Detection of *Chlamydia pneumoniae* DNA in CD3+ Lymphocytes From Healthy Blood Donors and Patients With Coronary Artery Disease

Ravi Kaul, PhD; Janet Uphoff, MSc; Jean Wiedeman, MD, PhD; Sanjay Yadlapalli, MD; Wanda M. Wenman, MD

**Background**—*Chlamydia pneumoniae* is an intracellular bacterium responsible for respiratory tract infections. Recent studies have implicated this organism in the pathogenesis of atherosclerosis.

**Methods and Results**—To address how the organism is transported from lungs to cardiac vessels, we characterized the cell population within peripheral blood mononuclear cells (PBMCs) that harbor *C pneumoniae* DNA. Adherent and nonadherent PBMCs from 28 patients with coronary artery disease (CAD) and 19 healthy blood donors were evaluated for the presence of *C pneumoniae* DNA by touchdown nested polymerase chain reaction (nPCR). Of the 28 patients, 10 (36%) had detectable PCR product in their nonadherent and 3 (10%) in their adherent PBMC population. *C pneumoniae*-specific PCR results were positive for 5 of 19 (26%) healthy blood donors. PCR positivity was detected only in the nonadherent cell population among this group of individuals. Fractionation of nonadherent PBMCs identified *C pneumoniae*-specific PCR signal among the CD3+ T-cell population exclusively. Of the 18 PCR-positive subjects (13 patients and 5 healthy control subjects), 67% (8 patients and 4 healthy blood donors) tested positive for *C pneumoniae*-specific IgG serology. Interestingly, 2 patients became PCR negative on a repeated blood draw 5 months after initial detection of *C pneumoniae* DNA despite retaining *C pneumoniae*-specific antibodies.

**Conclusions**—Our results demonstrate marginally significant prevalence of *C pneumoniae* DNA in patients with CAD compared with healthy subjects (*P*=0.082). In contrast, the prevalence of IgG seropositivity among the 2 groups did not reach statistical significance (*P*=0.306). We also provide unequivocal evidence for the presence of *C pneumoniae* DNA predominantly among the circulating CD3+ T-cell population. (*Circulation.* 2000;102:2341-2346.)

**Key Words:** *Chlamydia pneumoniae* □ atherosclerosis □ polymerase chain reaction □ cells □ lymphocytes

Atherosclerosis is a common pathological process associated with coronary heart disease, acute myocardial infarction, and ischemic stroke, the leading causes of death in the United States and other industrialized countries. An increasing body of evidence suggests that infections, either bacterial or viral, play an important role in the origin of atherosclerosis through inflammation and endothelial damage. One of the most compelling cases to be made linking infections to atherosclerotic heart disease is for *Chlamydia pneumoniae*, an agent responsible for upper and lower respiratory tract infections. Seroepidemiological studies have shown that >60% of adults are infected with this organism during their lifetime. Saikku et al reported a close association between high levels of *C pneumoniae* specific–IgA antibodies and an increased risk for myocardial infarction. It is unclear whether IgA antibody titers represent acute infection, reinfeciton, reactivation, or persistence.

The presence of *C pneumoniae* in atheromatous plaques is controversial. However, detection of the organism in plaques has provided direct evidence for an association between *C pneumoniae* and coronary heart disease. The involvement of *C pneumoniae* in the atherosclerotic process has been substantiated by recent reports showing reduced ischemic events after macrolide antibiotic treatment. The association of *C pneumoniae* with cardiac disease would entail transportation of this respiratory pathogen from the lungs to cardiac vessels. With the use of sensitive polymerase chain reaction (PCR) technology, a high prevalence of *C pneumoniae* DNA was found in peripheral blood mononuclear cells (PBMCs) among middle-aged blood donors and patients with cardiovascular disease, supporting a cellular route of infection. In patients with cardiovascular disease, 59% were PCR(+) /sero(+) and 32% were PCR(−)/sero(+) in contrast to healthy donors, of whom 46% were PCR(+) /sero(+) and 44% were PCR(−)/sero(+). These results reflect not only the prevalence of *C pneumoniae* DNA in the general population but also an upward bias among patients with cardiovascular disease.
disease. More recently, 2 other groups have reported the detection of \textit{C pneumoniae} DNA in PBMCs.\textsuperscript{11,12} In both studies, DNA extracted from the total PBMC fraction was used for PCR amplification; however, no attempt was made to identify PCR(+) cell type(s). Identification of the cell type(s) that harbor \textit{C pneumoniae} would help to clarify how \textit{Chlamydia} survive naturally in these cells and provide clues to subsequent interaction with other cells and tissues of the vascular system.

