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Signal Transduction Pathways Activated in Endothelial Cells Following Infection with Chlamydia pneumoniae

Matthias Krüll,* Andrea C. Klucken,* Frederik N. Wuppermann,† Oliver Fuhrmann,* Christian Magerl,* Joachim Seybold,* Stefan Hippenstiel,* Johannes H. Hegemann,‡ Christian A. Jantos,† and Norbert Suttorp²§

Chlamydia pneumoniae is an important respiratory pathogen. Recently, its presence has been demonstrated in atherosclerotic lesions. In this study, we characterized C. pneumoniae-mediated activation of endothelial cells and demonstrated an enhanced expression of endothelial adhesion molecules followed by subsequent rolling, adhesion, and transmigration of leukocytes (monocytes, granulocytes). These effects were blocked by mAbs against endothelial and/or leukocyte adhesion molecules (β1 and β2 integrins). Additionally, activation of different signal transduction pathways in C. pneumoniae-infected endothelial cells was shown: protein tyrosine phosphorylation, up-regulation of phosphorylated p42/p44 mitogen-activated protein kinase, and NF-κB activation/translocation occurred within 10–15 min. Increased mRNA and surface expression of E-selectin, ICAM-1, and VCAM-1 were noted within hours. Thus, C. pneumoniae triggers a cascade of events that could lead to endothelial activation, inflammation, and thrombosis, which in turn may result in or may promote atherosclerosis. The Journal of Immunology, 1999, 162: 4834–4841.

Chlamydia pneumoniae, a Gram-negative obligate intracellular bacterium, is a widespread respiratory pathogen causing pneumonia, bronchitis, sinusitis, and pharyngitis (1–3). Recently, chronic C. pneumoniae infection has been suggested as a trigger and promoter of inflammatory reactions and development of vascular lesions. This notion is supported by the demonstration (electron microscopy, immunocytochemistry, PCR) of C. pneumoniae in atherosclerotic plaques and the serological association between C. pneumoniae infection and coronary heart disease (4–8).

The role of C. pneumoniae in atheroma formation has not been studied in detail. Monocytes, macrophages, and smooth muscle cells have been shown to be susceptible for C. pneumoniae infection (9–11). Chlamydiae may reside and replicate in monocytes/macrophages and induce a chronic immune activation resulting in TNF-α, IL-1β, IL-6, and IFN-γ release as well as up-regulation of CD14 (12, 13). Little is known about C. pneumoniae-induced endothelial cell alterations and C. pneumoniae-mediated interactions among all cell types involved in the progress of atherosclerosis.

Airway-derived organisms may be able to spread systemically via at least two different ways: 1) carried within monocytes following pulmonary infection or 2) by direct access to the blood stream causing a short interval of chlamydial bacteremia. Chronic monocyte/macrophage infection as well as direct activation of endothelial cells may contribute to local inflammation by inducing cytokine release and increased expression of adhesion molecules, which in turn may result in enhanced rolling, adhesion, and transendothelial migration of leukocytes.

Adhesion of circulating leukocytes to endothelial cells is an early step in an inflammatory reaction that is regulated by a complex communication between the cell types involved. Recent studies revealed that multiple receptor-ligand pairs act sequentially and in an overlapping manner to effect initial attachment, rolling, firm adhesion, and finally transmigration of leukocytes (14–16). For leukocyte adhesion to activated endothelial cells, separate receptor-ligand pairs are involved: ICAM-1 and β2 integrins (CD11a/CD18, CD11b/CD18), VCAM-1 and very late Ag-4 (VLA 4), P-selectin and P-selectin-glycoprotein ligand-1, E-selectin and E-selectin ligand-1, as well as sialyl LewisX and related carbohydrates on leukocytes (14, 17–19).

Therefore, the first objective of the present study was to assess C. pneumoniae’s capability to infect and activate HUVEC and human aortic endothelial cells (HAEC). Enhanced expression of endothelial adhesion molecules was accompanied by an increased rolling, adhesion, and transmigration of polymorphonuclear leukocytes (PMN) and monocytes. These effects were reduced by mAbs directed against different adhesion molecules on leukocytes and/or endothelial cells.

