**Chlamydia pneumoniae** Binds to Platelets and Triggers P-Selectin Expression and Aggregation 

A Causal Role in Cardiovascular Disease?

Hanna Kälvegren, Meytham Majeed, Torbjörn Bengtsson

**Objective**—Evidence linking *Chlamydia pneumoniae* to atherosclerotic cardiovascular disease is expanding. Platelets are considered to play an essential role in cardiovascular diseases; however, so far platelets have not been associated with an infectious cause of atherosclerosis. This study aims to clarify the interaction between *C pneumoniae* and platelets and possibly present a novel mechanism in the pathogenesis of atherosclerosis.

**Methods and Results**—The effects of *C pneumoniae* on platelet aggregation and secretion were assessed with lumiaggregometry, and the ability of *C pneumoniae* to bind to platelets and stimulate expression of P-selectin was analyzed with flow cytometry. We found that *C pneumoniae*, at a chlamydia:platelet ratio of 1:15, adheres to platelets and triggers P-selectin expression after 1 minute and causes an extensive aggregation and ATP secretion after 20 minutes of incubation. Inhibition of glycoprotein IIb/IIa with Arg-Gly-Asp-Ser or abciximab markedly reduced *C pneumoniae*-induced platelet aggregation. Exposure of *C pneumoniae* to polymyxin B, but not elevated temperature, abolished the stimulatory effects on platelet activation, suggesting that chlamydial lipopolysaccharide has an active role. In contrast, other tested bacteria had no or only moderate effects on platelet functions.

**Conclusion**—Our findings demonstrate a new concept of how *C pneumoniae* activates platelets and thereby may cause atherosclerosis and thrombotic vascular occlusion. (*Arterioscler Thromb Vasc Biol. 2003;23:1677-1683.)*

**Key Words:** atherosclerosis □ bacteria–cell interaction □ LPS □ thrombosis

An association between *C pneumoniae* infections and atherosclerosis has been demonstrated in a number of epidemiological, serological, immunohistochemical, and molecular biological investigations. Furthermore, in vitro studies have revealed that *C pneumoniae* is able to infect and replicate in the major cell types found within the atherosclerotic lesion, such as macrophages, endothelial cells, and smooth muscle cells. However, the interaction between *C pneumoniae* and platelets, other important actors in atherogenesis, has to our knowledge not been investigated.

Platelets are specialized in the processes of hemostasis and thrombus formation after an endothelial injury but are also considered to be involved in inflammatory reactions and the development of atherosclerosis. We have previously reported inflammatory properties of platelets by demonstrating their regulatory effects on leukocyte function. The α-granule constituent P-selectin is considered as a reliable marker of platelet activation and as a predictor of acute coronary heart disease. Several studies indicate that P-selectin mediates adhesion and recruitment of leukocytes to activated platelets, exerts procoagulant activity, and facilitates atherosclerotic development.
In this study, we evaluated the interaction between C. pneumoniae and human platelets and found that C. pneumoniae binds to platelets and effectively stimulates aggregation, secretion, and surface expression of P-selectin. These observations introduce new possible mechanisms involved in the development of cardiovascular diseases.

Methods

Cells and Bacteria
C. pneumoniae (strain T45) was cultured in HEp2 cells essentially as described by Redecke et al. The bacteria and cells were tested for mycoplasma contamination by using mycoplasma-specific polymerase chain reaction essentially according to van Kuppeveld et al. Platelets and neutrophils were isolated from human blood as previously described. For a detailed description of the preparation of cells and bacteria, please see http://atvb.ahajournals.org.

Platelet Aggregation and ATP Secretion

Aggregation and ATP secretion were analyzed under stirring conditions by using a calibrated two-sample Lumi-Aggregometer model 560 (ChronoLog Corp). Aggregation was measured as the change in light transmission, where the unstimulated platelet suspension was set to 0% and the buffer (KRG) to 100%. ATP secretion was measured in parallel as change in bioluminescence when ATP interacts with a luciferin–luciferase mixture (1.6 µg/mL luciferin and 176 U/mL luciferase; ChronoLog Corp). Calibration was performed for each test by adding a known amount of ATP.