The purpose of the present study was to investigate the prevalence of \textit{C pneumoniae} among patients with cardiovascular disease and to identify the specific cell types in the peripheral blood that harbor \textit{C pneumoniae} DNA. We present evidence showing the presence of \textit{C pneumoniae} DNA among patients with coronary artery disease (CAD) and healthy blood donors. Furthermore, \textit{C pneumoniae} DNA was detected predominantly in circulating CD3+ T cells and not adherent CD14+ monocyte/dendritic cell populations.

**Methods**

**Samples**

Venous blood was collected from patients attending the cardiology clinic at the University of California Davis Medical Center and healthy adult volunteers at the Sacramento Blood Center according to institutional review board guidelines. All samples were collected between March and December 1999. Sex and age of each donor were recorded at the time of initial blood draw. Of the 30 patients with cardiovascular disease who consented to donate blood, 19 were men and 11 were women. All patients enrolled in this study had a diagnosis of CAD. The control group comprised 19 healthy blood donors: 11 men and 8 women. There was no evidence of a \textit{C pneumoniae} epidemic in the community during collection of samples. Serology for \textit{C pneumoniae} was performed using a microimmunofluorescence test (Labsystems). A titer of $\geq$1:32 was defined as positive for IgG antibodies according to the manufacturer’s recommendation.

**Isolation of Adherent and Nonadherent PBMCs**

Venous blood (20 to 30 mL) was collected by percutaneous venipuncture in EDTA-treated tubes and layered over Ficoll-Paque (Pharmacia Biotech). Cells were centrifuged at 2000 rpm for 15 minutes, and the PBMC band was aspirated, washed twice, suspended in PBS, and plated on tissue culture-grade Petri dishes, followed by incubation at 37°C for 1 hour. At the end of incubation, nonadherent cells were aspirated, and adherent cells were washed 3 times. Nonadherent cells and all washes were pooled and pelleted at 2000 rpm. Adherent cells were gently scraped and pelleted, and purity analysis was performed by flow cytometry with FITC anti-CD14 clone MIP9 (Becton Dickinson).

**Selection of CD3+, CD14+, CD4+, and CD8+ Cell Populations**

The nonadherent cells collected above were subjected to CD3+ positive selection by magnetic cell sorting with microbeads (Milteneyi Biotec) according to the manufacturer’s instructions. Similarly, total PBMCs were used for positive selection of CD14+, CD4+, and CD8+ cell populations. The purity of isolated cells was determined by flow cytometry with fluorochrome-labeled antibodies (Becton Dickinson).

