Chlamydia pneumoniae Activates Epithelial Cell Proliferation via NF- κ B and the Glucocorticoid Receptor

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Chlamydia pneumoniae is an obligate intracellular eubacterium and a common cause of acute and chronic respiratory tract infections. This study was designed to show the effect of *C. pneumoniae* on transcription factor activation in epithelial cells. The activation of transcription factors by *C. pneumoniae* was determined in human epithelial cell lines (HL and Calu3) by electrophoretic DNA mobility shift assay, Western blotting, and luciferase reporter gene assay. The activation of transcription factors was further confirmed by immunostaining of *C. pneumoniae*-infected HL cells and mock-infected controls. The effect of transcription factors on *C. pneumoniae*-induced host cell proliferation was assessed by [³H]thymidine incorporation and direct cell counting in the presence and absence of antisense oligonucleotides targeting transcription factors or the glucocorticoid receptor (GR) antagonist RU486. The activation of the GR, CCAAT-enhancer binding protein (C/EBP), and NF- κ B was induced within 1 to 6 h by *C. pneumoniae*. While the interleukin-6 promoter was not activated by *C. pneumoniae*, the GR-driven p21^(Waf1/Cip1) promoter was increased 2.5- to 3-fold over controls 24 h after infection. *C. pneumoniae* dose-dependently increased the DNA synthesis of the host cells 2.5- to 2.9-fold, which was partly inhibited either by RU486 or by NF- κ B antisense oligonucleotides. Furthermore, we provide evidence that heat-inactivated *C. pneumoniae* does not cause a significant increase in cell proliferation. Our results demonstrate that *C. pneumoniae* activates C/EBP- β , NF- κ B, and the GR in infected cells. However, only NF- κ B and the GR were involved in *C. pneumoniae*-induced proliferation of epithelial cells.

Asthma, chronic obstructive pulmonary disease (COPD), and bronchiolitis obliterans are inflammatory diseases of the lung to which multiple risk factors have been linked. Asthma exacerbations often develop following acute *Chlamydia pneumoniae* infection (8, 9, 13, 15, 33). *C. pneumoniae* infection has also been associated with COPD and has been discussed as an important cofactor causing chronic airway inflammation (6, 9, 13, 15, 17, 30, 31, 33, 37), one of the central characteristics of asthma and COPD. A recent study demonstrated the presence of *C. pneumoniae* in lung tissue of patients with COPD by immunohistochemical staining (39).

C. pneumoniae is known to infect and replicate in macrophages and epithelial cells, where it uses several surface proteins and peptidoglycans to attach to and infect the host cells (4, 32, 40, 41). Both its intracellular mode of growth and its ability to modulate host cell protein synthesis enable *C. pneumoniae* to escape the host's immune system. *C. pneumoniae* can divide without killing the host cell and allows infected cells to proliferate. Therefore, *C. pneumoniae* infection can become slowly spreading and latent (14, 41). It is also known that bacterial infection may cause apoptosis in the host cell (43). If *Chlamydia*-infected cells fail to keep the balance between cell death and cell division, this in turn may cause unlimited proliferation of infected cells. We suggest that *C. pneumoniae* infection in epithelial cells may lead to increased proliferation

and therefore contribute to local inflammation or to modification of the thickness of the airway wall and function.

Recent studies have suggested that *C. pneumoniae* heat shock proteins (hsp) interfere with some of the host's signal transducers and transcription factors (12, 29). *C. pneumoniae* hsp60 and human hsp60 affect the activation of the transcription factor NF- κ B in endothelial and smooth muscle cells (18). Furthermore, hsp need nucleic acid triphosphate, such as ATP, for their interaction with other signaling proteins, including the glucocorticoid receptor (GR) (20, 21, 25). HSP function as chaperones and interact with the GR, thereby controlling activation of the GR (20, 21). The GR plays a central role in lung embryology and lung cell differentiation (2, 11); its modulation by *C. pneumoniae* would be important in explaining the role of *C. pneumoniae* in the pathogenesis of inflammatory lung diseases.

MATERIALS AND METHODS

Cell lines and infection with *C. pneumoniae* in cell culture. Two human epithelial cell lines were used in these experiments: HL and Calu3. Both cell lines were cultured in minimal essential medium supplemented with 10% fetal bovine serum, 8 mM stabilized L-glutamine, 2% minimal essential medium-vitamin mix, and 20 mM HEPES (Gibco BRL, Paisley, United Kingdom).

