Chlamydia pneumoniae Infection in Circulating Human Monocytes Is Refractory to Antibiotic Treatment

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Background—Recovery of the intracellular bacterium Chlamydia pneumoniae from atherosclerotic plaques has initiated large studies on antimicrobial therapy in coronary artery disease. The basic concept that antibiotic therapy may eliminate and prevent vascular infection was evaluated in vitro and in vivo by examining the antibiotic susceptibility of C pneumoniae in circulating human monocytes, which are thought to transport chlamydiae from the respiratory tract to the vascular wall.

Methods and Results—Blood monocytes (CD14+) from 2 healthy volunteers were obtained before and after oral treatment with azithromycin or rifampin and then inoculated with a vascular C pneumoniae strain and continuously cultured in the presence of the respective antibiotic. Progress of infection and chlamydial viability was assessed by immunogold-labeling and detection of C pneumoniae–specific mRNA transcripts. Circulating monocytes from patients undergoing treatment with experimental azithromycin for coronary artery disease were examined for C pneumoniae infection by cell culture. Antibiotics did not inhibit chlamydial growth within monocytes. Electron microscopy showed development of chlamydial inclusion bodies. Reverse transcription–polymerase chain reaction demonstrated continuous synthesis of chlamydial mRNA for 10 days without lysis of the monocytes. The in vivo presence of viable pathogen not eliminated by azithromycin was shown by cultural recovery of C pneumoniae from the circulating monocytes of 2 patients with coronary artery disease.

Conclusions—C pneumoniae uses monocytes as a transport system for systemic dissemination and enters a persistent state not covered by an otherwise effective antichlamydial treatment. Prevention of vascular infection by antichlamydial treatment may be problematic: circulating monocytes carrying a pathogen with reduced antimicrobial susceptibility might initiate reinfection or promote atherosclerosis by the release of proinflammatory mediators. (Circulation. 2001;103:351-356.)

Key Words: Chlamydia pneumoniae ■ atherosclerosis ■ infection ■ azithromycin ■ rifampin

The obligate intracellular pathogen Chlamydia pneumoniae has been related to atherosclerosis by seroepidemiology, detection of chlamydial structures and, most recently, recovery of viable chlamydiae from atherosclerotic plaques.1,2 Chlamydiae may thus have a role in the multifactorial pathogenesis of atherosclerosis, a chronic inflammatory condition of the vascular wall.3,4 On the basis of these findings, antimicrobial treatment studies are currently underway, with the intention to improve the clinical condition of patients with coronary artery disease (CAD) by chlamydial eradication from atheromatous plaques.5 Endothelial and smooth muscle cells can acquire a productive chlamydial infection that can be inhibited in vitro by common antichlamydial antibiotics.6 However, chlamydiae are notorious for causing chronic infections,7 and treatment failure is common. These infections and failures may be due to the establishment of a nonreplicating but viable state of chlamydiae in the host cells.8

Systemic dissemination of C pneumoniae from the respiratory tract to the cells of the vascular wall requires a cellular transport system, because chlamydiae replicate exclusively within their host cells. The elementary body, the metabolically inactive extracellular life stage of chlamydiae, has never been found circulating free within the bloodstream. Circulating monocytes, which are pivotal to the development of atherosclerosis, are known to migrate into the vascular wall. Recent studies have described the in vitro infection of monocyte-derived macrophages with C pneumoniae9 and the presence of chlamydial DNA within peripheral blood mononuclear cells from patients with CAD.10,11 Furthermore, C pneumoniae resists digestion in monocytes for 3 days, at least
under in vitro cell culture conditions.\textsuperscript{12} Thus, monocytes are a potential carrier system for \textit{C pneumoniae}.

