Infliximab Improves Endothelial Dysfunction in Systemic Vasculitis
A Model of Vascular Inflammation

A.D. Booth, MRCP; D.R.W. Jayne, FRCP; R.K. Kharbanda, MRCP; C.M. McEniery, PhD; I.S. Mackenzie, MRCP; J. Brown, FRCP; I.B. Wilkinson, MRCP

Background—Endothelial vasomotor dysfunction and markers of systemic inflammation are independent determinants of cardiovascular risk. However, the link between clinical inflammation and endothelial dysfunction is unclear. The aim of this study was to use anti–neutrophil cytoplasmic antibody–associated systemic vasculitis (AASV) as a model of systemic inflammation in which to test the hypothesis that inflammation is associated with endothelial dysfunction and can be reversed with anti–tumor necrosis factor–α (TNF–α) therapy.

Methods and Results—Fourteen patients with active AASV and 21 age-matched control subjects were studied. Endothelial function was assessed through the use of forearm plethysmography and related to clinical disease activity: Birmingham Vasculitis Activity Score (BVAS) and serum levels of C-reactive protein (CRP), interleukin-6 (IL-6), and TNF–α. The effects of anti–TNF–α therapy (infliximab), either alone (n=6) or in combination with standard treatment (n=4), on endothelial function were subsequently determined. Patients had a mean BVAS of 11±1, and CRP and IL-6 were higher in the AASV group than in control subjects (34.8±10.5 versus 1.6±0.2 pg/mL, P<0.001; 9.0±0.7 versus 6.7±0.6 pg/mL, P=0.02). Forearm blood flow response to acetylcholine (ACh) was reduced in the patients compared with control subjects (P=0.002), but sodium nitroprusside (SNP) responses were not (P=0.3). The response to ACh improved with infliximab treatment (P=0.004) in particular, with infliximab alone (P=0.03).

Conclusions—AASV is associated with endothelial dysfunction. Anti–TNF–α therapy, alone or in combination with standard treatment, results in clinical remission, reduced inflammation, and improved endothelium-dependent vasomotor responses. (Circulation. 2004;109:1718-1723.)

Key Words: endothelium ■ inflammation ■ vasculature ■ antibodies
The investigations conformed to the principles outlined in the Declaration of Helsinki.

Study Population
A sequential cohort of 14 patients, between 18 and 85 years of age, with a diagnosis of AASV according to international consensus definitions,\textsuperscript{14} were recruited from the vasculitis clinic at Addenbrooke’s Hospital, Cambridge, United Kingdom, over an 8-month period. Disease activity was scored through the use of the validated Birmingham Vasculitis Scoring Index (BVAS): BVAS ≥1 defined remission and active disease was defined by a BVAS >8.\textsuperscript{19} Concomitantly, 21 healthy volunteers from the community were also recruited into the study. The groups were matched for age, gender, body composition, and lipid profiles. Subjects with coronary artery disease, hypertension (blood pressure ≥140/90 mm Hg), diabetes, hypercholesterolemia (total cholesterol >6.5 mmol/L), current smokers, a calculated creatinine clearance of <50 mL/min, and those taking vasoactive drugs were excluded.

Assessment of Resistance Vessel Endothelial Function
All studies were performed in the morning in a quiet, temperature-controlled room (22° to 24°C). Subjects abstained from alcohol for 24 hours and from caffeine-containing food and beverages for at least 5 hours before each study. After an overnight fast, subjects rested quietly in the supine position for 30 minutes, and forearm blood flow (FBF) was measured by venous occlusion plethysmography with calibrated mercury-in-silastic strain gauges applied to the widest part of the forearm, as previously described.\textsuperscript{16,17} Upper-arm cuffs were intermittently inflated to 40 mm Hg for 10 seconds every 15 seconds to temporarily prevent venous outflow. During FBF measurement, circulation to the hand was excluded by a wrist cuff that was inflated to 50 mm Hg above systolic pressure for a period of 3 minutes during the plethysmographic readings. Both arms were elevated above the level of the right atrium. Blood pressure was monitored in the noninfused arm, through the use of a validated oscillometric sphygmomanometer (HEM-705CP, Omron Corporation).\textsuperscript{18}