Flow Cytometry

C. pneumoniae–Platelet Interaction

Platelets (2×10^9/mL) were preincubated for 5 minutes at 37°C under stirring conditions in a 24-well plate (Nunc) before being mixed with various concentrations of C. pneumoniae. Samples were taken immediately before, and 1 minute, 5 minutes, 10 minutes, and 20 minutes after adding C. pneumoniae to the platelet suspension. In some experiments, viable C. pneumoniae was replaced with heat-inactivated (70°C, 30 minutes) C. pneumoniae, HEp2 debris, or collagen (2 µg/mL). The role of platelet adhesion proteins was tested by preincubating the platelets with blocking antibodies against CD42b (3.6 µg/mL) and CD41 (4.3 µg/mL). The involvement of chlamydial lipopolysaccharide (LPS) was evaluated by treating C. pneumoniae with polymyxin B (100 µg/mL) for 30 minutes at room temperature. Unspecific effects of polymyxin B on platelet activity were tested during collagen-induced activation.

Immunoﬂuorescence staining of platelets and C. pneumoniae was performed by incubation with saturating concentrations of monoclonal ﬂuorescein isothiocyanate (FITC)-conjugated anti P-selectin (CD62p; BD Biosciences, Pharmigen) or phycocerythrin-conjugated anti-GpIIb (CD42b; Dakopatts), and monoclonal FITC-labeled anti-chlamydia LPS (Boyle Diagnostics) at room temperature for 10 minutes in the dark. The samples were then fixed with Polylose (with 2.5% formaldehyde; Immunotech) under the same conditions and diluted in distilled H2O. Phycocerythrin- or FITC-labeled irrelevant isotype-matched monoclonal antibodies were used as controls for nonspecific staining. Immediately after staining, the samples were analyzed with flow cytometry in a Becton Dickinson FACS Calibur. The platelet population was identified by means of its light-scatter characteristics and by confirming that more than 99% of analyzed events stained positive for platelet and C. pneumoniae antigens (GpIIb and LPS, respectively) were considered to represent platelet–chlamydia complexes and were distinguishable from events stained positive for GpIIb alone. The extent of platelet activation was assessed by analyzing anti–P-selectin FITC fluorescence in the platelet gate. The mean fluorescence value of each sample was determined from cells counted during a time period of 20 seconds or from 500 000 counted cells at most.

Binding of C. pneumoniae to Neutrophils

Neutrophils were preincubated for 5 minutes at 37°C and then mixed with C. pneumoniae at a C. pneumoniae:neutrophil ratio of 1:5. After 5 minutes of coincubation, the samples were stained with monoclonal phycocerythrin–conjugated anti-CD11b (Dakopatts) and monoclonal FITC-labeled anti-chlamydia LPS antibodies. Neutrophil events stained positive for C. pneumoniae LPS were considered to represent neutrophil–chlamydia complexes. Unspecific binding of the FITC-labeled anti-chlamydia LPS antibody to the neutrophil control was subtracted from the fluorescence value of neutrophils incubated with C. pneumoniae.

Results

C. pneumoniae Triggers Platelet Aggregation and Secretion

The interaction between C. pneumoniae and platelets was assessed by using lumiaaggregometry, which enables a simultaneous analysis of platelet aggregation and ATP secretion. We found that the addition of C. pneumoniae to a pure platelet suspension (2×10^9 platelets/mL) induced aggregation (Fig. 1A and 1B) and ATP secretion (Fig. 1C) in a dose- and time-dependent manner. The responses were triggered at an infection forming unit chlamydia:platelet (C/p) ratio of 1:30. The chlamydia-induced platelet aggregation occurred in an all-or-nothing manner and increases in the bacteria concentration affected the lag period (time between addition of bacteria and onset of aggregation) but not the extent of aggregation (Fig. 1A and 1B). Once begun, the aggregation proceeded at about the same rate when using different C/p ratios, as indicated by the slope of the tracings (Fig. 1A). The C. pneumoniae-induced platelet aggregation and secretion were comparable with the responses triggered by collagen (Fig. 1C). No aggregation or ATP secretion was observed in stirred, unstimulated platelet suspensions or in samples with platelets incubated with cell debris of uninfected HEp2 cells (data not shown).