There is limited knowledge of the mechanisms of C. pneumoniae entry into endothelial cells. The chlamydial growth cycle is initiated when an infectious elementary body (EB) attaches to a susceptible target cell, promoting entry into a host cell-derived phagocytic vesicle. EB develop into reticular bodies, a process that could be detected metabolically within 15 min and microscopically 12–15 h after addition of Chlamydiae to Hep-2 and Hela-229 cells (20, 21). The length of the complete developmental cycle, as studied in cell culture models, is 48–72 h (22–24).

*Department of Internal Medicine, Justus-Liebig-University, Giessen, Germany; †Institute of Medical Microbiology, Justus-Liebig-University, Giessen, Germany; ‡Institute of Microbiology, Heinrich-Heine-University, Düsseldorf, Germany; and §Charité Department of Internal Medicine, Humboldt-University, Berlin, Germany

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‡ Address correspondence and reprint requests to Dr. Norbert Suttorp, Charité, Department of Internal Medicine/Infectious Diseases Humboldt-University, Augustenburger Platz 1, 13353 Berlin, Germany. E-mail address: norbert.suttorp@charite.de

3 Abbreviations used in this paper: VLA, very late Ag; HAEC, human aortic endothelial cells; PMN, polymorphonuclear leukocytes; MAPK, mitogen activated protein kinase; IFU, inclusion forming unit; EB, elementary body; EMSA, electrophoretic mobility shift assay.

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Almost nothing is known about the signal transduction pathways initiated in the host cell upon *Chlamydiae*-target cell interaction (intracellular Ca\(^{2+}\) increase, activation of protein and tyrosine kinases, mitogen-activated protein kinases (MAPK), NF-κB translocation). Therefore, the second objective of the present study was to assess *C. pneumoniae*’s capability to activate host cell signal transduction pathways. *C. pneumoniae* induced an immediate increase of total protein tyrosine phosphorylation in HUVEC and HAEC, especially of phosphorylated p42/p44 MAPK and an activation/translocation of endothelial cell NF-κB with subsequently increased transcription and translation of E-selectin, ICAM-1, and VCAM-1.

Overall, the data presented indicate that *C. pneumoniae* can infect and activate human endothelial cells and suggest that *C. pneumoniae*-induced endothelial cell alterations may promote atherosclerosis.

### Materials and Methods

#### Materials

Tissue culture plasticware was obtained from Becton Dickinson (Heidelberg, Germany). MCDB131 medium, PBS, trypsin-EDTA solution, HEPES, and FCS were from Life Technologies (Karlsruhe, Germany). MCDB131 medium, PBS, trypsin-EDTA solution, HEPES, and FCS were from Life Technologies (Karlsruhe, Germany). MCDB131/5% FCS, and seeded into tissue culture flasks (80 cm\(^2\)), well plates, or glass coverslips.

#### Isolation of human PMN.

Heparinized human donor blood was centrifuged in a discontinuous Percoll gradient to yield a PMN fraction of 90\% purity as described (28, 29).

#### Preparations of HUVEC.

Cells were isolated from umbilical cord veins and identified according to the method of Jaffe et al. (25). Briefly, cells obtained from collagenase digestion were washed, resuspended in MCM/15% FCS, and seeded into tissue culture flasks (80 cm\(^2\)), well plates, or glass coverslips (26, 27).

#### HAEC.

HAEC were obtained from Clonetics (San Diego, CA). HAEC were grown to confluence using EGM-2/2% FCS (Clonetics) in tissue culture flasks, or glass coverslips (26, 27).

#### Isolation of human PMN.

Heparinized human donor blood was centrifuged in a discontinuous Percoll gradient to yield a PMN fraction of 90\% purity as described (28, 29).

#### Isolation and labeling of human monocytes.

Human monocytes were isolated from anti-coagulated whole blood or from buffy coats by centrifugation on Ficoll-Paque density gradient followed by counterflow centrifugation elutriation in a Beckman J-6 MC centrifuge using a JE-6 elutriation rotor and a 5.5-ml Sanderson chamber (30). Monocyte suspension was 90 ± 4\% (n = 20) pure with 7\% lymphocytes and <2\% granulocytes as determined by light scatter (FACScan; Becton Dickinson).

#### Chlamydia strain

*C. pneumoniae* strain GiD was used. This strain was originally isolated from a patient with bronchitis. The isolate was identified to be *C. pneumoniae*. Isolation and purification of *C. pneumoniae* was performed as described (31).

