

Chlamydia pneumoniae infection of microglial cells *in vitro*: a model of microbial infection for neurological disease

Hideaki Ikejima,^{1,2} Herman Friedman¹ and Yoshimasa Yamamoto^{1,3}

Correspondence

Yoshimasa Yamamoto
yyamamoto@sahs.med.osaka-u.
ac.jp

¹Department of Medical Microbiology and Immunology, University of South Florida College of Medicine, Tampa, FL 33612, USA

²Department of Internal Medicine, St. Marianna University School of Medicine, Kawasaki 216-8511, Japan

³Laboratory of Molecular Microbiology, Department of Basic Laboratory Sciences, Osaka University Graduate School of Medicine, 1-7 Yamadaoka, Suita, Osaka 565-0871, Japan

Chlamydia pneumoniae is the aetiological cause of a wide variety of chronic inflammatory diseases and may be associated with neurological disease. Microbiological and immunological aspects of the interaction between *C. pneumoniae* and the central nervous system (CNS) are not well understood because of the lack of a suitable infection model for neuronal studies. In the present study, an *in vitro* *C. pneumoniae* infection model was developed in the established microglial cell line EOC 20. Infection of the cells resulted in obvious induction of proinflammatory cytokines. The infection also selectively induced matrix metalloproteinase-9 (MMP-9) but not MMP-2. Moreover, beta interferon, which is known to modulate CNS disease, inhibited induction of MMP-9 following *C. pneumoniae* infection. These results support the view that *C. pneumoniae* infection may be associated with marked alteration of the ability of microglial cells to enhance cytokine production as well as induction of an MMP.

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INTRODUCTION

Chlamydia (Chlamydophila) pneumoniae is an obligate intracellular Gram-negative bacterium that causes a wide spectrum of respiratory tract infections. The pathogen is associated with not only respiratory disease but also chronic inflammatory disease (Grayston, 1996; Hahn *et al.*, 1991; Saario & Toivanen, 1993). In this regard, it has been proposed recently that some diseases of the central nervous system (CNS), such as multiple sclerosis (MS), may be linked to *C. pneumoniae* infection, because this pathogen can be detected in the cerebrospinal fluid of some patients with such diseases (Ikejima *et al.*, 2001; Sriram *et al.*, 1999, 2002). It is well known that this bacterium infects and replicates in epithelial cells as well as monocytes and macrophages, but the susceptibility and immune responses of microglial cells, which are important immune cells of the CNS (Benveniste, 1997), to *C. pneumoniae* infection is not yet clear. Therefore, in the present study, an established microglial cell line was examined as a possible host cell for this bacterium in order to develop a model for infection. In addition, matrix metalloproteinases (MMPs), which are a

key factor in the development of neuronal loss in CNS diseases (Bar-Or *et al.*, 1999), and the inflammatory cytokine response of microglial cells to *C. pneumoniae* infection were analysed using the model.

METHODS

Microglial cells. The mouse microglial cell line EOC 20 was obtained from the American Type Culture Collection (ATCC). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma), supplemented with 10% heat-inactivated fetal calf serum (FCS), 20% conditioned medium from LADMAC cells and antibiotics, as described elsewhere (O'Keefe *et al.*, 1999; Olivas *et al.*, 1995).

Bacteria. *C. pneumoniae* (TW 183) was obtained from the ATCC and propagated in a HEp-2 cell culture system using methods described previously (Roblin *et al.*, 1992). The bacteria were suspended in sucrose-phosphate-glutamic acid buffer (0.2 M sucrose, 3.8 mM KH₂PO₄, 6.7 mM Na₂HPO₄, 5 mM L-glutamic acid, pH 7.4) and stored at -70 °C until used. Inclusion-forming unit (IFU) counts of the prepared bacteria were determined by counting chlamydial inclusions in HEp-2 cell monolayers (Summersgill *et al.*, 1995). Heat-killed *C. pneumoniae* was prepared by heating at 70 °C for 45 min. Assessment of the viability of the heat-killed bacteria, as determined by specific inclusion formation, showed no viable bacteria.

Infection with *C. pneumoniae*. The microglial cells (1 × 10⁶ cells per well) were dispensed in 6-well culture plates and incubated for

Abbreviations: CNS, central nervous system; IFN-β, beta interferon; IFU, inclusion-forming units; IL-10, interleukin-10; IL-12, interleukin-12; MMP-9, matrix metalloproteinase-9; MS, multiple sclerosis; TIMP-1, tissue inhibitor of metalloproteinase 1; TNF-α, tumour necrosis factor α.