**Nested PCR Amplification Strategy**

Total DNA isolated from purified cells by SDS–proteinase K treatment was subjected to touchdown nested PCR (nPCR) analysis as described previously using \textit{C pneumoniae}–specific outer membrane protein (omp1)\textsuperscript{13,14} or heat shock protein (hsp\textsuperscript{60})\textsuperscript{15} primers. DNA concentration was measured by comparing the density of samples with DNA quantification standards (GIBCO-BRL) with the use of Alphalager software (Alpha Innotech Corp). The primary amplification was carried out with either omp1-specific 5’-TGGCAAAGAGCTGGGT-3’ and 5’-AGCCCTAAGCTAGAGTCTGACT-3’ or hsp\textsuperscript{60}-specific 5’-ACGTACGTAGTTATAGAG-3’ and 5’-AAGTAGCTGGAGAGTATCCACGG-3’ forward and reverse primers, respectively. The PCR product amplified by outer primers was diluted 10-fold before a nested reaction was begun. Amplification of nPCR was carried out with either omp1-specific 5’-TGGCAACATCATCTCATG-3’ and 5’-TCTGAAGTGGACC-AGATACGT-3’ or hsp\textsuperscript{60}-specific 5’-CTCCCAAGTGACTAAAGATGTG-3’ and 5’-TCCCTGCTACAAAGCTGGAGAG-3’ inner primer sets, respectively. For touchdown nPCR, the annealing temperature was lowered 1°C every 2 cycles from 65°C to 55°C, at which point 20 more cycles were performed. The denaturation and extension temperatures stayed constant at 94°C and 72°C, respectively. To test the authenticity of PCR reactions, amplified products were Southern blotted and hybridized, respectively, to an omp1-specific 5’-AGAAAGTGGCAGAGCTT-3’ or hsp\textsuperscript{60}-specific primers 5’-TCCTGCGGCTGGGCTTCA-3’ internal to the amplified sequence. Two positive (\textit{C pneumoniae} DNA) and 2 negative (sterile water) samples were included for each assay run simultaneously, along with duplicate subject samples comprising template DNA isolated from adherent, nonadherent, CD14+, CD4+, and CD8+ sorted cell populations. To minimize the risk of contamination, sample preparation, PCR amplification, and product analysis were performed in separate rooms with dedicated equipment. Aerosol-resistant pipette tips were always used.

**DNase Digestion**

The 500 copies of \textit{C pneumoniae}–template DNA in a background of 1 μg DNA isolated from CD14+ cells of sero(−)/PCR(−) subjects were digested with RQ1 DNase (Promega). After incubation, the enzyme was heat inactivated (94°C for 15 minutes), and one fifth of the original reaction volume was used for PCR amplification. Heat-inactivated DNase before its incubation with DNA served as a positive control.

**Results**

Table 1 shows the age and sex distribution of patients with cardiovascular disease and healthy blood donors. Of 30 patients with CAD, 19 (63%) were male and 11 (37%) were female. The average age of study patients was 67.9 years (range, 48 to 84 years), whereas the average age of the control group was 57.6 years (range, 31 to 83 years). \textit{C pneumoniae}–specific IgG antibodies were found in 18 of 30 patients (60%) and 10 of 19 healthy blood donors (53%; $P = 0.306$).

To determine which enriched cell population within PBMCs harbors \textit{C pneumoniae} DNA, nPCR analysis was performed on both adherent and nonadherent PBMCs. The sensitivity of nPCR was tested with a known copy number of \textit{C pneumoniae} genomic DNA template in the background of 1 to 5 μg cellular DNA from PCR(−)/sero(−) individuals. Figure 1A shows the results of such analysis with the use of 500, 50, 10, and 1 copy of \textit{C pneumoniae} DNA using either omp1- or hsp\textsuperscript{60}-specific primers. As demonstrated, as few as 10 copies of \textit{C pneumoniae} DNA were visualized on an ethidium bromide–stained agarose gel with either primer set

<table>
<thead>
<tr>
<th>Table 1. Age and Sex Distribution of Study Patients</th>
</tr>
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<tbody>
<tr>
<td><strong>Group</strong></td>
</tr>
<tr>
<td>Heart patients</td>
</tr>
<tr>
<td>Healthy donors</td>
</tr>
</tbody>
</table>
Table 2. Distribution of C pneumoniae DNA Among Adherent and Nonadherent Cell Populations

<table>
<thead>
<tr>
<th>Group*</th>
<th>Subjects, n</th>
<th>Adherent</th>
<th>Nonadherent</th>
<th>Negative†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart patients</td>
<td>30‡</td>
<td>3</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>Healthy donors</td>
<td>19</td>
<td>0</td>
<td>5</td>
<td>14</td>
</tr>
</tbody>
</table>

*Ps = 0.082; power of analysis 0.3974 (prevalence of DNA in 2 groups).
†All true-negatives showed no inhibition when spiked with a low copy number positive DNA.
‡Two heart patients (1 seropositive and 1 seronegative) exhibited PCR-specific inhibition.