Study Drugs
The brachial artery of the nondominant arm was cannulated with a 27-gauge needle under local anesthesia (0.1% lignocaine; Antigen Pharmaceuticals) to allow infusions of drugs. All drugs were prepared aseptically from freshly opened vials with saline (0.9%; Baxter) as a diluent. Saline was infused for 30 minutes to establish a stable baseline. Endothelium-dependent and endothelium-independent vasodilation was assessed by incremental infusions of acetylcholine (ACh, 7.5 and 15 μg/min) and sodium nitroprusside (SNP, 3.0 and 10.0 μg/min), respectively. Basal release of NO was assessed by infusing the specific l-arginine analogue NO synthase inhibitor LNMMA at 2.0 and 4.0 μmol/min. All drugs were infused for 6 minutes at each dose, and FBF was recorded in both arms for the last 3 minutes. A 20-minute saline washout period allowed blood flow to return to baseline levels after each infusion condition. The drugs were infused at a rate of 1.0 mL/min. The analysis of the plethysmographic readings was performed by a technician unaware of the subject status and blood results.

Biochemical Markers
The CRP and cytokines (IL-6, TNF-α) were determined by ELISA, as described previously.\textsuperscript{19} In addition, serum cholesterol, triglycerides, LDL, HDL, and serum creatinine were determined. ANCA was measured by means of indirect immunofluorescence and ELISA, by conventional methodology.

Experimental Protocols

\begin{table}
\centering
\caption{Demographic Variables and Baseline Biochemical Characteristics of Patients and Control Subjects}
\begin{tabular}{lccc}
\hline
 & Patients & Control Subjects & Significance \\
 & (n=14) & (n=21) & \\
\hline
Age, y & 54±4 & 53±3 & 0.9 \\
Gender, female/male, n (%) & 9/14 (64) & 13/21 (62) & 0.9 \\
Body mass index, kg/m² & 26.4±1.1 & 25.4±1.1 & 0.5 \\
BVAS & 11±1 & ... & \\
CRP, mg/L & 34.8±10.5 & 1.6±0.2 & <0.0001 \\
IL-6, pg/mL & 9.0±0.7 & 6.7±0.6 & 0.02 \\
TNF-α, pg/mL & 11.0±3.4 & 8.1±0.7 & 0.4 \\
ANCA level, IU/mL & 32.7±8.5 & 0.7±0.3 & <0.0001 \\
Creatinine, μmol/L & 138±35 & 79±4 & 0.1 \\
Glucose, mmol/L & 5.0±0.3 & 4.7±0.2 & 0.3 \\
Cholesterol, mmol/L & 5.9±0.4 & 5.5±0.3 & 0.4 \\
LDL, mmol/L & 3.5±0.3 & 3.5±0.3 & 0.8 \\
HDL, mmol/L & 1.6±0.1 & 1.4±0.1 & 0.3 \\
TG, mmol/L & 1.8±0.2 & 1.3±0.2 & 0.1 \\
\hline
Values represent mean±SEM.
\end{tabular}
\end{table}

Study 1: Assessment of Endothelial Function in Patients With Active Vasculitis
Baseline demographics were recorded for each subject. Venous blood (30 mL) was drawn into EDTA tubes and immediately centrifuged, and the supernatant was stored at −80°C for subsequent analysis. Resistance vessel endothelial function was assessed as described above. At the time of study, 14 patients had active AASV. Matched control subjects were studied concomitantly with the use of an identical protocol.

\textbf{Study 2: Effects of Treatment on Endothelial Function}
Ten of 14 patients with active disease agreed to enter an established clinical pilot study,\textsuperscript{20} in which patients received 5 infusions of the human-mouse chimeric monoclonal antibody infliximab, directed against TNF-α (5 mg/kg) at 0, 2, 6, and 10 weeks. Infliximab was given either alone (n=6) to patients receiving stable prednisolone doses (unchanged for a month) and mycophenolate mofetil or in addition to standard induction therapy of prednisolone and cyclophosphamide (n=4). Resistance vessel endothelial function was assessed at baseline and again 12 weeks later.

Statistical Analysis
All results are expressed as mean±SEM. Data were analyzed with the use of the SPSS PC statistical package (version 11). Group comparisons were made by means of repeated-measures ANOVA followed by post hoc Student’s t tests with a Bonferroni correction applied for multiple comparisons. FBF data are presented either as the percentage change from baseline in the ratio of blood flow between the infused and noninfused arms or as the area under the curve (AUC) expressed in arbitrary units. Linear regression analysis and bivariate correlation were used to compare FBF responses and inflammatory markers. Values of IL-6 and CRP were significantly skewed and therefore a log transformation was applied before performing bivariate correlation. A probability value of <0.05 was considered significant.

