Progression of Early Carotid Atherosclerosis Is Only Temporarily Reduced After Antibiotic Treatment of Chlamydia pneumoniae Seropositivity

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Background—Chlamydia pneumoniae (Cp) infection has been associated with atherosclerosis and cardiovascular events. There are controversial results regarding the beneficial effects of antibiotic therapy on future cardiovascular end points.

Methods and Results—We determined the long-term effect of a 30-day roxithromycin therapy on intima-to-media thickness (IMT) progression of the common carotid artery in 272 consecutive Cp-positive and Cp-negative patients with ischemic stroke in a prospective, double-blind, randomized trial with a follow-up of 4 years. Cp IgG (>1:64) or IgA (>1:16) antibodies were initially found in 125 (46%) patients. During the 3 years before antibiotic therapy, Cp-positive patients showed an enhanced IMT progression even after adjustment for other cardiovascular risk factors (0.12 [0.11 to 0.14] versus 0.07 [0.05 to 0.09] mm/year; \(P<0.005\)). The 62 Cp-positive patients given roxithromycin showed a reduced IMT progression during the first 2 years compared with the Cp-positive patients without therapy (0.07 [0.045 to 0.095] versus 0.11 [0.088 to 0.132] mm/year; \(P<0.01\)). However, IMT progression increased again during the third and fourth year to similar values as before treatment. No significant difference in the occurrence of future cardiovascular events was found between both groups during follow-up.

Conclusions—The only limited positive impact of antibiotic therapy on early atherosclerosis progression in Cp-positive patients observed in our study may explain the negative results of most antibiotic trials on clinical end points.

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Key Words: atherosclerosis ■ infection ■ stroke ■ ultrasonics ■ inflammation

Increasing evidence has linked infection to atherosclerosis, myocardial infarction, and stroke.1,2 Present research on the infectious hypothesis has focused mainly on Chlamydia pneumoniae (Cp) and Helicobacter pylori. The evidence for Cp as a potential causative agent is based on findings of seroepidemiological studies,3 histopathological examinations,4 and in vitro animal models.5 Therefore, therapy with antibiotics may favorably influence the natural course in patients with cardiovascular disease. Pilot antichlamydial antibiotic intervention trials showed a potential beneficial effect of such treatment.6 However, several larger antibiotic studies found no effect on clinical events,7-10 and some investigators hypothesized that Cp may be refractory to treatment11 and that longer treatment periods or repeated treatment is necessary.6 It has been suggested that the detection of a positive effect of antibiotic therapy on clinical end points may need even longer patient numbers or longer follow-up periods12 and that it therefore seems reasonable to analyze the impact of antibiotic therapy on surrogate parameters of atherosclerosis formation.

High-resolution B-mode ultrasonography has proved to be a valid and reliable method of detecting initial structural atherosclerotic changes. Increased intima-to-media thickness (IMT) of the common carotid artery (CCA) is a powerful predictor of the presence of coronary atherosclerosis and its clinical sequelae.13 We demonstrated an enhanced IMT progression of the CCA in Cp-positive patients during 3 years of follow-up14 and a positive effect of antibiotic therapy with roxithromycin on IMT progression during the first 2 years after treatment.15 In this investigation, we reported the 4-year follow-up data to determine the long-term effects of antibiotic therapy on the progression of CCA IMT and inflammatory markers.

Methods

Subjects

From a series of 290 initially evaluated inpatients >55 years of age (mean age, 64 years [95% CI, 62 to 67]; 161 men) admitted to the Department of Neurology, Technical University of Munich, because of a first-ever acute cerebral ischemia, 272 patients with transient ischemic attacks (n=71) or minor ischemic stroke (n=201) were randomized. Eighteen patients died during the 3-year baseline period (myocardial infarction, n=11; sudden death, n=1; stroke, n=6).
Baseline characteristics of the randomized patients are given in the Table.

**Study Design**
All 272 patients were studied for 3 years before the initiation of antibiotic treatment. The baseline study was followed by antibiotic treatment and 4 years of follow-up. Details of the baseline investigation and the results of the first 2 years after antibiotic treatment are given elsewhere. A complete 4-year follow-up after antibiotic treatment and 4 years of follow-up are performed by the same investigators using a computerized questionnaire every year. All fatal and nonfatal events were independently coded by 2 physicians (D.S. or J.K.). Copies of hospital records, autopsy records, and death certificates were available. Risk factors were determined as previously described in detail.