Of known positive. Two samples showed PCR-specific inhibition and were excluded from the study (Table 2). Thus, of 28 patients, there were 15 true negatives (54%). Of the 13 positive patients (46%), 10 (36% of the total or 77% of the total positive) revealed C pneumoniae-specific DNA in their nonadherent PBMC population (lanes 6, 14, 16, 26, 30, 32, 40, 54, 56, and 58). In the other 3 (11% of the total or 23% of the total positive), PCR product was amplified from adherent PBMC DNA only (lanes 3, 7, and 35). A positive correlation was observed between ethidium bromide–stained Southern blot and the number of male and female healthy donors who tested positive for C pneumoniae DNA. The percentage of male subjects with CAD who tested positive for C pneumoniae DNA was 44% (8 of 18) compared with 50% (5 of 10) for female subjects (Table 3). Only 8 of 13 PCR-positive patients (62%) were found to be IgG seropositive (Table 4). Interestingly, repeated blood draw from 2 sero+(+)PCR(+) patients 5 months after initial screening tested PCR negative. No C pneumoniae-specific DNA was detected in their CD14+, CD4+, or CD8+ cell fractions at the repeated test despite the fact that both patients maintained seropositivity (data not shown).

C pneumoniae-specific PCR results were positive for 3 of 19 healthy blood donors (26% of the total), and the percentage of male and female healthy donors who tested positive for C pneumoniae DNA was 27% (3 of 11) and 25% (2 of 8), respectively (Table 3). Of the 5 PCR-positive blood donors, 3 (60%) were IgG seropositive (Table 4). Figure 3A shows the results of nPCR analysis performed on the initial group of 8 healthy individuals, with 4 exhibiting C pneumoniae-specific DNA in their nonadherent PBMC population (lanes 2, 4, 6, and 8). A positive correlation was again observed between the ethidium bromide–stained PCR product and its corresponding Southern blot (Figures 2B).

Figure 1. A, Ethidium bromide–stained agarose gel showing nested amplification of 500 (lanes 1 and 6), 50 (lanes 2 and 7), 10 (lanes 3 and 8), and 1 (lanes 4 and 9) copy of C pneumoniae genomic DNA. Lanes 5 and 10 show amplification from template DNA containing 5 μg background cellular DNA from sero(-)/PCR(-) subjects without exogenous C pneumoniae DNA. Amplification was carried out with either omp1 (lanes 1 through 5) or hsp60 (lanes 6 through 10) gene-specific primer sets. Water was used as template in (−) controls. B, Southern blot analysis of gel in A probed with omp1- or hsp60-specific oligonucleotides, respectively. M represents 1-kb DNA ladder.

Figure 2 shows the results of PCR analysis performed on fractionated PBMCs. Seventy of 30 patients (57%) with CAD did not show C pneumoniae-specific 385-bp omp1 amplification product with either adherent or nonadherent PBMC DNA used as a template (Figure 2A). To exclude the possibility of any PCR inhibitors in this group, we spiked all negative samples with a low copy number (10 to 50 copies) of known positive. Two samples showed PCR-specific inhibition and were excluded from the study (Table 2). Thus, of 28 patients, there were 15 true negatives (54%). Of the 13 positive patients (46%), 10 (36% of the total or 77% of the total positive) revealed C pneumoniae-specific DNA in their nonadherent PBMC population (lanes 6, 14, 16, 26, 30, 32, 40, 54, 56, and 58). In the other 3 (11% of the total or 23% of the total positive), PCR product was amplified from adherent PBMC DNA only (lanes 3, 7, and 35). A positive correlation was observed between ethidium bromide–stained Southern blot (Figure 2B). The percentage of male subjects with CAD who tested positive for C pneumoniae DNA was 44% (8 of 18) compared with 50% (5 of 10) for female subjects (Table 3). Only 8 of 13 PCR-positive patients (62%) were found to be IgG seropositive (Table 4). Interestingly, repeated blood draw from 2 sero(+)PCR(+) patients 5 months after initial screening tested PCR negative. No C pneumoniae-specific DNA was detected in their CD14+, CD4+, or CD8+ cell fractions at the repeated test despite the fact that both patients maintained seropositivity (data not shown).