C. pneumoniae Binds to Platelets

The capacity of C. pneumoniae to bind and activate platelets was studied by using flow cytometry. Platelet events positive for FITC-conjugated anti-chlamydia LPS represent platelets with bound chlamydiae. We found that exposing platelets to C. pneumoniae caused a rapid and pronounced increase in platelet-associated FITC fluorescence. Scatterplots on platelets (Fig. 2A) with or without chlamydiae illustrated a change in size distribution, proposing the formation of platelet–chlamydia complexes. Figure 2B illustrates a time-dependent increase in bound chlamydiae as revealed by elevated FITC fluorescence in the platelet gate. Approximately 10% of the platelet population bound to C. pneumoniae (C/p 1:10) after 1 minute, 20% after 5 minutes, 40% after 10 minutes, and 45% after 20 minutes of coincubation (Fig. 2B). Control experiments did not reveal any unspecific binding of anti-LPS antibodies to platelets and only a negligible binding of irrelevant isotype control antibodies. The wide distribution in size and fluorescence intensity indicates that platelets and platelet aggregates bound varying numbers of chlamydiae. The chlamydiae themselves did not form aggregates during incubation at 37°C under stirring conditions (data not shown).
We found that \textit{C pneumoniae} at a C/p ratio of 1:15 markedly increased the expression of P-selectin, whereas no effects were detected on platelets incubated with uninfected HEp2 cell debris or 2-sp buffer. The P-selectin expression increased dramatically already after 1 minute and reached a maximum after 10 minutes of coincubation (Fig. 3A and 3B). We found a chlamydia-induced increase in platelet P-selectin at C/p ratios as low as 1:60, and the effects of \textit{C pneumoniae} were comparable with those induced by collagen (2 \(\mu\)g/mL; Fig. 3C).

**Effects of Other Bacteria on Platelet Activity**

The ability of a number of other bacteria to induce platelet aggregation and secretion was tested. Neither \textit{Staphylococcus aureus}, \textit{Staphylococcus epidermidis}, \textit{Salmonella typhimurium}, nor \textit{Escherichia coli} stimulated platelet aggregation, ATP secretion, or P-selectin expression when using similar bacteria:platelet ratios as in the experiments of \textit{C pneumoniae}. An irreversible, incomplete aggregation was triggered by \textit{S. aureus} at a considerable higher bacteria:platelet ratio (2:1).

**Role of Chlamydial LPS in Platelet Activation**

To determine whether platelet activation required viable bacteria, an active release of chlamydial cell components, and/or binding to heat-labile chlamydial surface structures, experiments by using heat-inactivated \textit{C pneumoniae} were performed. We found that heat treatment of \textit{C pneumoniae} did not change the platelet binding capacity nor the stimulatory effects on platelet aggregation and P-selectin expression. To study the role of LPS in the interaction between \textit{C pneumoniae} and platelets, \textit{C pneumoniae} was preincubated with polymyxin B (100 \(\mu\)g/mL) for 30 minutes at room temperature. Figure 4 shows that polymyxin B–treated \textit{C pneumoniae} was unable to stimulate platelet P-selectin expression (C/p 1:20). To elucidate whether polymyxin B unspecifically affected platelet activation, we studied the surface expression of P-selectin on platelets exposed to a mixture of polymyxin B (100 \(\mu\)g/mL) and collagen (2 \(\mu\)g/mL). We found that polymyxin B only slightly reduced the collagen-triggered increase of P-selectin (not shown).