The cell lines were checked for *Mycoplasma* contamination using PCR as described earlier (26). *C. pneumoniae* (isolate K-6, obtained from the Department of Virology, University of Helsinki, Helsinki, Finland) was grown in cycloheximide-treated HL and Calu3 cells, purified through 30% Urografin (Schering, Berlin, Germany) centrifugation, and resuspended in cycloheximide-free medium. For storage, SPG (250 mM sucrose–10 mM sodium phosphate–5 mM L-glutamic acid)-diluted stock was kept frozen at –80°C. The purified *C. pneumoniae* stock was titrated in both the Calu3 and HL cell lines, and the titer was expressed as inclusion-forming units (IFU) per milliliter or multiplicities of infection (MOI). Purified *C. pneumoniae* stock was used in the corresponding

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cells for all experiments at a concentration of 5×10^8 IFU/ml. Mock-infected controls were prepared in the same way as infected cells.

Cells were grown in six-well plates (10^4 cells per well) and were infected with the *C. pneumoniae* stock (5×10^8 IFU/ml) by centrifugation (1 h; $3,000 \times g$; 32°C). Mock-infected controls were prepared by the same procedure. To determine the growth of *C. pneumoniae* in infected cells, we used fluorescein-labeled monoclonal antibodies (Cellabs, Sydney, Australia).

Preparation of nuclear and cytosolic proteins. Nuclear and cytosolic proteins from infected or mock-infected cells were extracted from six-well plates (10^4 cells per well) at 0, 0.5, 1, 3, 6, and 24 h postinfection. In brief, the cells were washed twice with 15 ml of ice-cold phosphate-buffered saline (PBS) and collected by centrifugation (5 min; $600 \times g$). The supernatant was discarded, and the cells were resuspended in 150 μl of low-salt buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM NaVO_4 , 1 mM EDTA, 1 mM EGTA, 0.2% Nonidet P-40, 10% glycerol, $1 \times$ Boehringer Complete), vortexed (1 min), and incubated on ice (5 min). Following centrifugation (2 min; $13,000 \times g$), the supernatant was transferred into a new tube and kept as a cytosolic fraction at -70°C . The remaining pellet was resuspended in 60 μl of high-salt buffer B (20 mM HEPES, pH 7.9, 420 mM NaCl, 10 mM KCl, 0.1 mM NaVO_4 , 1 mM EDTA, 1 mM EGTA, 20% glycerol, $1 \times$ Boehringer Complete), kept on ice (30 min), and vortexed every 5 min. Nuclear membrane fragments were removed by centrifugation (10 min; $13,000 \times g$; 4°C), and the supernatant was stored as a nuclear fraction at -70°C (7).

Western blotting for transcription factors. Samples of nuclear and cytosolic fractions containing 5 μg of protein (determined by Bradford assay) were size fractionated by denaturing sodium dodecyl sulfate–8% polyacrylamide gel electrophoresis. The proteins were transferred onto a polyvinylidene difluoride membrane, and protein levels were confirmed by Ponceau staining (28). The membranes were blocked (Tris-buffered saline [TBS] plus 2% skim milk) for 30 min. Protein analysis by Western blotting was performed using antibodies against GR, CCAAT-enhancer binding protein α (C/EBP- α), C/EBP- β , NF- κB , and AP-1 (all from Santa Cruz Biotechnology, Santa Cruz, Calif.) (28). The membranes were incubated with one of the primary antibodies overnight at 4°C and subsequently washed three times with blocking solution before being incubated with peroxidase-labeled sheep anti-rabbit (Chemicon International, Temecula, Calif.) or rabbit anti-goat (Calbiochem, Darmstadt, Germany) secondary antibody for 1 h at room temperature. Protein bands were visualized using a chemiluminescence detection system (ECL-advanced; Amersham) (7).