We were concerned that a persistent chlamydial infection with reduced antibiotic susceptibility might exist in monocytes in vivo and that it might interfere with the basic concept of antimicrobial treatment in coronary heart disease. If monocyte infection is not eliminated by therapeutic intervention, continuous transmission to vascular cells after a decline of the antibiotic tissue levels might affect long-term benefits. Therefore, we studied chlamydial growth and activity within monocytes in the presence of rifampin, the most effective antichlamydial drug in vitro,\textsuperscript{13} and azithromycin, a macrolide widely used in current treatment trials.\textsuperscript{3} To detect the pathogen, we used immunoelectron microscopy and Chlamydia-specific mRNA transcripts, which are markers of bacterial viability due to their short half-life. In addition, we attempted to culture \textit{C pneumoniae} from monocytes of patients with unstable angina pectoris who were enrolled in a current trial on the benefit of azithromycin in CAD.

**Methods**

**In Vitro and Ex Vivo Infection of Human Monocytes in the Presence of Antibiotics**

To assess the in vitro effect of antibiotics on the development of \textit{C pneumoniae} in human blood monocytes, CD-14+ positive cells were infected with the CV-3 coronary artery isolate of \textit{C pneumoniae}\textsuperscript{1} in the presence of rifampin or azithromycin. Human blood monocytes from 2 healthy male volunteers (28 and 36 years) were separated from 2 healthy male volunteers (28 and 36 years) were separated using a Ficoll-Histopaque density gradient (Sigma) and subsequent positive selection with anti CD-14 microbeads (MACS system, Miltenyi Biotec GmbH). A total of 10^6 CD-14+ positive cells per well were inoculated with 10^6 inclusion-forming units of \textit{C pneumoniae} strain CV-3 and continuously cultivated in 12-well tissue culture plates for up to 10 days in RPMI medium (10% FCS; Gibco/BRL) without antibiotics, with 10 \mu g/mL azithromycin (Pfizer), or with 10 \mu g/mL rifampin (Sigma).

Antibiotics were added simultaneously with the chlamydial. Two hours after inoculation, monocytes were washed once with PBS to remove nonphagocytized elementary bodies, and fresh medium with the respective antibiotic was added. For immunoelectron microscopy, cells were grown on Thermofax cover slips (Nalgene Nunc International) and collected at day 3. For reverse transcription–polymerase chain reaction (RT-PCR) detection of chlamydial mRNA synthesis, 10^6 cells were collected after 2 hours and 1, 3, and 10 days. Experiments were made in duplicate. The culture assay was repeated after oral treatment of the monocyte donors with azithromycin (Zithromax, Pfizer GmbH; 500 mg/d for 3 days) or rifampin (Rifa, after oral treatment of the monocyte donors with azithromycin. Experiments were made in duplicate. The culture assay was repeated

**Immunoelectron Microscopy**

Cells grown on Thermofax slides were fixed with 2% paraformaldehyde and 0.1% glutaraldehyde in PBS (pH 7.4) for 1 hour at 4°C and contrasted with 2% uranyl acetate in cacodylate buffer. After dehydration in a graded acetone series, cells were embedded in LR White (London Resin Co). Ultrathin sections were mounted on 300 mesh nickel grids and were blocked for 30 minutes with 0.5% bovine serum albumin (Sigma) in Tris-buffered saline (TBS). The sections were incubated for 16 hours with chlamydia genus–specific mouse anti-HSP60 IgG (Affinity BioReagents) diluted 1:250 with TBS. After rinsing with TBS, sections were incubated for 2 hours with donkey anti-mouse IgG coupled to 12-nm gold particles (Jackson ImmunoResearch) diluted 1:100 with TBS. Sections were contrasted with uranyl acetate and lead citrate and were examined for immunogold-stained chlamydial structures with a Philips EM 400 electron microscope. In control incubations, normal mouse serum was substituted for the primary antibody.

**Chlamydial mRNA Synthesis in the Presence of Antibiotics**

\textit{C pneumoniae}–specific mRNA was extracted by standard methods using TRIZOL Reagent (Gibco/BRL). RT of RNA and cDNA amplification was performed using the Access RT-PCR System (Promega) according to the manufacturer’s instructions. cDNA synthesis was performed at 48°C for 50 minutes. After 2 minutes of initial denaturation at 95°C, the samples were subjected to 40 cycles of denaturation (95°C, 40 s), annealing (55°C, 90 s), and extension (70°C, 120 s), followed by a final extension at 70°C for 10 minutes. Targets were the genes of the outer membrane protein (MOMP) and the 60-kDa heat shock protein (HSP60). Primers for MOMP mRNA were Cpn 201 (’5’TGTTCTCGAAGACATTITTT-GATG3’) and Cpn 202 (’5’AGCTTCTACGATACTCCCA3’), as originally described by Gassen et al.\textsuperscript{15} Primers for the HSP60 mRNA were GE-1 (’5’AGTCTACGATTATGATAGAAG3’) and GE-2 (’5’AATGACTGAGAGGATCCAGCG3’), as described by Airet et al.\textsuperscript{16}