**Role of Platelet Surface Structures in the Interaction with \textit{C pneumoniae}**

Experiments were also performed to search for the platelet surface components involved in the interaction with \textit{C pneumoniae}. Neither monoclonal antibodies directed against GpIIb or P-selectin nor the peptide glycocalicin (blocking the von Willenbrand factor binding site on GpIIb) antagonized the effects of \textit{C pneumoniae} on platelet activity (data not shown). However, preincubation of platelets with Arg-Gly-Asp-Ser (RGDS; 1 mg/mL) or the monoclonal Gp IIb/IIIa antibody abciximab (Reopro; 40 \(\mu\)g/mL), significantly inhibited platelet aggregation triggered by \textit{C pneumoniae} (Fig. 5).

**Discussion**

A growing amount of evidence suggests that \textit{C pneumoniae} has a role in the development of atherosclerosis.\textsuperscript{1,2} However, it is uncertain whether a \textit{C pneumoniae} infection is a triggering event of atherosclerosis or a secondary infectious
complication of an already-formed atherosclerotic plaque. Several studies have investigated \textit{C pneumoniae} interaction with different cell types involved in the atherosclerotic process, for example, monocytes/macrophages, smooth muscle cells, and endothelial cells.\textsuperscript{10,18} However, it is not known whether \textit{C pneumoniae} affects platelets. In this study, we evaluated \textit{C pneumoniae} interaction with platelets by studying binding, aggregation, secretion, and surface expression of P-selectin.

We found that \textit{C pneumoniae} was highly adhesive to platelets and triggered aggregation and secretion in a time- and concentration-dependent manner. An extensive \textit{C pneumoniae}-platelet binding was observed already after 1 minute of coincubation and increased significantly during 10 to 25 minutes, whereupon an irreversible complete aggregation was obtained. Flow cytometric analysis shows a continuous increase in the size distribution of the platelet microaggregates during the lag period. The low number of \textit{C pneumoniae} elementary bodies in relation to the platelet concentration (C/p 1:20) and the kinetics of the chlamydial-induced lumiaggregometry response with a lag period of 20 to 25 minutes suggest a cascade effect, where the chlamydiae initially stimulate few platelets, which activate neighboring cells through paracrine-signaling mechanisms. The ability of \textit{C pneumoniae} to cross-link platelets and support formation of microaggregates may constitute a mechanism by which \textit{C pneumoniae} relocalizes from the infected lung epithelium into the circulation. Earlier studies have demonstrated that bacteria can survive inside platelet aggregates, which protect the bacteria from the host defense and spread the bacteria in the circulation.\textsuperscript{20}

Several studies implicate an important role of P-selectin in atherosclerosis and thrombosis, shown by elevated levels of P-selectin in patients with congestive heart failure, stroke, peripheral artery disease, and acute coronary syndromes.\textsuperscript{21,22} Furthermore, ongoing \textit{C pneumoniae} infection and the occur-
ence of myocardial infarction is related to increased plasma levels of soluble P-selectin. In this study, we demonstrated that \( C. pneurnoniae \) rapidly increases the surface expression of platelet P-selectin. The degree of P-selectin expression triggered by \( C. pneurnoniae \) was comparable with the effects of collagen, which is a potent platelet activator. P-selectin mediates the interaction of activated platelets with neutrophils and monocytes, which may be important in the pathophysiology of cardiovascular disease. Several observations suggest that platelets deposited at sites of thrombosis and vascular injury serve as surrogates for endothelium by recruiting circulating leukocytes. Tsuji et al demonstrated that platelet P-selectin directly triggers oxygen radical production in neutrophils, and we have recently shown that collagen-activated platelets stimulate, via P-selectin, leukocyte reactive oxygen species production in whole blood. In addition, raised expression of P-selectin reflects platelet activation involving secretion of a broad range of adhesive proteins, procoagulants, cytokines, and growth factors stored in the \( \alpha \)-granules.

After percutaneous coronary intervention, arterial damage takes place that can lead to restenosis. Adhesion and aggregation of platelets on the damaged arterial wall and expression of P-selectin represent the first steps in these pathophysiological reactions. \( C. pneurnoniae \) is suggested to be involved in the progress of restenosis, which is supported by this study showing that \( C. pneurnoniae \) stimulates both P-selectin expression and platelet aggregation.