DNA mobility shift assay for activated glucocorticoid receptor and C/EBPs. DNA mobility shift analysis for each of the above-mentioned transcription factors was performed using commercially available DNA consensus sequences (Santa Cruz Biotechnology) as described earlier (7). Equal amounts of either cytosolic or nuclear protein preparations were incubated with 10 fM radioactively labeled DNA consensus sequences: GR, 5'-AGA GGA TCT GTA CAG GAT GTT CTA GAT-3'; CCAAT, 5'-TGC AGA TTG CGC AAT CTG CA-3'; NF- κB , 5'-AGT TGA GGG GAC TTT CCC AGG C-3' (all from Santa Cruz Biotechnology). The mixtures were incubated for 30 min at room temperature and were then loaded onto a nondenaturing 10% polyacrylamide gel and size fractionated by gel electrophoresis for 2 h (50 mA; 4°C). The gels were dried for 1 h, and the bands of DNA-protein complexes were visualized using a phosphor-imager system.

Unlabeled DNA consensus fragments were used in 10-fold excess to specify protein-DNA bands, and specific antibodies were used for supershift characterization of DNA-protein bands as described earlier (7).

Luciferase reporter gene constructs. Two different human luciferase reporter gene constructs were used to determine the functionality of the *C. pneumoniae*-induced transcription factors: (i) interleukin-6 (IL-6) promoter and (ii) p21(Waf1/Cip1) promoter. Subconfluent cell layers (10^4 cells per well) were transiently transfected with one of the two reporter gene constructs, and gene activation was assessed 24 h postinfection as described earlier (7, 28).

Antisense oligonucleotides. To down regulate transcription factors, the cells were incubated with thioated antisense oligonucleotides (1 μM) for 24 h prior to infection with *C. pneumoniae*, and 0.5 μmol of antisense oligonucleotides was added to the cultures every 24 h for the duration of the experiments. The oligonucleotide sequences were as follows: C/EBP- α , 5'-GAA GGC CGC GGC GCT GCT GGG CGC GT-3'; scrambled C/EBP- α , 5'-AGC TCG GAT GCA TGG AGG AG-3'; C/EBP- β , 5'-GTG GTC GGG GGT GTG ATT AT-3'; scrambled C/EBP- β , 5'-GTG GGG GTC GTG GGT TTA AT-3'; NF- κB , 5'-GAA ACA GAT CGT CCA GGT-3'; and scrambled NF- κB probe, 5'-GAA GAT ACA CCA CGT GGT-3' (MWG, Ebersberg, Germany).

DNA synthesis. Cells (10^4 per well) were seeded in a 24-well tissue culture plate, grown overnight, and serum deprived (0.1% serum) for 24 h. The cells were infected with *C. pneumoniae* or with heat-inactivated (65°C for 60 min)

C. pneumoniae elementary bodies at MOI of 2,500, 1,250, 625, and 312 per well and were cultured for 24 or 48 h. [^3H]thymidine (1 $\mu\text{Ci/ml}$) was added for the final 5 h of culture. The cells were washed twice with ice-cold PBS and fixed with methanol-acetic acid (3:1) for 5 min. After an additional wash with PBS, the cells were lysed in 100 μl of 0.5 M NaOH, and the incorporated [^3H]thymidine was measured after the addition of scintillation liquid (7). [^3H]thymidine incorporation data were confirmed by counting infected and uninfected cells 3 days postinfection in a Neubauer chamber.

Immunohistochemistry. HL cells were grown overnight to confluency of 10^4 per well in 24-well plates and were infected with *C. pneumoniae* at an MOI of 2,500 per well. Mock-infected cells were used as a control. At appropriate times, the cells were fixed by adding 1 volume of 3:1 methanol-glacial acetic acid to 1 volume of growth medium. After incubation at room temperature (5 min), the cells were washed with the fixative and fixed for an additional 10 min, rinsed with TBS, and blocked for 30 min with 1% bovine serum albumin in TBS. The cells were stained with a mixture of 1:500-diluted anti-*Chlamydia* monoclonal antibody (Research Diagnostics Inc., Flanders, N.J.) and 1:400-diluted anti-GR rabbit polyclonal antibody (Santa Cruz Biotechnology Inc.) or a mixture of anti-*Chlamydia* antibody and 1:400-diluted anti-NF- κB p65 rabbit polyclonal antibody (Santa Cruz Biotechnology Inc.). Endogenous peroxidase activity was removed with 0.1% NaN_3 and 0.3% H_2O_2 (30 min). Samples were incubated with primary antibodies at $+4^\circ\text{C}$ overnight, washed, and stained using a Strept-ABCComplex/HRP Duet Mouse/Rabbit kit (Dako, Glostrup, Denmark) and then developed with a DAB substrate kit for peroxidase (Vector Laboratories, Burlingame, Calif.). All wells were examined using an Olympus microscope, and the cells with black nuclear (GR and NF- κB) or cytoplasmic (*C. pneumoniae*) staining were accepted as positive.