As a control for the absence of DNA in the RNA preparation, each sample was additionally amplified without prior RT. PCR products were separated on a 2% agarose gel and transferred on a nitrocellulose membrane by vacuum blotting. For improved sensitivity and specificity, nonradioactive DNA hybridization was performed using probes 3′-tailed with digoxigenin-11 dUTP/dATP (Roche Diagnostics), according to the manufacturer’s instructions. Probes were designed according to the sequence of the amplicons and checked for genus-specificity in a BLAST 2.0 search (’5’GCAATCTTATAGC-GAAGGTCTT3’ for HSP60 amplicons; ’5’TACACCGCAATGTCTCAGC3’ for MOMP amplicons).

**C pneumoniae Culture From Monocytes of CAD Patients Under Azithromycin Treatment**

Two male patients (68 and 57 years) admitted to our emergency room because of acute chest pain were diagnosed as having unstable angina pectoris on the basis of coronary angiography, electrocardiography, clinical presentation, and laboratory parameters. The first patient was admitted with a myocardial infarction of the inferior wall. Coronary angiography showed 2-vessel disease (right coronary artery and left circumflex artery). The occluded right coronary artery was reopened by PTCA. The patient’s risk factors were diabetes mellitus, arterial hypertension, and smoking. The second patient was admitted for a myocardial infarction of the anterior wall. Two-vessel disease (left anterior descending artery and left circumflex artery) was documented by coronary angiography, and the occluded left anterior descending artery was reopened by PTCA. Risk factors in this patient were arterial hypertension and obesity.

After informed consent was given, both patients were enrolled in an ongoing clinical trial on the benefit of azithromycin in CAD and received oral courses of 500 mg/d azithromycin on days 1 to 3 and
on days 14 to 16 after coronary angiography. These patients yielded a positive result for \textit{C} pneumoniae DNA in their peripheral blood mononuclear cells, which were collected at day 1, as determined in a previously described nested PCR technique.\textsuperscript{15} Thus, CD14-positive cells from 8 mL of blood were isolated on day 28, as described above, and subjected to \textit{C} pneumoniae culture. Separated CD14-positive cells were disrupted with glass beads on a vortex and centrifuged onto HEp-2-cell monolayers in 6-well tissue culture plates and cultured in serial subcultures, as described previously for vascular materials.\textsuperscript{1} Productive infection was detected by immunofluorescence using a \textit{C} pneumoniae monoclonal antibody (Syva, Dako).

## Results

### Chlamydial Growth in the Presence of Antibiotics

Rifampin and azithromycin had no discernible effect on chlamydial growth and metabolic activity within monocytes in vitro. Inoculation of the freshly isolated human monocytes resulted in the development of inclusion bodies after 3 days whether antibiotics were added or not. These inclusions were proven to be of chlamydial origin by staining with the anti-chlamydial HSP60 antibody using immunoelectron microscopy. However, the inclusions were frequently of aberrant morphology in comparison with those seen in epithelial cells. They contained fewer and less dense reticulate and elementary bodies (Figure 1).

### mRNA Synthesis in the Presence of Antibiotics and After Prior Systemic Antibiotic Treatment

Chlamydiae within monocytes were metabolically active and did not cause host cell lysis; thus, they were proven to persist. Infected CD14-positive cells were viable until the end of the experiment (10 days), as shown by Trypan blue and immunofluorescence staining. RT-PCR demonstrated chlamydial HSP60 and MOMP mRNA synthesis, which indicated the viability of the pathogen in the presence of azithromycin or rifampin at 2 hours and 1, 3, and 10 days after infection. Similarly, mRNA transcripts of HSP60 and MOMP were continuously expressed from 2 hours to 10 days, despite prior systemic treatment of the monocyte donors with conventional courses of the respective antibiotics and their permanent presence in the culture medium (Figure 2). In a control experiment, synthesis of HSP60 and MOMP mRNA ceased completely in infected HEp-2 cells treated with azithromycin or rifampin within 72 hours, thus demonstrating the in vitro efficiency of the antibiotics in epithelial cells in the same setting (Figure 3).