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To examine which specific cell type(s) among the nonadherent PBMC population harbored *C pneumoniae* DNA, we obtained fresh blood from the 4 healthy donors who tested positive during initial screening. Subsequently, adherent and nonadherent cells from these individuals were isolated, and nonadherent cells were further purified for CD3+ T-cell population. Table 5 shows the flow cytometry purity analysis after CD3+ selection. Generally, cells were ≥87% pure. Adherent PBMCs from these blood donors were 60% to 70% CD14+ when analyzed by flow cytometry. Figure 3C shows the results of PCR analysis performed on DNA isolated from adherent (lanes 1, 3, 5, and 7) and nonadherent CD3+ cells (lanes 2, 4, 6, and 8). No *C pneumoniae*–specific omp1 DNA was observed among adherent cells, as noted previously; however, a 385-bp PCR product was detected among CD3+ T cells. The amplified product probed positive on hybridization to an oligonucleotide probe (Figure 3D). Additional evidence for the presence of *C pneumoniae* genome among nonadherent CD3+ cells was obtained through amplification of a 443-bp region of the hsp60 gene. Using primers that specifically targeted the *C pneumoniae* hsp60 gene, we were able to detect a PCR-positive signal in DNA isolated from CD3+ cells from individuals who had also tested positive for the omp1 gene (Figure 3E). Again, a positive correlation was observed between the ethidium bromide–stained band and its corresponding Southern blot (Figure 3F). At the same time, no amplified product was visualized among DNA isolated from adherent cells (lanes 1, 3, 5, and 7). These studies not only reaffirm the presence of *C pneumoniae* genome but also rule out any omp1-specific carryover contamination in CD3+ cells.

After identifying *C pneumoniae* DNA in nonadherent CD3+ cells, we subfractionated PBMCs from an additional 11 healthy blood donors into CD14+ (primarily monocytes and dendritic cells), CD4+ (primarily T helper cells), and CD8+ (primarily cytotoxic and suppressor T cells) populations. *C pneumoniae*–specific DNA was detected in only 1 subject, and that was in the CD8+ population alone (Figure 4; lane 17), giving a grand total of 5 PCR-positive subjects of 19 screened (Figure 4). Flow cytometry analysis performed on sorted cells revealed 95% to 99% pure CD14+, 90% to 98% pure CD4+, and 84% to 98% pure CD8+ populations. In particular, this PCR-positive individual tested 98% pure for CD8+ selection.

### Discussion

Using PCR technology, we have shown the presence of *C pneumoniae*–specific signals in patients with CAD and healthy blood donors. We were able to detect as few as 10 copies of the *C pneumoniae* genome using nPCR to amplify either omp1 or hsp60 genes. Although the detection of circulating *C pneumoniae* DNA does not prove the presence of viable *Chlamydia* among our subjects, it is helpful in identifying carriers. Our data support previous work showing the prevalence of *C pneumoniae* DNA in PBMCs isolated from middle-aged blood donors and patients with cardiovascular disease. More recently, 2 other groups have reported
the presence of circulating \( C\) pneumoniae DNA among PBMCs in specific populations.\(^{11,12}\) Blasi et al\(^{11}\) found a positive correlation between the detectability of \( C\) pneumoniae DNA in abdominal aortic tissue from patients undergoing abdominal aneurysm surgery and circulating PBMCs. Wong et al\(^{12}\) reported a higher prevalence of circulating \( C\) pneumoniae DNA among men with CAD (OR, 3.4 for men compared with 0.7 for women). However, results from our laboratory do not support these observations. \( C\) pneumoniae–specific DNA was detected in 44% of male (8 of 18) and 50% of female (5 of 10) heart patients compared with 27% of healthy male (3 of 11) and 25% of healthy female (2 of 8) donors. Our studies rule out sex-based bias in the detection of \( C\) pneumoniae DNA; however, they support a marginally significant prevalence of circulatory \( C\) pneumoniae DNA among patients with CAD compared with healthy blood donors (overall, 46% versus 26%, respectively; \( P=0.082\)). Consistent with our findings, others have also found a non–sex-based upward bias for \( C\) pneumoniae DNA in patients with cardiovascular disease.\(^{10}\) Whether the discrepancy between our data and those reported by Wong et al\(^{12}\) is due to the detection techniques or to geographical differences is not clear at this time.