The *Chlamydia* infection rate in HL cells was assessed by staining with anti-*Chlamydia* monoclonal antibody (Research Diagnostics Inc.) and alkaline phosphatase-conjugated goat anti-mouse antibody (Zymed Laboratories, San Francisco, Calif.), and the red color was developed with a DAKO-APAAP kit for alkaline phosphatase conjugates (Dako). The cells were counterstained with hematoxylin.

Effect of dexamethasone treatment on *C. pneumoniae* infection rate. HL cells (5×10^4 per well) were grown overnight in 24-well plates, infected with *C. pneumoniae* at an MOI of 625 (3×10^7 IFU) per well, and grown for 72 h in antibiotic- and cycloheximide-free medium. The cells were pretreated with 10^{-8} , 10^{-10} , and 10^{-12} M dexamethasone (Calbiochem) for 30 min before infection. *C. pneumoniae* was prepared in 10% FCS RPMI medium containing 10^{-8} , 10^{-10} , or 10^{-12} M dexamethasone. Infected cells were grown for 72 h in cycloheximide under antibiotic-free conditions and stained using an alkaline phosphatase-based detection method as previously described.

Statistics. The null hypothesis was that *C. pneumoniae* does not affect the growth of epithelial cells or the activation of human transcription factors. We tested the null hypothesis by analysis of variance (ANOVA) and Student's paired *t* test.

RESULTS

***C. pneumoniae* infection in vitro.** Infection of a human lung epithelial cell line (HL) showed fast attachment (within 30 min) of the microorganism to the cells (Fig. 1A, I). Alkaline phosphatase-based immunohistochemistry staining for *C. pneumoniae* infection in vitro showed a constant increase of infected cells within the first 6 h, and after 3 h, *C. pneumoniae* had clearly invaded the host cell cytoplasm (Fig. 1A, III and IV).

***C. pneumoniae* activates transcription factors.** We tested the hypothesis that infection of human lung epithelial cells with *C. pneumoniae* modulates the activity of the host transcription factors that are related to proliferation in the host cells.

As shown in Fig. 1B, infection of HL cells with *C. pneumoniae* resulted in rapid activation and translocation of the GR into the nucleus, which started at 30 min and became maximal after 3 h (Fig. 1B, I to III). In uninfected cells, the GR was located in a zone around the nucleus (Fig. 1B, V to VIII).

Using Western blot analysis, we confirmed that infection of HL cells and Calu3 cells with *C. pneumoniae* caused translo-

FIG. 1. (A) Kinetics of *C. pneumoniae* infection in human epithelial cells (HL). *C. pneumoniae* was stained with a red substrate, and the cells were counterstained with hematoxylin. (B) GR location and *C. pneumoniae* infection by double immunohistochemical staining. Shown is the time course of *C. pneumoniae*-induced translocation and activation of the GR in infected cells (I to IV) and in uninfected control cells (V to VIII). Inactive GR is visible as a black-stained region around the nuclear membrane, and active GR is indicated by dark-grey to black nuclei (arrows). Some of the immunohistochemically stained *C. pneumoniae* in cells are circled in image I. (C) Representative Western blot assay of *C. pneumoniae* (*C. pneu*)-induced translocation of GR in HL cells. Similar results were obtained in Calu3 cells, and each experiment was repeated three times. (D) Functional activation of GR by *C. pneumoniae* is depicted as a representative EMSA over a period of 6 h. GR binding was monitored by a double-stranded GRE DNA consensus sequence.

FIG. 1—Continued.

cation of the GR from the cytosol into the nucleus, which was nearly complete at 3 h (Fig. 1C). Functional activation of the GR by *C. pneumoniae* infection was shown by using a DNA electrophoretic mobility shift assay (EMSA) demonstrating that the GR recognized and bound to its specific DNA consensus sequence. Figure 1D shows that *C. pneumoniae* induced binding of a protein to the glucocorticoid response element (GRE) within 3 h after infection, further increasing until 6 h in both cell lines. The specificity of the GR signal in EMSA was confirmed using HeLa cell extract (Fig. 1D, second lane from left) and a 10-fold excess of unlabeled GRE (fourth lane from left). We tested for nonspecific DNA interactions using Oct-1 (fifth lane from left) oligonucleotides as a negative control.