Results

\textbf{Study 1}
Of the 14 patients with AASV, 9 had a diagnosis of Wegener’s granulomatosis, 3 had microscopic polyangiitis, and 2 had Churg-Strauss angiitis. Table 1 shows the demographic variables and baseline biochemical characteristics of the patients and control subjects. One patient was free from medication, but otherwise treatment included daily prednisolone (n=11; mean dose, 13±2 mg) in addition to cyclo-
phosphamide (n=5), azathioprine (n=1), or mycophenolate mofetil (n=6).

The mean BVAS of the patients with active AASV was 11±1. CRP levels were significantly higher in the AASV group than in control subjects (34.8±10.5 versus 1.6±0.2 mg/L; P<0.001), as were serum IL-6 levels (9.0±0.7 versus 6.7±0.6 pg/mL; P=0.02). However, circulating TNF-α levels were not significantly different between the groups (P=0.4). The serum creatinine values were not statistically different between the study groups, and neither were the calculated creatinine clearance values (103.8±7.2 and 66.0±8.6 mL/min in control subjects and patients, respectively). Basal flow in the infused arm was similar in both active disease and control groups (2.4±0.7 and 3.1±1.3 mL/100 mL per minute, respectively; P=0.9), and there was no change in heart rate or blood pressure measured in the noninfused arm of subjects during each study. The FBF response to ACh was significantly reduced in subjects with active disease compared with control subjects (P=0.002) (Figure 1A), but there was no difference in the response to SNP between the groups (P=0.2) (Figure 1B). The response to LNMMA was not significantly different between diseased and control groups (P=0.3) (Figure 1C).

Study 2
Ten patients with active disease (BVAS, 12±1) subsequently received intravenous infliximab. Infliximab was added as additional therapy to existing prednisolone doses (mean dose, 13.0±2.5 mg) in 6 patients and accompanied by reducing doses of steroids (initially 1 mg/kg) and cyclophosphamide (1.5 mg/kg) in 4 patients, with the use of protocols previously described.21 Table 2 shows the effect of treatment on disease activity and circulating biochemical markers. All 10 patients achieved clinical remission (mean BVAS, 12±1, fell to 0). CRP and IL-6 levels fell with treatment (40.4±13.8 to 2.0±0.5 mg/L, P=0.01, and 10.8±0.8 to 6.3±0.9 pg/mL, P=0.002, respectively), but ANCA levels did not change significantly (P=0.1). There was no significant treatment-related change in baseline blood flow in the infused arm (2.5±0.9 to 2.2±0.8, P=0.9). However, the FBF response to ACh but not to SNP increased significantly with treatment (P=0.004 and P=0.2, respectively), compared with baseline response (Figure 2). Indeed, the posttreatment ACh and SNP responses did not differ significantly from those of the control group (P=0.9 and P=0.3, respectively). The 6 patients treated with infliximab alone also showed a significant improvement in the responses to ACh (P=0.03) but not SNP (P=0.9, ANOVA).

The responses of the 10 patients who subsequently received infliximab were not different from the active group as a whole.

**Relation Between Inflammation and FBF**
The degree of clinical inflammatory disease activity, through the use of BVAS, was inversely correlated with ACh response (AUC; r=-0.36, P=0.01) and CRP (r=0.35, P=0.01). Furthermore, log [CRP] was inversely correlated

**Figure 1.** A, Blood flow responses to 2 doses of ACh (7.5 and 15 μg/min); B, SNP (3.0 and 10.0 μg/min); and C, LNMMA (2.0 and 4.0 μmol/min) in patients with active disease (n=14 [○]; control subjects, n=21 [×]). Blood flow is expressed as change in FBF ratio (%). Response to ACh but not SNP or LNMMA was significantly reduced in active versus control group (P=0.002, P=0.2, and P=0.3, respectively) by 2-way ANOVA. **P<0.005.
TABLE 2. Effect of Treatment on Disease Activity and Circulating Biochemical Markers

<table>
<thead>
<tr>
<th></th>
<th>Before Treatment</th>
<th>After Treatment</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>BVAS</td>
<td>12 ± 1</td>
<td>0 ± 1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>40.4 ± 13.8</td>
<td>2.0 ± 0.5</td>
<td>0.01</td>
</tr>
<tr>
<td>IL-6, pg/mL</td>
<td>10.8 ± 0.8</td>
<td>6.3 ± 0.9</td>
<td>0.002</td>
</tr>
<tr>
<td>TNF-α, pg/mL</td>
<td>12.7 ± 4.8</td>
<td>15.0 ± 5.2</td>
<td>0.8</td>
</tr>
<tr>
<td>ANCA level, IU/mL</td>
<td>51 ± 13</td>
<td>23 ± 13</td>
<td>0.1</td>
</tr>
<tr>
<td>Creatinine, µmol/L</td>
<td>159 ± 48</td>
<td>126 ± 26</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM.