#### CFU determination

A suspension of 10\(^6\) IFU/ml was perfused through the chamber at a constant wall shear stress of 1.0 dynecm\(^{-2}\) (syringe pump sp100; World Precision Instruments, Sarasota, FL). Interactions were visualized using a phase contrast video microscope (IMT-2; Olympus Optical, Hamburg, Germany). A suspension of 10\(^9\) leukocytes/ml was perfused through the chamber at a constant wall shear stress of 1.0 dynecm\(^{-2}\) (syringe pump sp100; World Precision Instruments, Sarasota, FL). Interactions were visualized using a phase contrast video microscope (IMT-2; Olympus Optical, Hamburg, Germany). A suspension of 10\(^9\) leukocytes/ml was perfused through the chamber at a constant wall shear stress of 1.0 dynecm\(^{-2}\) (syringe pump sp100; World Precision Instruments, Sarasota, FL). Interactions were visualized using a phase contrast video microscope (IMT-2; Olympus Optical, Hamburg, Germany). A suspension of 10\(^9\) leukocytes/ml was perfused through the chamber at a constant wall shear stress of 1.0 dynecm\(^{-2}\) (syringe pump sp100; World Precision Instruments, Sarasota, FL). Interactions were visualized using a phase contrast video microscope (IMT-2; Olympus Optical, Hamburg, Germany).

#### Western blot analysis

For detection of tyrosine phosphorylation HUVEC or HAEC grown on six-well culture plates were stimulated with 6.5 × 10\(^5\) IFU/ml *C. pneumoniae*. Cell proteins (40 μg/lane) were separated by SDS-PAGE according to Laemmli et al. (34), blotted on Hybond enhanced chemiluminescence membranes (Amersham, Dreieich, Germany), blocked, incubated with rabbit-mAb against tyrosine-phosphorylated proteins (RC20; Transduction Laboratories, Lexington, Kentucky) or phosphorylated p42/p44 MAPK (New England Biolabs, Beverly, MA), and detected by enhanced chemiluminescence (Amersham).

#### Electroophoretic mobility shift assay (EMSA)

After stimulation with 6.5 × 10\(^5\) IFU/ml *C. pneumoniae* for different time points, nuclear protein was isolated as described by Newton et al. (35). The consensus NF-κB oligonucleotide (5′-AGT TGA GGC ATT CCC ACC TTT-3′) was end-labeled with [γ\(^{32}\)P]ATP using T4 polynucleotide kinase (Bioline, Berlin, Germany). Unincorporated nucleotides were separated on a Sephadex G-25 spin column (Pharmacia, Freiburg, Germany). EMSA binding reactions were performed by first preincubating 5 μg of nuclear extract with 1 μg of poly(dI-dC) in binding buffer (10 mM Tris, pH 7.7, 50 mM NaCl, 20% glycerol, 1 μM DTT, 0.5 mM EDTA) for 20 min. Approximately 10,000 cpm (0.2 ng) of [γ\(^{32}\)P]-labeled DNA probe was then added and allowed to bind for 20 min. The reaction mixture was subjected to electrophoresis on 7% native acrylamide gels before vacuum drying and exposing to a storage phosphor screen for quantification and documentation (PhosphorImager, Molecular Dynamics, Sunnyvale, CA). Competition experiments were performed as above except that 100-fold excess unlabeled competitor DNA was added to the incubations.

#### Immunofluorescence analysis of NF-κB nuclear translocation

After stimulation of HUVEC grown on glass chamber slides (Falcon CultureSlide, Becton Dickinson, Rutherford, NJ) with 6.5 × 10^4 IFU/ml *C. pneumoniae* suspensions were thawed, diluted in MCDB131 medium, and inoculated onto HUVEC or HAEC grown in 24- or 96-well tissue culture plates. Plates were centrifuged at 800 × g for 1 h at 35°C. After incubation at 37°C for 1 h, the supernatant was replaced by fresh medium and plates were processed for additional experiments at the times indicated in the figure legends.
C. pneumoniae for 30 min, cells were fixed and permeabilized with acetone/methanol (−20°C, 50:50 v/v) for 5 min. Human Ig was used to reduce nonspecific binding, and the primary Ab (polyclonal rabbit anti-human NF-κB p65 Ab; Santa Cruz Biotechnology, Santa Cruz, CA) was added for 30 min. Thereafter, cells were washed thrice and exposed to an ALEXA-488-conjugated goat anti-rabbit Ig Ab (Molecular Probes, Eugene, OR) for 15 min. After washing thrice with PBS, coverslips were sealed and examined in a Olympus IMT-2 fluorescence microscope (Olympus Optical, equipped with an Olympus OM-4 camera) with an Olympus 60X objective.