Infection with *C. pneumoniae* also activated C/EBP- α and C/EBP- β , two members of the C/EBPs (Fig. 2A). The activation of C/EBP- α by *C. pneumoniae* infection was not very strong, since we could not observe a significant decrease in the C/EBP- α signal in the cytosol and observed only a marginal increase of the signal in the nuclear protein samples (Fig. 2A). In contrast, the activation of C/EBP- β was significant within 1 h after infection with *C. pneumoniae* (Fig. 2A). Using a C/EBP binding DNA consensus sequence, we observed a protein-DNA complex 3 h after infection in HL cells; the signal reached maximal level at 6 h and was not detectable after 24 h (Fig. 2B). In Calu3 cells, the C/EBP signal appeared 1 h after *C. pneumoniae* infection, with maximal activity at 3 h and declining thereafter (Fig. 2B).

In addition, the translocation of NF- κ B was induced by *C. pneumoniae* infection in the two epithelial cell lines. As shown by immunohistochemistry (Fig. 3A), the activation of NF- κ B by *C. pneumoniae* did not occur before 1 h after infection, and maximal activation was observed after 6 h (Fig. 3A, I to IV). In uninfected cells, NF- κ B was located in the cytosol close to the nuclear membrane. The activation of NF- κ B by *C. pneumoniae* was confirmed by Western blotting, which revealed a time course similar to that shown by immunohistochemistry (Fig.

3B). NF- κ B signal decreased in the cytosol, increased after 1 h in the nucleus, and was maximal at 3 h (Fig. 3B). Furthermore, the functional activation of NF- κ B was demonstrated by EMSA, which also showed a slow increase in DNA binding of a protein to the NF- κ B oligonucleotide (Fig. 3C). We observed a slight difference when the binding kinetics of NF- κ B in the two cell lines were compared. While significant binding was observed in HL cells at 3 h, which increased until 6 h, in Calu 3 cells NF- κ B binding occurred at 30 min, peaked at 3 h, and then declined (Fig. 3C).

The DNA binding proteins were further characterized by an EMSA either in the presence of the GR inhibitor RU486 or after pretreatment (24 h) of the cells with antisense oligonucleotides (1 μ M) targeting C/EBP- α , C/EBP- β , or NF- κ B. As shown in Fig. 4, the signal for the GR-GRE complex was significantly reduced when the cells were preincubated for 2 h with the GR inhibitor RU486 (10^{-6} M) prior to infection with *C. pneumoniae* (Fig. 4, second lane from left). In the presence of an antisense oligonucleotide to NF- κ B, the *C. pneumoniae*-dependent NF- κ B-DNA complex was abolished in supershift EMSA (Fig. 4, fourth lane from left). In the presence of an antisense oligonucleotide specific for C/EBP- α , the C/EBP band was slightly reduced (Fig. 4, seventh lane from left), and in the presence of an antisense oligonucleotide specific for

FIG. 2. (A) Representative Western blot of *C. pneumoniae*-induced activation of C/EBP- α and C/EBP- β in HL cells. Similar results were obtained in triplicate experiments and in Calu3 cells. (B) Representative EMSA of *C. pneumoniae* (*C. pneu*)-activated CCAAT oligonucleotide binding nuclear proteins in HL and Calu3 cells. Similar results were obtained in triplicate experiments. The specificity of C/EBP proteins was demonstrated by 10-fold cold CCAAT oligonucleotide (oligo) and anti-C/EBP- α or C/EBP- β antibodies.

FIG. 3. (A) Cell compartment localization of NF- κ B in *C. pneumoniae*-infected HL cells (I to IV) and in uninfected HL cells (V to VIII) by double immunohistochemical staining. Inactive NF- κ B is visible as a black-stained region around the nuclear membrane, and active NF- κ B is indicated by dark-grey to black nuclei (arrows). Some of the immunohistochemically stained *C. pneumoniae* cells are circled in image I. (B) Representative Western blot assay of *C. pneumoniae*-induced translocation of NF- κ B in HL cells. Similar results were obtained in Calu3 cells, and each experiment was repeated three times. (C) Functional activation of NF- κ B by *C. pneumoniae* (*C. pneu*) depicted as a representative EMSA over a period of 6 h. Oligo, oligonucleotide.