### Cultural Recovery of \textit{C} pneumoniae Under Azithromycin Treatment

Viable \textit{C} pneumoniae was isolated and continuously cultured from CD14-positive cells of both CAD patients who had undergone azithromycin treatment. Prior treatment with azithromycin did not eradicate \textit{C} pneumoniae from circulating monocytes. The minimal inhibitory concentration of azithromycin, as determined in a standard system in laryngeal HEp-2 cells,\textsuperscript{13} was 0.08 \(\mu\text{g/mL}\) for both strains. There was no evidence that the patients suffered from a systemic inflammatory response syndrome, according to current consensus criteria.\textsuperscript{16}

## Discussion

There are 2 main findings in this study: (1) monocytes can disseminate the viable respiratory pathogen \textit{C} pneumoniae within the systemic circulation, and (2) the pathogen cannot be eliminated from its monocyte host by standard antichlamy-
dial treatment. The continuous presence of azithromycin or rifampin does not protect cultured human monocytes from *C. pneumoniae* infection in vitro. In addition, monocytes isolated after a standard course of azithromycin or rifampin treatment, and thus already replenished with antibiotics, still acquire *C. pneumoniae* infection when subsequently cultured in the presence of antibiotics. Intracellular development beyond mere phagocytic ingestion was shown electron-microscopically by immunogold-labeling of reticulate and elementary bodies within inclusions. These inclusions and their content were morphologically different from what is found in the acute infection of epithelial cells, but their viability and metabolic activity was proven by continuous detection of chlamydial mRNA transcripts. The pathogen remained viable without initiating the host cell lysis that otherwise marks the end of the regular chlamydial replication cycle. Thus, the monocytes acquire a persistent infection. Finally, the cultural retrieval of *C. pneumoniae* from circulating CD14-positive cells of CAD patients undergoing experimental azithromycin treatment for coronary sclerosis proved the presence of viable chlamydiae in the bloodstream, despite antichlamydial therapy.

In this study, we defined systemically circulating CD14-positive cells as hosts for *C. pneumoniae*. This fills the current gap between the respiratory epithelium, the primary target of this pathogen, and the vascular wall, where the chlamydiae were detected by various techniques, albeit their origin remained unexplained. The recovery of *C. pneumoniae* from monocytes now illustrates one method whereby the obligate intracellular organism may gain access to the vascular system. Other studies suggested that monocytes could be a potential vector system for chlamydial distribution on basis of DNA detection within peripheral blood mononuclear cells or CD14-positive cells, but these studies lacked evidence of viability. In the lung, *C. pneumoniae* enters epithelial cells and alveolar macrophages, but the exact site of transmission of chlamydiae to blood monocytes remains to be defined. In vitro data indicate that monocytes may transmit *C. pneumoniae* to vascular endothelial cells, but in vivo data are lacking. For initiation or promotion of atherogenesis, however, infection of the vascular wall does not seem mandatory because a release of proinflammatory mediators by circulating or transendothelially migrating infected monocytes might be sufficient.

In acute infection, host cells disintegrate and release newly produced elementary bodies within 3 days. In monocytes, however, a persistent infection was established for the observation period of 10 days. This persistent state seems to be typical of chlamydiae ingested by human monocytes under in vitro culture conditions and is not induced by antibiotics, because it occurs without any antibiotic supplementation. In a previous study, *C. trachomatis* serovar K–specific mRNA was detected in monocytes for 10 days. Airenne et al showed that *C. pneumoniae* was transcriptionally active for 3 days in vitro and did not develop infectious progeny in human monocytes. However, because we were able to culture the organism from monocytes from CAD patients, the data suggest that the ability to replicate is not lost within the monocytes. Interestingly, an establishment of the persistent infection could not be prevented by antichlamydial treatment. Although monocytes were subjected in vitro and in vivo to adequate amounts of antibiotic substance, they still promoted persistent infection with a vascular *C. pneumoniae* strain. When tested under standard susceptibility testing conditions,
the very same strain was highly sensitive to rifampin and azithromycin. This may explain the treatment failures seen in respiratory infections with strains that seemed susceptible in vitro. Current chlamydial susceptibility testing is focused on acute infection in epithelial cells and apparently does not apply to persistent infection.