**NF-κB reporter gene assay**

Six NF-κB DNA binding sites (5′-GGG GAC TTT CCC T-3′; italics indicates original binding site) were inserted into the Smal site in a pGL3-basic vector (Promega). Downstream of this six NF-κB binding region, a minimal β-globin promoter (containing a TATA box) was inserted into the Xhol/HindIII sites followed by the luciferase gene (pGL3.BG.6xkB). HUVEC were transiently transfected with 2 μg NF-κB plasmid using SuperFect transfection reagent (Qiagen, Hilden, Germany). Luciferase-assay was performed using a commercial kit (Promega). Luminescence was measured in a Lumat LB 9501 luminometer (Berthold, Wildbad, Germany). The 598-bp cDNA fragment of GAPDH was obtained as described by Chomczynski and Sacchi (36). cDNA probes were labeled with [α-32P]dCTP (3000 Ci/mmol) by random priming (Rediprime DNA labelling system; Amersham, Braunschweig, Germany), added to the prehybridization chambers, and incubated for 12–16 h at 42°C. E-selectin and VCAM-1 cDNA probes were a friendly gift of Dr. D. Simmons (Imperial Cancer Research Fund, Institute of Molecular Medicine, Oxford, U.K.), and the ICAM-1 probe was kindly provided by Dr. D. Vestweber (Department of Cell Biology, University of Münster, Münster, Germany). The 598-bp cDNA fragment of GAPDH was obtained as previously described (37).

**Northern blot analysis**

RNA was extracted using the guanidinium isothiocyanate method as described by Chomczynski and Sacchi (36). cDNA probes were labeled with [α-32P]dCTP (>3000 Ci/mmol) by random priming (Rediprime DNA labeling system; Amersham, Braunschweig, Germany), added to the prehybridization chambers, and incubated for 12–16 h at 42°C. E-selectin and VCAM-1 cDNA probes were a friendly gift of Dr. D. Simmons (Imperial Cancer Research Fund, Institute of Molecular Medicine, Oxford, U.K.), and the ICAM-1 probe was kindly provided by Dr. D. Vestweber (Department of Cell Biology, University of Münster, Münster, Germany). The 598-bp cDNA fragment of GAPDH was obtained as previously described (37).

**Statistical methods**

Depending on the number of groups (A) and the number of different time points studied (B), data of Figs. 1b and 2, α, c, and e were analyzed by a two-way ANOVA. An one-way ANOVA was used for data of Figs. 1, a and c, 2, b, d, and f, 3, a–c, and 4ab. Values of p < 0.05 were considered significant (38).

**Results**

C. pneumoniae strain GiD infects human endothelial cells

C. pneumoniae has been demonstrated to infect and thereby activate human endothelial cells (10, 11, 39). To investigate leukocyte-endothelial cell interaction, we infected HUVEC with the recently described C. pneumoniae strain GiD (31). GiD turned out to be a highly effective Chlamydia strain and was able to replicate and to form inclusions in HUVEC with a mean titer of 2.6 \times 10^3 IFU/ml as compared with growth in cyclohexamide-treated HEp-2 cells at a mean titer of 6.5 \times 10^4 IFU/ml. Inclusions were visualized using a FITC-conjugated genus-specific mAb (Chlamydia