Activated platelets can alter the chemotactic and adhesive properties of endothelial cells by stimulating release of the chemotactic monocyte chemoattractant protein 1 and a surface expression of intercellular adhesion molecule-1. Both monocyte chemoattractant protein-1 and intercellular adhesion molecule-1 have been detected in high concentrations in
E. coli aggregation. The fact that C pneumoniae infections and strengthens the pathogenic property linking activates platelets underlies the tropism of chlamydial infection with atherosclerosis. The low number of elementary bodies of C pneumoniae required for platelet activation may be caused by an extensive release of chlamydial LPS, which activates the major part of the platelet population in an aggregatory and secretory response. The finding that C pneumoniae but no other tested Gram-negative bacteria (E. coli and S. typhimurium) activates platelets suggests that differences in the chemical structure of LPS are essential. Chlamydial LPS contains a unique lipid A, lacks an O-chain, and exposes a genus-specific highly immunogenic epitope on the polysaccharide core. Similar LPS has also been identified in Porphyromonas gingivalis.

Interestingly, it has been shown that platelets are directly stimulated by lipid A through an activation of protein kinase C and that bacteria with modified LPS, for example, P. gingivalis, are much more potent activators of platelets than classic Gram-negative bacteria. P. gingivalis is a major pathogen of periodontal diseases and has also been associated with atherosclerosis.

A role for chlamydial LPS in atherogenesis has previously been reported by its ability to induce foam cell formation. C pneumoniae has considerably larger affinity to platelets than to neutrophils, which suggests recognition of specific receptors on the platelet surface. The counter receptors on the platelet surface involved in the interaction with C pneumoniae were studied by using specific blocking antibodies and peptides. We found that inhibition of GpIb/IIa, with RGDS or with the monoclonal Fab fragment abciximab, significantly lowered the aggregation induced by C pneumoniae, whereas blocking of GpIIb or P-selectin had no effects. Abciximab is used worldwide in patients with acute coronary syndromes and in those undergoing percutaneous coronary intervention. The rapid upregulation of platelet P-selectin induced by C pneumoniae reflects an early release of α-granule constituents, including fibrinogen. We suggest that the extensive delay (15 to 20 minutes) between α-granule secretion and platelet aggregation constitutes a period of transformation of GpIIb/IIa into a competent fibrinogen receptor. This process, supported by generation of intercellular mediators (eg, eicosanoids), engages more and more platelets forming microaggregates and leads finally to a complete aggregation.

C pneumoniae infection is very common among the human population, occurs early and several times in life, and the bacteria persist for long periods in tissues. Thus, there are probably several opportunities for bacteria–platelet interaction, which may stimulate both the early proliferative phases of atherosclerosis and the late thrombotic vascular occlusion. A crucial role for C pneumoniae-induced platelet aggregation in atherogenesis is supported by findings suggesting that recurrent thrombus incorporation into atherosclerotic lesions is fundamental in the pathogenesis and progression of atherosclerotic plaques.

This study supports the concept that C pneumoniae plays a major causative role in atherosclerosis and suggests that platelets are susceptible target cells. Antibiotics and vaccines against C pneumoniae infections might in the future be
complementary to, or even replace, classic antiplatelet and antiatherogenic drugs. We believe that an approach to specifically prevent C. pneumoniae binding to platelets and C. pneumoniae-induced activation of platelets can be a novel therapeutic tool for cardiovascular diseases.

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Preparation of cells and *Chlamydia pneumoniae*

**Cell culture**

HEp2 cells were grown and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10 mg/L gentamicin, and 2 mmol/L L-glutamine (Gibco, BRL, Life Technologies, Paisley, Scotland). The cells were incubated at 37°C and 5% CO₂ in 75 cm² culture flasks, and then subcultured in 6-well plates at a density of 0.7 x 10⁶ cells/well prior of infection with chlamydiae.