**Laboratory Examinations**
Nonfasting blood samples were drawn from each subject within 6 hours after hospitalization, and serum was separated by centrifugation within 6 hours and stored at −20°C until analysis. Cp (TWAR) titers were measured by microimmunofluorescence initially, before onset of antibiotic treatment, and after 2 and 4 years of follow-up, as previously described in detail. IgA titers ≥1:16 or IgG titers ≥1:64 were taken as positive and IgA titers ≤1:8 or IgG titers ≤1:32 were taken as negative according to previous studies and recommendations using microimmunofluorescence techniques to analyze the relationship between carotid atherosclerosis and Cp. Acute infection or reinfection just before testing was presumed to be indicated by titers of IgG ≥1:512 and IgM ≥1:8.

C-reactive protein (CRP) concentration (Dimension RxL clinical chemistry analyzer with CRP Flex reagent cartridges) was measured initially within 6 hours after hospitalization and every year at the day of the ultrasound examination. The assay range was 0.05 to 12 mg/dL. A concentration ≥0.5 mg/dL was defined as pathologically increased according to the reference values of our laboratory.

**Ultrasound Imaging**
The initial Duplex ultrasonography and the follow-up investigations (every year) were performed by the same investigators using a 7.5-MHz linear-array transducer. The measurements of CCA IMT were done as previously described in detail. The Spearman correlation between all the IMT measurements before treatment and 2 years later were 0.87 (Cp positive [IgG ≥1:64 or IgA ≥1:16]) and 0.85 (Cp negative [IgG ≤1:32 or IgA ≤1:8]), indicating a good reproducibility of the IMT measurements during follow-up. The progression of early carotid atherosclerosis was calculated as the difference between 2 consecutive IMT measurements (3 years during baseline and every year during follow-up) and was normalized as the change of IMT per year.

**Antibiotic Therapy**
After initiating conventional therapy and obtaining informed consent, patients were randomized to receive either the second-generation macrolide roxithromycin 150 mg twice daily or matching...
placebo twice daily for 30 days using a double-blind design. Sixty-two Cp-positive (IgG ≥1:64 or IgA ≥1:16) patients received roxithromycin, and 63 positive patients received placebo. Roxithromycin was given to 74 Cp-negative (IgG ≤1:32 or IgA ≤1:8) patients, and 73 negative patients were randomized to placebo. Follow-up visits were scheduled at day 31 and then every year. All patients were advised to take 2 study tablets per day. No patient received macrolide antibiotics outside the study. Additional other antibiotic medication (mainly short-period ciprofloxacin or amoxicillin) was prescribed during follow-up for 18 of 136 patients in the roxithromycin group and for 23 of 136 patients in the placebo group. This distribution in favor of the placebo group ruled out a possible beneficial effect in favor of the roxithromycin subgroup.

**Statistical Analysis**
A follow-up period of 4 years and 60 patients per group were planned to provide a statistical power of 90% using a 2-sided α of 0.05. Randomization lists were based on a computer-generated sheet of randomization numbers (StatMate, Graphpad Inc). Analysis of all end points was by intention to treat. All values are given as mean and 95% CI. Independent t tests were used to test differences between groups. Adjustment for multiple comparisons was done by using the Bonferroni method. The variation in IMT between subgroups according to age, pack-years of smoking, CRP, prevalent ischemic heart disease (IHD), cholesterol, diabetes as well as systolic and diastolic blood pressure was tested with an ANCOVA using SYSTAT (Systat Inc). Because the CRP was highly skewed (Kolmogorov-Smirnov test), the CRP levels were natural-log-transformed before additional analysis. Survival curves were estimated using the Kaplan-Meier product-limit method. Hazard ratios were calculated with the Cox proportional hazard regression model. A calculated difference of P<0.05 was considered to be statistically significant.

**Results**
Cp IgG antibodies (≥1:64) were initially found in 123 (45%) patients, and IgA antibodies (≥1:16) were found in 119 (41%) patients. Overall, 125 patients (46%) were Cp positive (IgG ≥1:64 or IgA ≥1:16). During the 3 years before onset of treatment, Cp-positive patients showed a significant enhanced IMT progression, even after adjustment for other cardiovascular risk factors (0.12 mm/year [95% CI, 0.11 to 0.14] versus 0.07 mm/year [0.05 to 0.09]; P=0.0048). No significant differences between the corresponding patient subgroups were found for several cardiovascular risk factors (Table).