**FIGURE 1.** Infection of endothelial cells increased monocyte rolling, adhesion, and transendothelial migration. a, Endothelial cells grown on Thermox coverslips were infected with C. pneumoniae (for details, see Materials and Methods). After 4 h, coverslips were processed for laminar flow assay. A total of 3 \times 10^6 monocytes/ml was injected into the flow system and perfused over endothelial cell monolayer for 5 min using a high precision syringe pump. Rolling monocytes (for definition and details, see Materials and Methods) were counted over a 3-min observation period. Note that monocyte rolling on C. pneumoniae-infected endothelial cells (6.5 \times 10^6 IFU/ml, 4 h, open bars and 24 h, filled bars) at a shear rate of 1 dyne/cm² was significantly increased. C. pneumoniae-mediated rolling after 4 h was reduced by anti-E-selectin mAb (50 μg/ml, 60 min) and after 24 h by anti-VCAM-1 mAb on HUVEC (50 μg/ml, 60 min) or anti-CD49d mAb on monocytes (10 μg/ml, 10 min). b, C. pneumoniae-induced leukocyte adhesion to endothelial monolayer under flow conditions. Adhesion was determined after 5 min of perfusion by analysis of 10 random high power fields (20×) from videotape, and results are expressed as mean of total adherent leukocytes. c, Note that endothelial cell infection with 6.5 \times 10^2-6.5 \times 10^4 IFU/ml C. pneumoniae dose-dependently increased monocyte transmigration after 4 and 24 h. Data presented (α–c) are mean ± SEM of five separate experiments.
culture confirmation system; Sanofi Diagnostics Pasteur, Freiburg, Germany) (data not shown).

C. pneumoniae-mediated leukocyte endothelial cell interaction

To investigate C. pneumoniae-mediated leukocyte-endothelial cell interaction, HUVEC were stimulated with different concentrations of strain GiD. Intracellular infection resulted in a profound cell stress of 1.0 dyn/cm^2. Alternatively, leukocytes were pretreated with mAb against β1 or β2 integrins for 10 min and then added to C. pneumoniae-stimulated HUVEC. Leukocyte adhesion to C. pneumoniae-infected HUVEC increased after 1 h and remained elevated up to 72 h. Data are indicated as percent inhibition of adherent leukocytes/mm^2 ± SEM (n = 5).

Table I. Modification of C. pneumoniae-induced leukocyte adhesion to HUVEC by mAbs directed against different adhesion molecules

<table>
<thead>
<tr>
<th>mAb Added</th>
<th>4 h incubation</th>
<th>24 h incubation</th>
<th>4 h incubation</th>
<th>24 h incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control^a</td>
<td>4.0 ± 2.0</td>
<td>3.0 ± 1.7</td>
<td>3.2 ± 1.2</td>
<td>2.4 ± 1.1</td>
</tr>
<tr>
<td>CD11a</td>
<td>85.7 ± 4.1</td>
<td>27.6 ± 3.4</td>
<td>71.5 ± 3.8</td>
<td>85.1 ± 4.1</td>
</tr>
<tr>
<td>CD11b</td>
<td>60.9 ± 1.8</td>
<td>56.3 ± 1.9</td>
<td>55.9 ± 2.2</td>
<td>60.6 ± 3.3</td>
</tr>
<tr>
<td>CD11c</td>
<td>36.5 ± 2.2</td>
<td>73.2 ± 2.2</td>
<td>17.8 ± 3.5</td>
<td>35.0 ± 0.8</td>
</tr>
<tr>
<td>CD18</td>
<td>95.5 ± 5.6</td>
<td>60.6 ± 3.6</td>
<td>68.8 ± 4.1</td>
<td>68.7 ± 4.2</td>
</tr>
<tr>
<td>CD49d</td>
<td>22.1 ± 2.3</td>
<td>40.2 ± 4.3</td>
<td>34.1 ± 3.4</td>
<td>56.8 ± 3.3</td>
</tr>
<tr>
<td>E-selectin</td>
<td>45.5 ± 2.2</td>
<td>53.5 ± 1.5</td>
<td>58.3 ± 3.6</td>
<td>9.3 ± 1.7</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>42.8 ± 4.2</td>
<td>43.6 ± 3.5</td>
<td>63.2 ± 1.9</td>
<td>84.2 ± 3.9</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>3.3 ± 0.9</td>
<td>24.2 ± 1.4</td>
<td>28.5 ± 3.4</td>
<td>83.8 ± 2.5</td>
</tr>
</tbody>
</table>

^a HUVEC on glass coverslips were incubated with 6.5 × 10^8 IFU/ml C. pneumoniae for 4 or 24 h. Cells were then washed, exposed to 50 μg/ml mAb against E-selectin, ICAM-1, or VCAM-1 for 1 h, and mounted in a parallel plate flow chamber. Then, 1 × 10^6 leukocytes/ml (PMN or monocytes) were perfused at a wall shear stress of 1.0 dyn/cm^2. Alternatively, leukocytes were pretreated with mAb against β1 or β2 integrins for 10 min and then added to C. pneumoniae-stimulated HUVEC. Leukocyte adhesion to C. pneumoniae-infected HUVEC increased after 1 h and remained elevated up to 72 h. Data are indicated as percent inhibition of adherent leukocytes/mm^2 ± SEM (n = 5).