The present study suggests that chlamydiae can survive an antichlamydial therapy within monocytes in vitro and in vivo. Optimal regimens for chlamydial eradication from monocytes are not known, but cultural recovery of the pathogen from circulating monocytes after 2 conventional courses of azithromycin treatment clearly indicates that a standard approach, as used in acute lung infection, may not be successful. Thus, endogenous reinfection of vascular cells after a decline of the antibiotic tissue levels cannot be excluded. In fact, this may seriously affect the current efforts made in large prospective trials to alleviate clinical CAD symptoms by antichlamydial treatment. This notion is supported by recent data from ongoing treatment trials. In the largest study on antimicrobial treatment in CAD patients published to date, azithromycin treatment had no significant effects on clinical events after 6 months and 2 years. A statistically significant benefit among roxithromycin-treated CAD patients seen after 30 days in the Roxithromycin Ischemic Syndromes (ROXIS) study was not reproduced after 6 months. Erythromycin or tetracycline treatment within the last 5 years had no effect on the risk of having a first myocardial infarction in a retrospective study. In contrast, another retrospective analysis of first-time myocardial infarction cases and controls reported a favorable effect of tetracycline and quinolone antibiotics but not macrolides. In a rabbit model on the acceleration of atherogenesis by chlamydial infection, azithromycin treatment had a documented protective effect. However, C pneumoniae antigen was still detected in the vessel walls after a 7-week azithromycin course, indicating persistence.

It is important to note that antibiotics can inhibit chlamydial growth in the atherogenetically relevant endothelial and smooth muscle cells. Thus, chlamydial eradication from cells other than monocytes/macrophages is potentially feasible. However, there is evidence suggesting that persistence may be established in those cells, too. In a recently described in vitro model of continuous C pneumoniae infection, epithelial cells sustained infection for 2 years without an addition of fresh host cells or chlamydiae. Six-day courses of azithromycin or ofloxacin did not eliminate the infection. It is unknown if this corresponds to the in vivo situation in pulmonary or vascular tissue.

In vivo, chlamydial persistence has been observed in only monocytes thus far, but it can be induced in endothelial and epithelial cells in vitro by tryptophan depletion, interferon-γ, or tumor necrosis factor-α. When a corresponding mechanism was studied for monocytes, growth inhibition could not be neutralized by tryptophan or interferon-γ antibodies. Apparently, the persistent state can be entered spontaneously in monocytes/macrophages but is dependent on the cytokine network in other cell populations. Nevertheless, the absence of essential nutrients in the vacuole of the intracellular parasite may be the cause of growth arrest. Persistence seems to be the result of a stress response, as indicated by the prominent production of HSP60. We speculate that the altered metabolic condition in this state is also the cause of the ineffectiveness of the antimicrobial agents described in this study and that this state is based on differentially displayed chlamydial genes for cell differentiation, cytokinesis, and stress response. Therefore, we suggest the use of infected monocytes as a model system for further examination of the genetic background of chlamydial persistence and for the definition of targets for the eradication of persisting chlamydiae.

An infectious component in the chronic inflammatory condition of atherosclerosis may provide additional explanations for unclear phenomena of atherogenesis, such as mesenchymal cell proliferation and its distinct inflammatory component. The obvious appeal of treating a bacterial pathogen in this setting, the leading cause of death in the industrialized nations, has initiated a variety of prospective antimicrobial intervention studies with >10 000 patients enrolled. However, evidence is increasing that eradication may face enormous problems due to the chlamydial ability to enter a refractory state of persistent infection that seems unique to chlamydiae.

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