In the present study, \( C\) pneumoniae–specific IgG antibodies were detected in 67% of PCR-positive subjects, including healthy blood donors and patients with CAD. Although reports of PCR positivity but seronegativity or antigen negativity have been published previously for both \( C\) pneumoniae and viral carriers,\(^{10,11,16,17}\) 2 groups have recently reported that as many as 95% of their PCR-positive subjects possess \( C\) pneumoniae–specific antibodies. Given the high sensitivity of nPCR, the presence of \( C\) pneumoniae DNA in the absence of antibody response may simply reflect an early stage of the infection or very low levels of IgG antibodies. In contrast, failure to detect \( C\) pneumoniae DNA in circulatory cells among seropositive patients (56% of CAD patients and 70% of healthy blood donors) may reflect very low levels of DNA or clearance of \( C\) pneumoniae DNA from circulating cells over a period of time. The latter possibility is supported by the finding that 2 seropositive patients who initially tested positive for \( C\) pneumoniae DNA were found to be PCR negative on repeated blood draw after an interval of 5 months. However, both patients continued to exhibit \( C\) pneumoniae–specific IgG antibodies.

The predominance of \( C\) pneumoniae DNA in nonadherent PBMCs (composed predominantly of CD3+ T-cell and CD19+ B-cell populations) compared with the adherent population (composed predominantly of CD14+ monocytes/dendritic cells) is interesting, especially because most of the published work has focused on CD14+ monocytes or macrophages.\(^{15,18–21}\) It has been suggested that macrophages can serve as vehicles for systemic dissemination on the basis of the detection of \( C\) pneumoniae DNA among alveolar and peritoneal macrophages of intranasally and intraperitoneally inoculated mice.\(^{20}\) However, only 3 of our 13 positive patients had \( C\) pneumoniae DNA in their adherent PBMCs. It is doubtful that these results reflect CD3+ cell contamination because DNA isolated from the corresponding nonadherent population failed to exhibit \( C\) pneumoniae–specific DNA. It is noteworthy that all 3 subjects were seronegative. The significance of these results remains to be defined. However, our results support the hypothesis that \( C\) pneumoniae is transported from the lungs through the cellular route.

To characterize the cell population(s) within nonadherent PBMCs that harbor \( C\) pneumoniae DNA, we fractionated total PBMCs into adherent and nonadherent (CD3+) populations. In 4 subjects, \( C\) pneumoniae omp1 and hsp\(^{60}\) genes

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**TABLE 5. Flow Cytometry Purity Analysis After CD3+ Selection**

<table>
<thead>
<tr>
<th>Donor</th>
<th>CD3</th>
<th>CD14</th>
<th>CD15</th>
<th>CD19</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>87</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>95</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>90</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>95</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
were amplified from the CD3+ T-cell population only; no PCR signal was observed from adherent PBMCs. Amplification ofomp1 and hsp60 genes that span 2 different regions of the Chlamydia pneumoniae genome rule out any carryover contamination while at the same time support the presence of C pneumoniae DNA among CD3+ cells. We excluded the possibility of contaminating CD14+ cells as the source of the PCR signal in our CD3+ selected population on the basis of the purity analysis of PCR-negative adherent PBMCs. To further characterize the T-cell population that is PCR positive, we subfractionated PBMCs into CD4+ (primarily T helper cells), CD8+ (primarily cytotoxic and suppressor T cells), and CD14+ populations. C pneumoniae-specific DNA was identified in the CD8+ selected population in 1 of the subjects. Once again, these results argue strongly in favor of circulating T cells harboring C pneumoniae naturally. The present work represents the first report of C pneumoniae or any other chlamydial species infecting CD3+ cells naturally. The significance of C pneumoniae DNA in CD3+ cells is unknown. However, T cells are known to accumulate and multiply within atherosclerotic plaque as part of the inflammatory response.22

Acknowledgments

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References

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