with the response to ACh (AUC: \( r = -0.35, P = 0.009 \)) but not baseline flow or responses to LNMMA or SNP. Log [IL-6] correlated with Log [CRP] (\( r = 0.43, P = 0.006 \)) but not the ACh response (AUC; \( P = 0.2 \)). Log [TNF-α] levels were not found to correlate with any of the measures of systemic or clinical inflammation or the response to ACh (AUC; \( P = 0.2 \)). There was no linear or curvilinear relation between the measures of renal function and endothelial function (percent change or AUC).

**Discussion**

In the present study, the hypothesis that systemic inflammation is associated with reversible endothelial dysfunction was tested in a cohort of subjects with AASV. The main findings were that active disease was associated with resistance vessel endothelial dysfunction and, as expected, raised inflammatory markers. Moreover, both clinical disease activity (BVAS) and more direct measures of systemic inflammation (CRP) correlated with the degree of endothelial dysfunction. Induction of remission through the use of anti–TNF-α therapy resulted in a reduction in inflammation and normalization of endothelium-dependent vasomotor responses. Together, these data suggest that the impaired endothelial vasomotor function in AASV is related to clinical disease activity and to the degree of systemic inflammatory response and that endothelial dysfunction can be reversed through the use of specific anti–TNF-α treatment.

Endothelial dysfunction, reflecting reduced bioavailability of NO, is a key early finding in atheroma formation. Although atherosclerosis is principally a conductance vessel phenomenon, endothelial dysfunction is present in both resistance vessels and conduit vessels of patients with, or at risk of, coronary artery disease. Furthermore, endothelial dysfunction in either vessel type independently predicts cardiovascular risk. Circulating measures of systemic inflammation, such as CRP, IL-6, and TNF-α, also predict cardiovascular events.\(^{22}\) A link between chronic low-grade inflammation and the slow process of atherosclerosis has been reported. Indeed, patients with chronic inflammatory autoimmune disease such as rheumatoid arthritis are at increased cardiovascular risk,\(^{23}\) and preliminary studies suggest this is also true for systemic vasculitis.\(^{24}\) Moreover, in patients with acute coronary disease, an association between systemic inflammation and a transiently increased risk of future cardiovascular events has also been reported. Understanding the link between systemic inflammation and endothelial dysfunction in vivo may therefore improve our understanding of the process of atheroma formation.

Acute experimental inflammation, induced by typhoid vaccination in healthy volunteers, is associated with endothelial dysfunction in both large (conduit) and small (resistance) vessels.\(^{13,25}\) Although vasomotor function in subjects with chronic inflammatory diseases such as rheumatoid arthritis or systemic vasculitis has been studied previously, the results are conflicting. In patients with rheumatoid arthritis, both normal and abnormal endothelial function has been described in conduit vessels.\(^{26,27}\) Moreover, one study of resistance vessel function demonstrated blunted endothelium-dependent

![Figure 2. Responses to ACh (A) and SNP (B) in 10 patients with active disease are shown before (○) and after (●) treatment. Response to ACh but not SNP improved significantly with treatment (\( P = 0.004 \) and \( P = 0.2 \), respectively). **\( P < 0.005 \).](http://circ.ahajournals.org/)
responses, but another demonstrated blunted endothelium-dependent and endothelium-independent responses.\textsuperscript{20,29} AASV encompasses a group of systemic vasculitides, predominantly characterized by inflammation of microscopic vessels. It is invariably associated with an elevated acute-phase response, but effective treatment results in >90% of patients achieving clinical remission.\textsuperscript{21} TNF-\(\alpha\) is considered to play a pivotal role in the pathogenesis of AASV.\textsuperscript{30} Interestingly, in cultured cells, TNF-\(\alpha\) reduces endothelial NO bioavailability, and in vivo TNF-\(\alpha\) may also directly alter endothelial vasomotor function.\textsuperscript{31} Therefore, we elected to use primary systemic vasculitides as a model of clinical inflammation. Although two previous studies have investigated vasomotor function in patients with AASV, they provide contradictory results. In one large study of conduit vessel function, impaired endothelium-dependent dilation (using the technique of flow-mediated dilation) was reported.\textsuperscript{32} However, in another small study of 10 patients (7 with AASV), an enhanced vasodilator response to ACh in resistance vessels using forearm plethysmography was demonstrated.\textsuperscript{33} Neither study reported a relationship between vasomotor responses and the degree of inflammation.