The human IL-6 promoter was not activated by *C. pneumoniae* in any cell line within 24 h. In contrast, the GR-inducible p21^(Waf1/Cip1) promoter was activated in a dose-dependent manner 24 h after *C. pneumoniae* infection (Fig. 5A). In HL cells, we observed a significant increase in luciferase activity with *C. pneumoniae* at an MOI of 1,250 per well (ANOVA, $P \leq 0.05$) (Fig. 5A). In Calu3 cells, *C. pneumoniae* infection activated the p21^(Waf1/Cip1) promoter at an MOI of 625 (ANOVA, $P \leq 0.05$) (Fig. 5A).

Figure 5B shows that infection with *C. pneumoniae* induced the activity of the p21^(Waf1/Cip1) promoter luciferase construct in both cell lines. The GR antagonist RU486 had no effect on luciferase activity in uninfected cells and reduced the *C. pneumoniae*-induced luciferase activity by $56.1\% \pm 5.2\%$ in HL cells (ANOVA, $P \leq 0.01$) and by $63.8\% \pm 5.2\%$ in Calu3 cells (ANOVA, $P \leq 0.05$). When the cells were pretreated with antisense oligonucleotides targeting NF- κ B p65, we observed no inhibition of *C. pneumoniae*-induced luciferase activity. Similarly, antisense oligonucleotides inhibiting C/EBP- β did not affect *C. pneumoniae*-inducible luciferase activity. The control oligonucleotides for either NF- κ B or C/EBP- β did not inhibit the activation of the p21^(Waf1/Cip1) promoter luciferase construct by *C. pneumoniae* (Fig. 5B).

C/EBP- β , the signal of the protein-DNA complex was clearly reduced (Fig. 4, right lane).

***C. pneumoniae* activates a glucocorticoid-inducible promoter.** To further assess the functions of transcription factors activated by *C. pneumoniae*, we transfected HL and Calu3 cells either with a luciferase-coupled human IL-6 promoter or with the human GR-dependent p21^(Waf1/Cip1) promoter.

FIG. 4. Characterization of transcription factors activated by *C. pneumoniae* infection in HL cells shown as a representative EMSA supershift assay. Nuclear extracts from HL cells infected with *C. pneumoniae* for 3 h were used for the analysis. To characterize GR, cells were pretreated with RU486 (10^{-6} M) for 2 h prior to infection (two left lanes). For NF- κ B characterization, we incubated the nuclear extract that was isolated 3 h after *C. pneumoniae* infection overnight in the presence or absence of an NF- κ B binding DNA oligonucleotide (lanes 3 and 4 from left). Lane 5 from the left is empty, and C/EBP- α and - β were characterized using CCAAT DNA sequence without (lane 6) or with a specific anti-C/EBP- α or anti-C/EBP- β antibody (two right lanes). Similar results were obtained in Calu3 cells.

We also assessed the transcription factor AP-1, but we did not observe its activation by *C. pneumoniae* infection under any culture conditions (data not shown).

***C. pneumoniae* increases epithelial cell proliferation via GR and NF- κ B activation.** Infection with *C. pneumoniae* elementary bodies stimulated the proliferation of Calu3 and HL epithelial cells compared to uninfected cells in 0.1% fetal bovine serum (Fig. 6A). The proliferative effect of *C. pneumoniae* was determined by both thymidine incorporation and direct cell counting. Both methods showed a significant increase of proliferation in the presence of *C. pneumoniae* at an MOI of 1,250 (ANOVA, $P \leq 0.01$) (Fig. 6A).

To assess whether one or more of the above-described host transcription factors is involved in *C. pneumoniae*-induced cell proliferation, we pretreated the cells for 2 h with RU486 (10^{-6} M). As shown in Fig. 6B, Ru486 had no significant effect on uninfected cells but reduced the proliferation of *C. pneumoniae*-infected HL cells by $70.1\% \pm 8.2\%$ (ANOVA, $P \leq 0.01$) and that of Calu3 cells by $62.5\% \pm 8.2\%$ (ANOVA, $P \leq 0.01$).