C. pneumoniae increased E-selectin, ICAM-1, and VCAM-1 expression in endothelial cells

Cell surface ELISA for endothelial adhesion molecule expression was performed to further characterize C. pneumoniae-mediated leukocyte-endothelial cell interaction. Infection of HUVEC and HAEC with C. pneumoniae dose- and time-dependently increased expression of E-selectin, ICAM-1, and VCAM-1 (Fig. 3) on endothelial cells (HUVEC and HAEC). Maximal effects in this study occurred in the presence of 6.5 × 10^8 IFU/ml (HAEC, data not shown). E-selectin expression in C. pneumoniae-infected endothelial cells increased 2 h postinfection, peaked at 4 h, and declined to almost baseline after 18–24 h. Even the lowest Chlamydia concentration tested was able to induce a significant E-selectin response (Fig. 2a). ICAM-1 and VCAM-1 surface expression in C. pneumoniae stimulated cells increased 4–8 h postinfection, peaked at 12–24 h (HUVEC) and persisted up to 72 h (Fig. 2, c and e). Compared with HUVEC, the kinetics of the ICAM-1 and VCAM-1 response in HAEC were delayed, and absolute levels of protein expression diminished.

Northern blot analysis of C. pneumoniae-pretreated HUVEC

Northern blot analysis was performed to verify mRNA up-regulation for E-selectin, ICAM-1, and VCAM-1 (Fig. 3). E-selectin mRNA peaked at 2 h of C. pneumoniae stimulation and almost completely disappeared after 8 h (Fig. 3a). ICAM-1 and VCAM-1 mRNA peaked at 2 h, declined to almost baseline after 8 h, and was detectable again after 24 h (Fig. 3, b and c).

Table II. Modification of C. pneumoniae-induced monocyte transmigration through the HUVEC monolayer by mAbs directed against different adhesion molecules

<table>
<thead>
<tr>
<th>mAb Added</th>
<th>4 h incubation</th>
<th>24 h incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control^b</td>
<td>1.3 ± 0.7</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>CD11b</td>
<td>62.3 ± 5.2</td>
<td>70.4 ± 3.7</td>
</tr>
<tr>
<td>CD18</td>
<td>80.1 ± 4.4</td>
<td>85.2 ± 5.2</td>
</tr>
<tr>
<td>CD11b + CD18</td>
<td>88.4 ± 5.3</td>
<td>90.5 ± 4.3</td>
</tr>
<tr>
<td>E-selectin</td>
<td>12.2 ± 1.2</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>55.3 ± 4.2</td>
<td>47.5 ± 3.3</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>1.3 ± 0.5</td>
<td>2.1 ± 0.7</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>50.9 ± 3.5</td>
<td>46.4 ± 6.8</td>
</tr>
</tbody>
</table>

^b Use of nonspecific IgG-isotype control.
Role of NF-κB in C. pneumoniae-mediated endothelial cell activation

Previous studies have elaborated the importance of NF-κB for the regulation of the transcriptional activities of the E-selectin, ICAM-1, and VCAM-1 genes (40). Multiple NF-κB binding sites have been located in the promoters of all three genes (41–43).

NF-κB activation in C. pneumoniae-infected endothelial cells was demonstrated by the enhanced binding-capacity of NF-κB to corresponding consensus oligonucleotides using band shift assays (Fig. 4a). This point was verified by immunofluorescence, which indicated increased NF-κB translocation within 15–30 min of C. pneumoniae expression was determined at 12 h (d and f). Open symbols (a, c, and e) or bars (b, d, and f) indicate unstimulated endothelial cells. Data presented (a–f) are mean ± SEM of five separate experiments.

