use, and oral hypoglycemic use</td>
<td>1.40</td>
<td>0.88–2.21</td>
<td>0.158</td>
</tr>
<tr>
<td>Age, current smoking, aspirin, oral hypoglycemic, and statin use</td>
<td>1.30</td>
<td>0.84–2.02</td>
<td>0.240</td>
</tr>
<tr>
<td>Ln(IL-6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>1.13</td>
<td>0.97–1.33</td>
<td>0.124</td>
</tr>
<tr>
<td>Age, current smoking, aspirin and oral hypoglycemic use</td>
<td>1.14</td>
<td>0.92–1.43</td>
<td>0.237</td>
</tr>
<tr>
<td>Age, current smoking, aspirin, oral hypoglycemic, and statin use</td>
<td>1.07</td>
<td>0.86–1.35</td>
<td>0.546</td>
</tr>
</tbody>
</table>

*Covariate was statistically significant in the model. $P<0.05$.

$e^{\text{CAAD}}$, the regression coefficient, is interpretable as the fold increase in response for men with CAAD vs men without CAAD.

Discussion

In this study, we took advantage of the seasonal influenza vaccination to investigate whether in vivo vaccine response was predicted by vascular disease status. Our results are in agreement with previous studies showing that vaccination does induce a mild acute immune response. However, previous studies were conducted in small numbers of healthy volunteers. Our study is unique in contrasting the APR among diseased and healthy men. We observed a larger inflammatory response in men with extant severe CAAD than in men without severe CAAD. Although our study was limited in size, we found a consistently increased response in men with versus without CAAD across different inflammation markers, but only
SAA was significantly higher in men with CAAD. Normal plasma levels of SAA are estimated to be approximately 3 mg/L in individuals without chronic inflammatory conditions but may increase to >1000 mg/L within 24 to 48 hours of an acute stimulus. Our results suggest that the magnitude of SAA response after flu vaccination is approximately 60% higher in men with versus without severe CAAD. Although the degree of immune response may depend on vaccine formulation, all participants in this study received the same dose of inactivated flu vaccine. Because of study logistics, we may have failed to measure IL-6 at its peak. We observed a larger change in IL-6 at 24 than 4 hours (data not shown). Tsai et al found a significant increase in IL-6 1 day after flu vaccination in healthy individuals, but did not serially measure for the peak. In a study of cytokine levels after surgery in middle-aged subjects, IL-6 was found to peak after 4 to 6 hours, whereas in a small study of yellow fever vaccination, daily measures of IL-6 indicated a peak at 5 days after vaccination. Because of the large individual variability in IL-6, we may have needed additional measurements to best characterize the IL-6 response.

Previous research has reported increased levels of inflammatory markers during convalescence, after surgery, and during stroke events. In particular, research on stroke victims has suggested that levels of CRP after a stroke event may predict severity and recurrence of future events within 1 year, independent of other risk factors. While these results may be caused by pre-event elevations or post-event responses, our results suggest that APR measurement may provide different information about the burden of inflammation than measurement of chronic levels of inflammatory markers. Using Bland-Altman plots, we did not see evidence that the magnitude of change during the acute phase response was dependent on level of the marker. Baseline levels, although higher in men with CAAD, were not positively correlated with response, which suggests that the magnitude of APR, if a vascular disease risk factor, may predict vascular disease independent of baseline levels of inflammatory markers. Using logistic regression models in which we assessed the associations between CAAD status and the SAA response and baseline level independently and together in the same model, we provided additional evidence that the APR variable is associated with CAAD status independently of baseline SAA level.

Men with previous severe stenosis were very homogeneous with respect to disease severity and the majority were taking statins and low-dose aspirin. Both statins (3-hydroxy-3-methylglutaryl (HMG) coenzyme A (CoA) reductase inhibitors) and aspirin have anti-inflammatory effects, with aspirin known to impede the NFkB response. Statins, which are commonly prescribed as lipid-lowering agents, have also been reported to lower CRP levels independent of their effects on lipids. In contrast to the anti-platelet clotting effect, which persists until the turnover of platelets (~7 days), the anti-inflammatory effects of aspirin are largely transient, although in one controlled trial, high doses (300 mg/d) of aspirin were reported to modestly decrease levels of IL-6 and CRP after 6 weeks of treatment. Most subjects in our study were on low-dose aspirin. When 15 men on high-dose aspirin were excluded from the analysis, we found slightly higher and more significant responses in men with CAAD than men without for all markers. We were unable to do a similar sensitivity analysis excluding men on statins because of the high prevalence of use in men with CAAD. Inclusion of the 3 subjects on insulin did not influence the results.
APR-related HDL changes may provide a mechanism for the association between APR and risk of CAAD. Unlike many inflammatory markers, HDL levels decrease during acute inflammation.34 HDL is believed to be protective against atherosclerosis because its major protein component, apolipoprotein AI (apoAI), also mediates cholesterol transport from cells.35 However, HDL loses its anti-inflammatory properties during infection, as measured by a decreased ability of the HDL to inhibit oxidation of LDL and LDL-induced monocyte migration in the arterial wall.36 HDL is also a transporter of SAA.37 During the APR, increased plasma SAA associates with HDL and may displace apoAI.34,38 HDL has been found in plaque39,40 and is reported to co-localize in plaque with SAA, suggesting that SAA-enriched HDL contributes to atherosclerotic plaque.41 Consequently, it has been hypothesized that under circumstances in which SAA is elevated on HDL, such as the APR, the ability of HDL to achieve cholesterol transport is diminished; HDL and the cholesterol it transports may be incorporated into plaque and, thus, contribute to atherosclerosis.34,41 Given this hypothesis, it is intriguing that the SAA APR was more strongly associated with CAAD status than were the other factors.

The APR may trigger both systemic and local (at the vessel wall) inflammation. Both types of inflammation may contribute to cardiovascular disease, but local inflammation is difficult to measure and quantify in large populations. In our study we investigated whether the increased APR is associated with a history of severe vascular disease. Although the acute response to illness or injury may be etiologically relevant for cardiovascular disease, the speed and degree of recovery are also important because chronic inflammation is known to be a risk factor for cardiovascular disease.32,42 The acute response and recovery are likely to be genetically modulated. In a small study, Verschuer et al found some evidence to attribute inter-individual variation in APR among healthy volunteers to genetic differences.9 Future studies aimed at describing the relationship between the magnitude of the APR and genetic variation in acute phase reactants may contribute to our understanding of the role of interindividual variation in disease susceptibility.

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Disclosures

None.

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