When cells were treated for 24 h with NF- κ B antisense oligonucleotides (10^{-6} M) prior to infection with *C. pneumoniae*, proliferation was reduced by $19.2\% \pm 2.5\%$ in HL cells (ANOVA, $P \leq 0.05$) and by $34.9\% \pm 5.0\%$ in Calu3 cells (ANOVA, $P \leq 0.01$) (Fig. 5B). In contrast, C/EBP- β antisense oligonucleotides (10^{-6} M) did not alter the proliferation of *C. pneumoniae*-infected epithelial cells (Fig. 5B). The control oligonucleotides for either NF- κ B or C/EBP- β did not inhibit the proliferative effect of *C. pneumoniae* (Fig. 5B).

To prove that live *C. pneumoniae* is essential for the observed proliferation of the host cell, we incubated HL cells with heat-inactivated and undiluted *C. pneumoniae* at 5×10^8 IFU/

FIG. 5. (A) *C. pneumoniae* stimulates the human promoter for p21^(Waf1/Cip1) in epithelial cells in a dose-dependent manner. Each bar represents the mean $\pm$ standard error of three independent experiments. *, $P \leq 0.05$ (ANOVA); **, $P \leq 0.01$ (ANOVA). FBS, fetal bovine serum. The numbers below the bars are MOI. (B) Involvement of transcription factors in *C. pneumoniae*-induced p21^(Waf1/Cip1) promoter activity. Each bar represents the mean $\pm$ standard error of three independent experiments. **, $P \leq 0.01$ (ANOVA). +, present; -, absent; scramb, scrambled.

FIG. 6. (A) Effect of diluted *C. pneumoniae* stock on epithelial cell proliferation. Proliferation was determined by direct cell counting after 3 days of culture of infected and uninfected cells. Each bar represents the mean + standard error of three independent experiments. **, $P \leq 0.01$ (ANOVA). FBS, fetal bovine serum. The numbers below the bars are MOI. (B) Involvement of transcription factors in *C. pneumoniae*-dependent epithelial cell proliferation determined by [3 H]thymidine incorporation after 24 h of culture. Each bar represents the mean + standard error of three independent experiments. *, $P \leq 0.05$ (ANOVA); **, $P \leq 0.01$ (ANOVA). +, present; -, absent; scramb, scrambled.

well. While *C. pneumoniae* at an MOI of 2,500 induced a 2.61-fold increase in cell proliferation, heat-inactivated *C. pneumoniae* and undiluted *C. pneumoniae* stock caused only a 1.47-fold increase.

Effect of glucocorticoids on *C. pneumoniae* infection. In addition to the modulation of the host transcription factors by *C. pneumoniae*, we investigated whether the GR may also affect the intracellular growth of *C. pneumoniae*. To test this,

FIG. 7. Dose-dependent support of dexamethasone for *C. pneumoniae* propagation in infected in HL cells. *C. pneumoniae* was stained with a red substrate, and immunohistochemical analysis was performed 72 h after infection with *C. pneumoniae* at an MOI of 625.

we infected HL cells (5×10^4 per well) with *C. pneumoniae* at an MOI of 625 in the presence and absence of various concentrations of dexamethasone (10^{-12} , 10^{-10} , and 10^{-8} M). The infected cells were further cultured for 72 h and then stained for *C. pneumoniae* as shown in Fig. 7. Dexamethasone at concentrations of 10^{-12} or 10^{-10} M did not increase staining for *C. pneumoniae* inclusion bodies, while 10^{-8} M dexamethasone significantly enhanced *C. pneumoniae* staining in infected cells (Fig. 7, I to IV).

DISCUSSION

In this study, we demonstrated that *C. pneumoniae* infection alters the activity of the transcription factors GR, C/EBP- β , and NF- κ B in human epithelial cells. Furthermore, the numbers of infected cells increased significantly within 72 h compared to those of uninfected cells. We showed that *C. pneumoniae* induces epithelial cell proliferation via the action of the GR and NF- κ B, while C/EBP- β had no effect on the proliferation of infected cells. Heat-inactivated *C. pneumoniae* did not increase epithelial cell proliferation. We provide evidence that the activation of the GR supports the propagation of *C. pneumoniae* in the infected cells.