Discussion

The study presented demonstrates that C. pneumoniae can infect and activate endothelial cells. The C. pneumoniae strain GiD, isolated from a man with bronchitis, was used and shown to up-regulate endothelial adhesion molecules followed by increased rolling, adhesion, and transendothelial migration of human leukocytes (PMN and monocytes). These effects were reduced by mAb directed against different adhesion molecules on PMN (β2 integrins), monocytes (β2 integrins, VLA4), and endothelial cells (ICAM-1, E-selectin, VCAM-1). Overall, these observations add important new properties to this bacterium regarding its capacity to
initiate a cascade of events leading to endothelial cell activation, inflammation, and thrombosis.

In vivo, endothelial cell infection may occur directly via blood-borne *C. pneumoniae* or indirectly as shown by recent work by Gaydos et al. demonstrating that *C. pneumoniae* transfer to human endothelial cells may proceed by cell-to-cell spread from infected adherent mononuclear phagocytes (44). Moreover, mice intranasally infected with *C. pneumoniae* showed evidence of systemic chlamydial dissemination via macrophages (45).

Almost nothing is known about the signal transduction pathways activated upon target cell infection. Our studies, aimed to identify possible intracellular signaling steps involved, indicated that protein tyrosine phosphorylation, MAPK activation, and NF-κB activation/translocation occurred within 10–15 min of

**FIGURE 4.** Role of NF-κB in *C. pneumoniae*-mediated endothelial cell activation. This aspect was analyzed using (a) EMSA, (b) NF-κB reporter gene assay, and (c) immunofluorescence. *a*, HUVEC were stimulated with $6.5 \times 10^4$ IFU/ml *C. pneumoniae* for times indicated. Nuclear proteins were extracted and processed for EMSA. Note enhanced binding of NF-κB to corresponding consensus oligonucleotides starting after 15 min of *C. pneumoniae* addition. A representative gel (of three) is demonstrated. *b*, NF-κB activation was confirmed by enhanced NF-κB-dependent transcription of a luciferase gene transiently transfected into HUVEC (*b*) (see Materials and Methods for details). A total of 10 ng/ml IL-1β was used as positive control in both assay systems. Data presented are mean ± SEM of three separate experiments. *c*, HUVEC were grown on glass chamber slides and stimulated with $6.5 \times 10^4$ IFU/ml *C. pneumoniae* for 30 min. Cells were permeabilized with acetone/methanol and incubated with a polyclonal rabbit anti-human NF-κB p65 Ab followed by the addition of an ALEXA-488-conjugated goat anti-rabbit Ig Ab. The upper picture (I) shows unstimulated control cells. Note that increased immunofluorescence in cell nuclei (i.e., nuclear NF-κB translocation) is demonstrable 30 min after addition of *Chlamydia* (*II*, magnification x600). Representative pictures (of three independent experiments) are demonstrated.
believe that bacteria-induced target cell activation results from stimulation of common and distinct (dependent on pathogen-specific virulence factors) signaling pathways in host cells.

The interpretation of our study is limited to cultured human large vessel endothelial cells. For an exact analysis of *C. pneumoniae*-related alterations of endothelial function in clinical disorders, it would be desirable to also study human small vessel endothelial cells of different organs. However, the isolation and culture of these cells in sufficient quantities is difficult, and therefore the applicability of the data presented to human disease must be verified in further studies. At least with respect to large vessel endothelium, we provided data demonstrating no substantial difference between cultured human large vein and aortic endothelial cells.

In conclusion, we present evidence that *C. pneumoniae* can infect HUVEC and HAEC and activate different signal transduction pathways: protein tyrosine phosphorylation, MAPK stimulation, and activation/translocation of NF-κB occurred within minutes. Within hours, increased mRNA and surface expression of E-selectin, ICAM-1, and VCAM-1 was noted, which in turn resulted in enhanced leukocyte (monocytes, PMN)-HUVEC interaction. Overall, the data presented suggest that *C. pneumoniae* infection triggers a cascade of events that could lead to endothelial damage, inflammation, and thrombosis.

**Acknowledgments**

We thank H. Geisel for technical assistance. Parts of this work will be included in the MD thesis of A. C. Klucken and C. Magerl. We also thank the staff of the Delivery-Services of the Krankenhaus Ehringshausen, Dillingen, and Lich for invaluable help in collecting umbilical cords.

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