**Effect of Roxithromycin on IMT Progression**
A total of 272 patients were randomized. The baseline IMT progression of the placebo and roxithromycin subgroup was comparable in the Cp-positive (IgG ≥1:64 or IgA ≥1:16) and Cp-negative (IgG ≤1:32 or IgA ≤1:8) patients (Figure 1). The 62 Cp-positive patients given roxithromycin showed a reduced IMT progression after 1 year (0.06 mm/year [0.04 to 0.08]; P=0.0008) and 2 years (0.07 mm/year [0.048 to 0.09]; P=0.009) compared with the baseline values as well as compared with the corresponding values of the placebo-treated subgroup (Figure 2, top). The IMT progression increased after 3 years and was comparable to baseline values and the placebo-treated group after 4 years (Figure 1, top). Additionally, the proportion of patients with an enhanced IMT progression >0.1 mm/year was significantly reduced because of antibiotic treatment compared with baseline (77.4%) only during the first (29%; P<0.001) and second year (33.9%; P<0.005) but not the third (59.7%) and fourth (72.6%) years. No significant reduction was seen throughout the follow-up in the placebo-treated group. No change of IMT progression was observed in Cp-negative (IgG ≤1:32 or IgA ≤1:8) patients given roxithromycin compared with those given placebo (Figure 1, bottom).

**Effect of Roxithromycin on Cp Antibodies**
The treatment with roxithromycin did not change the prevalence of IgG or IgA antibodies during follow-up.

**Effect of Roxithromycin on CRP**
Before treatment, CRP levels were significantly increased (P<0.001) in Cp-positive patients (IgG ≥1:64 or IgA ≥1:16) compared with Cp-negative patients (IgG ≤1:32 or IgA ≤1:8) (Figure 2). Treatment with roxithromycin significantly decreased the CRP in Cp-positive patients compared with the pretreatment value during the first 2 years and to a smaller extent after 3 years, whereas no significant change of CRP could be found in the Cp-positive placebo group (Figure 2, top). This positive effect of roxithromycin therapy on CRP remained nearly unchanged even after adjustment for age,
diabetes, pack-years of smoking, systolic blood pressure, cholesterol, and IHD using an ANCOVA procedure. After 4 years of follow-up, CRP values were again comparable to the pretreatment values (Figure 2, top). In the Cp-negative group, antibiotic therapy led to a slight but significant decrease of CRP compared with the pretreatment value only after 2 years. However, this difference was not significant if compared with the corresponding Cp-negative placebo group (Figure 2, bottom).

**Effect of Roxithromycin on Outcome Events**

During the 4-year follow-up, 35 (12.9%) of the 272 patients developed 11 fatal and 30 nonfatal cardiovascular (myocardial infarction [n=110057] and cerebrovascular events (recurrent transient ischemic attack [n=110058] or stroke [n=1100515]). Kaplan-Meier survival analysis (Figure 3) revealed a significantly higher rate of secondary end points (vascular morbidity and death) in Cp-positive (IgG ≥1:64 or IgA ≥1:16; top) and Cp-negative (IgG ≤1:32 or IgA ≤1:8; bottom) patients given roxithromycin compared with patients given placebo during baseline (3 years) and follow-up (4 years). Hazard ratios are adjusted for CRP, age, systolic and diastolic blood pressure, smoking, diabetes, cholesterol, and prevalent IHD.

**Discussion**

We recently demonstrated a significant reduction of carotid IMT progression in patients with Cp positivity (IgG ≥1:64 or IgA ≥1:16) and antibiotic treatment with roxithromycin for 30 days after 2 years of follow-up.15 These data implied that antibiotic treatment in Cp-positive patients older than 55 years of age with prevalent cerebrovascular disease is associated with a reduced progression of early stages of carotid atherosclerosis and were in accordance with several clinical and experimental findings.17–19 However, the combined frequency of stroke, myocardial infarction, or vascular death was not diminished during the 2 years of follow-up by roxithromycin. Apart from considerations of sample size and duration of follow-up, these findings are comparable with several other larger antibiotic trials performed that also revealed no significant impact of antibiotic therapy on clinical end points.6,10