*Chlamydia pneumoniae propagation*

*Chlamydia pneumoniae* (strain T45) was cultured in HEp2 cells, grown and maintained in RPMI 1640, essentially as described by Redecke *et al.* (18). The bacteria were added to subconfluent monolayers of HEp2 cells in 6-well plates. The plates were centrifuged at 3000 x g for 45 min at 25°C, and incubated for 2 h at 37°C and 5% CO₂. Nonadherent bacteria were removed and infected cells were incubated in fresh RPMI 1640, supplemented with 1 μg/mL cyclohexamide (ICN Biomedicals Inc, Aurora, OH). Infected cells were incubated for 72 h as mentioned above to allow development of characteristic chlamydial inclusions. The chlamydiae were harvested by disrupting HEp2 cells with glass beads followed by sonication and centrifugation at 900 x g for 10 min at 4°C to remove cellular debris. Supernatants were centrifuged at 12 000 x g for 30 min at 4°C, and the bacteria were suspended in sucrose-phosphate buffer, supplemented with FBS (10 %) (sp-2-buffer), counted by immunofluorescence staining and then stored at -70°C until use. The chlamydiae are expressed as inclusion forming units (IFU) throughout the study. Uninfected HEp2 cells (HEp2 cell debris) were handled exactly as chlamydia-infected cells and used as a control. To
study the involvement of heat-lable structures, *C. pneumoniae* was incubated at 70°C for 30 min.

The bacteria and cells were tested for mycoplasma contamination by using mycoplasma specific PCR essentially according to van Kuppeveld *et al.* (19). The nucleotide sequences of primers used in the Mycoplasma group-specific PCR assay were as follows: upstream primer GPO-3 5´-GGGAGCAAAACAGGATTAGATACCCT-3´ and downstream primer MGSO 5´-TGCACCATCTGTCACTCTGTTAACCTC-3´ (SGS AB, Köping, Sweden). In short, the cells were centrifuged at 300 x g at 5 min and the DNA of the cells and *C. pneumoniae* culture was extracted by using QIAamp® DNA Mini Kit according to QIAGen Blood & Body fluid Protocol. Sample (1 µL) was added to 25 µL of the following PCR mixture: 1.5 units of Taq DNA polymerase, 10 mmol/L Tris-HCL (pH 9.0 at room temperature), 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 200 µmol/L of each dNTP and stabilizers, including BSA, 0.8 µmol/L upstream primer GPO-3, and 0.8 µmol/L downstream primer MGSO. The PCR protocol used in a PTC-100™ (SDS, Falkenberg, Sweden) was as follows: denaturation at 94 °C for 40 seconds, annealing temperature at 55 °C for 40 seconds, extension at 72 °C for 1 min at 25 cycles.

**Preparation of platelets and neutrophils**

Platelets and neutrophils were isolated from freshly drawn heparinized human peripheral blood, donated by apparently healthy and drug free adult volunteers at the blood bank at Linköping University Hospital, Linköping, as previously described (13). Five parts of blood were mixed with one part of an acid citrate/dextrose solution (85 mmol/L trisodium citrate dihydrate, 71 mmol/L citric acid hydrate and 111 mmol/L D-glucose), followed by centrifugation at room temperature for 20 min at 220 x g to obtain platelet rich plasma (PRP).
The PRP was centrifuged for 20 min at 480 x g, and the platelets were then gently washed and resuspended in calcium-free buffer (final cell density 2x10^8/mL) and stored in plastic tubes at room temperature before use. To obtain functional but non-activated platelets, the isolation was performed without any specific platelet inhibitors, and, due to this, extra care was taken when handling the cells. Morphological studies showed discoid, solitary platelets displaying no signs of activation due to the preparation procedure. The contamination of other blood cells was negligible. The extracellular calcium concentration was adjusted to 1 mmol/L immediately before each experiment.

In short, neutrophils were isolated by layering one part of fresh whole blood on one part of lymphoprep over four parts of Polymorphprep (Nycomed Pharma AS, Olso, Norway) followed by centrifugation for 40 min at 480 x g. The resulting band of neutrophils was harvested and washed, and remaining red blood cells were eliminated by brief hypotonic lysis at 4°C followed by washing in calcium-free buffer.