In the present study, patients with active clinical disease had elevated systemic inflammatory markers (CRP and IL-6). Indeed, CRP and IL-6 were strongly correlated with each other, which is perhaps not unexpected, given that IL-6 induces synthesis of acute-phase proteins, including CRP. Furthermore, CRP was positively correlated with the degree of inflammatory disease activity (BVAS). Active disease was associated with endothelial dysfunction, characterized by a blunted response to ACH and preserved SNP response. The degree of endothelial dysfunction was correlated with both clinical disease activity, assessed with BVAS, and also CRP, a measure of the systemic inflammatory response. Interestingly, CRP and IL-6 have been shown to alter endothelial NO bioavailability in vitro,\textsuperscript{34,35} and IL-6 and TNF-\(\alpha\) may also directly alter vasomotor function in vivo. Taken together, these results suggest that inflammation and endothelial dysfunction are not only associated in AASV but that the acute-phase response may directly affect vasomotor function.

Although not significant, serum creatinine values were slightly higher in the patients than in control subjects. An association between endothelial dysfunction and renal impairment/failure has been demonstrated previously, but only in patients with more severe renal impairment (mean creatinine clearance, 30 mL/min)\textsuperscript{36} and concomitant additional cardiovascular risk factors. We are unaware of any such relation within the clearance range reported in the present study. Indeed, in the present study, there was no linear or curvilinear relation between serum creatinine and endothelial function. Therefore, differences in renal function are unlikely to account for the differences reported.

Despite demonstrating endothelial dysfunction, the vasomotor response to LNMMA was no different between patients with AASVs and control subjects, suggesting similar basal NO production. However, LNMMA is a nonspecific inhibitor of NO synthase (NOS), blocking both endothelial (eNOS) and inducible (iNOS) isoforms, and ACh only stimulates eNOS. Therefore, one potential explanation for this paradox is a relative increase in vascular iNOS activity. Indeed, histologically increased iNOS expression has been demonstrated in patients with active AASV.\textsuperscript{37} However, whether such a situation of normal basal but reduced stimulated NO synthesis is detrimental remains unclear, as does whether stimulated rather than basal NO best correlates with risk.

The present study also addressed the effects of anti-inflammatory therapy on inflammation and endothelial dysfunction. Interestingly, anti–TNF-\(\alpha\) therapy not only significantly reduced disease activity and suppressed serum inflammatory markers (CRP and IL-6) but also led to a significant improvement in endothelial function. This indicates that endothelial dysfunction is potentially reversible in AASV and further strengthens the link between inflammation and endothelial dysfunction in this model of clinical inflammation. Only one previous study in patients with rheumatoid arthritis has investigated the effect of infliximab on endothelial function.\textsuperscript{37} Although treatment was associated with improvement in endothelial conduit vessel function and disease activity, CRP did not change, and cytokine levels were not reported in this study.

Although TNF-\(\alpha\) is thought to play a pivotal role in the pathogenesis of AASV, treatment with anti–TNF-\(\alpha\) antibodies did not result in any change in serum TNF-\(\alpha\) levels, despite improving endothelial function. This is perhaps surprising, but previous studies measuring circulating TNF-\(\alpha\) in AASV have provided inconsistent results and trials of infliximab in patients with rheumatoid arthritis have failed to demonstrate a reduction in circulating TNF-\(\alpha\) levels. Moreover, tissue levels of cytokines are likely to be more important in the disease process, and currently available ELISAs measure total TNF-\(\alpha\) but not free TNF-\(\alpha\), which may be more closely related to disease activity. Nevertheless, treatment with anti–TNF-\(\alpha\) therapy resulted in clinical remission. TNF-\(\alpha\) is thought to drive IL-6 expression, and the clinical benefit in treated patients with AASV may arise from the resulting reduction of IL-6 and CRP.

In summary, we have used AASV as a model of systemic inflammation with which to show that endothelial dysfunction in resistance vessels parallels disease activity and systemic inflammation. Treatment involving infliximab reduces systemic inflammation and improves endothelial function and therefore may improve systemic vascular health and reduce cardiovascular risk.

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