Chlamydiae are known to use the host's energy system, specifically glucose and ATP metabolism, for its own propagation (10, 24, 35). However, the use of the host's energy system cannot explain why *Chlamydia* infection causes host cell proliferation; it would rather suggest the opposite. Several studies have implied that intracellular parasites are capable of activating the host defense system via the host transcription factors, and our data provide evidence that *C. pneumoniae* manipulates the transcription factors of the host to modify the behavior of the infected cells.

Other intracellular microorganisms, including *Neisseria gonorrhoeae*, have been shown to activate NF- κ B and AP-1, thereby inducing the infected cells to produce granulocyte-macrophage colony-stimulating factor, tumor necrosis factor alpha, IL-1 β , IL-8, monocyte chemotactic protein, and transforming growth factor β , while no activation of C/EBPs or the cyclic AMP response element was observed (5, 12, 18, 16, 36). *Leishmania donovani* affects the activation of STAT1 and also counteracts the host defense system by decreasing MAP kinase activity and inhibiting Elk-1 activation. MAP kinase, c-FOS, and iNOS were markedly increased in *Leishmania*-infected macrophages through activation of cellular phosphotyrosine phosphatases, resulting in the deactivation of macrophages during intracellular infection (19, 22, 23, 34). *Mycobacterium tuberculosis* induced STAT1 and increased its interaction with the cyclic AMP response element binding protein (CREB) and p300, thereby suppressing the induction of gamma interferon in macrophages (19, 22, 23). Similarly, *C. pneumoniae* was reported to inhibit the immune response by down regulation of gamma interferon-inducible major histocompatibility complex class II (42). There is evidence that *C. pneumoniae* activates NF- κ B in endothelial cells (18), in vascular smooth muscle cells (16), and in Mono Mac 6 cells (38). NF- κ B activation by *C. pneumoniae* may prevent the host cells from undergoing apoptosis, as suggested by the data presented by Wahl et al. (38). The activation of NF- κ B by *C. pneumoniae* seems to be a

general response in many cell types and was also observed in the epithelial cells used in this study.

In agreement with the results of other studies, we demonstrated that *C. pneumoniae* enhances the proliferation of infected host cells. Such an effect of *C. pneumoniae* infection has been shown in vascular smooth muscle cells (29), where the proproliferative effect of *C. pneumoniae* infection involved NF- κ B and AP-1. In our studies, the GR and NF- κ B were necessary to stimulate the proliferation of epithelial cells. Epithelial cells require an active GR to proliferate (16), in contrast with mesenchymal cells, where activation of the GR arrests cell proliferation (28). Furthermore, our data indicates that GR activation may ensure the survival and propagation of *C. pneumoniae* in the infected host cells.

Comparing the two human epithelial cell lines to each other, our data indicate that there might be a cell line- or cell-type-specific response for transcription factor modulation by *C. pneumoniae*. In our experimental settings, NF- κ B activation was faster in Calu3 cells and GR activity was prolonged. Calu3 cells also showed greater response to luciferase activation by *C. pneumoniae*. However, further experiments would be needed to confirm whether the observed differences are cell line specific.

What are the consequences of the activation of the host cell transcription factors by *C. pneumoniae* in the lung? As chronic inflammation of the airway wall is a major pathological feature in asthma, it could be that chronic infection with *C. pneumoniae* contributes to the severity of an asthma attack via modification of the host's transcription factor activity. *C. pneumoniae*-dependent proliferation of the host cell, as well as cell damage, may alter the structure and function of the lung. Several studies indicate that *C. pneumoniae* inhibits apoptosis of monocytes and epithelial cells (1, 3, 27). Our data support these findings and furthermore suggest that *C. pneumoniae* infection leads to increased proliferation in epithelial cells. It might also be possible that infection with *C. pneumoniae* and the subsequent activation of the host cell's transcription factors change the stage of differentiation and therefore the response of the cell to exogenous signals, such as cytokines and growth factors.

In summary, our data demonstrate that *C. pneumoniae* is a microorganism that not only uses the host's energy system for its own benefit but also forces epithelial cells to proliferate. The data further show that *C. pneumoniae* uses cell-type-specific signaling pathways to activate cell proliferation to support its own survival. Future studies must determine whether inhibition of the signaling pathway by nonantibiotic drugs could effect *C. pneumoniae* infection.

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