It has been suggested that permanent eradication might be needed for sustained prevention of atheroma progression and ischemic complications. In the present investigation we therefore evaluated whether our treatment regimen (1-shot 30-day application of roxithromycin) is sufficed to eradicate, rather than temporarily suppress, vascular infection with Cp by analyzing the long-term impact of this therapy on IMT progression.
progression and CRP changes. Ultrasonographically determined increased IMT of the CCA was identified as a strong predictor of stroke and myocardial infarction\textsuperscript{13} and has been used to monitor beneficial effects of medical treatment on atherosclerosis in addition to clinical end points in several large trials. Our present results of a reduced IMT progression for only 2 years after initial therapy and a progression rate comparable to baseline after 4 years imply that our treatment regimen, which is presently used similarly in several ongoing clinical trials, has only a temporary effect on early atherosclerotic formation. These findings are in accordance with several experimental data. Gieffers et al\textsuperscript{11} showed that Cp uses monocytes as a transport system for systemic dissemination and enters a persistent state not covered by an otherwise effective antimicrobial treatment. Thus, it is conceivable that circulating monocytes carrying a pathogen with reduced antimicrobial susceptibility might initiate reinfection or promote atherosclerosis by the release of proinflammatory mediators even after antibiotic treatment. In fact, these data and our findings may have serious implications for additional large prospective trials to alleviate clinical end points by antimicrobial treatment. This notion is supported by recent data from clinical treatment trials. A statistically significant benefit among roxithromycin-treated patients was seen after 30 days but not reproduced after 6 months.\textsuperscript{20} In a rabbit model on the acceleration of atherogenesis by Cp infection, azithromycin treatment had a documented protective effect, but Cp antigen was still detected in the vessel walls after a 7-week azithromycin course, indicating persistence.\textsuperscript{21} The large WIZARD trial was considered negative, because the 7\% reduction in events after a follow-up of 1 to 4 years was not statistically significant. However, in the treated group there was a significant 33\% reduction in death and MI during the first 6 months of the trial \((P=0.02)\).\textsuperscript{6} Interestingly, the CLARIFY trial with a longer treatment period of 3 months using clarithromycin observed a reduction of the risk of ischemic cardiovascular events.\textsuperscript{22}

An additional important finding of our study was that the progression of IMT is closely related to the course of CRP levels, which is a serological marker of chronic inflammation. There is increasing evidence that one of the primary mechanisms in atherogenesis is inflammation. Recent in vitro studies have shown that Cp infection in human smooth muscle cells results in production of interleukin-6,\textsuperscript{23} which is the major regulator of CRP production.\textsuperscript{24} If chronic vascular infection with Cp is responsible for CRP elevation, successful treatment should lead to reduced CRP levels. We observed a significant reduction of CRP in the Cp-positive group treated with roxithromycin during the first 2 years of follow-up. In contrast, there was no significant change of CRP in the placebo-treated Cp-positive group and only a slight reduction of CRP after 2 years in the roxithromycin-treated Cp-negative group. However, after 3 and 4 years, there was a steady increase of CRP levels to values comparable to baseline, indicating a reincrease of inflammatory activity most probably attributable to a reactivation of Cp infection. We recently demonstrated the most enhanced IMT progression in patients with both Cp positivity and elevated CRP levels.\textsuperscript{14} Our present results imply a close relation between CRP levels and IMT progression over a longer follow-up period and underscore the importance of inflammatory activity even for early atherosclerotic formation. Recent results of the Helsinki Heart Study showed that persistently elevated levels of Cp-IgA or human heat-shock protein 60 antibodies predicted new coronary events, especially when present together with elevated CRP levels.\textsuperscript{25}

The concept of an infectious component in the chronic inflammatory condition of atherosclerosis is intriguing, because it may provide additional explanations for unclear phenomena of atherogenesis. However, evidence is increasing that eradication might be difficult because of the ability of Cp to enter a refractory state of persistent infection. Based on our findings of a temporarily beneficial effect of antibiotic treatment (30 days of roxithromycin) on IMT progression and CRP, we hypothesize that it is necessary to use longer treatment periods or, in our opinion, more promising repeated treatment regimes in future trials to preserve the beneficial short-term effects of antibiotic therapy seen in several clinical investigations. Two large ongoing clinical trials\textsuperscript{6} with longer treatment periods (ACES, 12 months) or repeated regimes (PROVE IT, intermittent for 2 years) are presently underway and might additionally clarify whether there is an adequate treatment regimen for Cp infection.

\section*{References}
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