24 h in 5% CO₂ at 37 °C. The cells were then infected with either viable or heat-killed bacteria at an m.o.i. of 10:1 by centrifugation at 900 g for 60 min followed by incubation for 60 min at 37 °C, washed twice with Hanks' balanced salt solution and incubated further in the medium (10% FCS-DMEM). In some experiments, the cells were treated with 100 U mouse recombinant beta interferon (IFN- β) (R & D Systems) ml⁻¹ for 24 h at 37 °C, infected with bacteria and then incubated further in the presence or absence of IFN- β . The cells were also treated with 100 ng phorbol myristate acetate (PMA; Sigma) ml⁻¹ as a positive control for induction of MMPs.

Assessment of *Chlamydia* inclusions. Isolated microglial cells were centrifuged on to glass slides in a Cytospin (Shandon). After fixation with ethanol, cells were stained with *Chlamydia* genus-specific fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody (specific to *Chlamydia* lipopolysaccharide; Research Diagnostics). The presence of bacteria in cells was then determined with a fluorescence microscope.

RNA extraction and RT-PCR. Total RNA was extracted from cells using an RNeasy Mini kit (Qiagen) according to the manufacturer's instructions with the protocol for bacterial cells. The concentration of RNA was measured by spectrophotometry and the samples were stored at -70 °C until used. The extracted RNAs were treated with DNase (DNA-free; Ambion) to eliminate contaminating DNA. The resulting DNA-free RNAs were confirmed by PCR without RT. The reverse transcription of 2 μ g RNA was performed with avian myeloblastosis virus RT (Promega) with random primers in a commercial reaction mixture (20 μ l) (Reverse Transcription System; Promega). The resulting cDNAs (2 μ l) were then subjected to either conventional or real-time PCR with primers specific for *C. pneumoniae omcB*, 16S rRNA, TNF- α (tumour necrosis factor α), IL-10 (interleukin-10), IL-12 p40, MMP-2, MMP-9, TIMP-1 (tissue inhibitor of metalloprotease 1) and β -actin. The primer sequences for 16S rRNA, β -actin, TNF- α , MMP-2, MMP-9 and TIMP-1 are shown in Table 1. The primers for *omcB*, IL-10 and IL-12 p40 were synthesized on the basis of sequences described previously (Haranaga *et al.*, 2003; Yamamoto *et al.*, 1995). The real-time PCR was performed in the master mixture (SYBR Green PCR Master Mix; Applied Biosystems) with primers and cDNAs in an iCycler thermal cycler (Bio-Rad Laboratories). The thermal cycling conditions were 95 °C for 10 min and 50 cycles of 95 °C for 15 s, 60 °C for 1 min and 72 °C for 20 s. The melting temperature profile was assessed for each PCR run to confirm the specificity of PCR products. As a standard for *C. pneumoniae* genes, a dilution series of bacterial DNA extracted from

purified *C. pneumoniae* elementary bodies was used. The relative concentrations of bacterial genes (number of copies) were calculated from the standard curve. In the case of TNF- α , MMP-9 and β -actin, cDNAs of these messages generated by PCR were purified from gels with the QiAquick Gel Extraction kit (Qiagen) and were utilized for standard curves of real-time PCR. The levels of transcripts were normalized to host cell β -actin mRNA expression and were represented as an x -fold increase in the expression. In the case of conventional PCR, the PCR products were visualized by 2% agarose gel electrophoresis staining with ethidium bromide.

ELISA. The amount of TNF- α in the culture supernatants of cells was determined by ELISA using matched antibody pairs and protein standards for ELISA (R & D Systems). Concentrations were calculated from the standard curve produced for each plate.

Statistical analysis. Statistical analysis was performed with the paired Student's t test.

RESULTS AND DISCUSSION

Infection of microglial cells with *C. pneumoniae*

C. pneumoniae infection of EOC 20 cells, which maintain the phenotype of microglial cells (Walker *et al.*, 1995), resulted in rapid intracellular replication of the bacteria. The infected cells showed many chlamydiae, as determined by immunostaining with FITC-labelled anti-*Chlamydia* antibody at 72 h after infection, whereas immediately after infection the cells showed only a few bacteria (data not shown). The uninfected control cells did not stain positive for bacteria. Furthermore, as shown in Fig. 1(a), the number of infective progenies in lysates of the *C. pneumoniae*-infected microglial cells increased rapidly as determined by standard IFU assays with susceptible HEp-2 cells. Within 24 h after infection, there was an approximately eight- to ninefold increase in the number of infective progenies and the number remained stable for the next few days.

Bacterial gene transcripts in the infected cells assessed by real-time PCR showed the viability of bacteria in microglial cells. *C. pneumoniae* 16S rRNA transcripts are known to be stably expressed in bacteria throughout the developmental cycle (Shaw *et al.*, 2000). *omcB*, which encodes the 60 kDa cysteine-rich outer-membrane protein, is transcribed during the mid and the late developmental cycle (Shaw *et al.*, 2000). As shown in Fig. 1(b), 16S rRNA gene transcript levels increased immediately after infection. In the case of *omcB* transcripts, the levels increased slowly but continuously during infection.

These results indicate that the opportunistic pathogen *C. pneumoniae*, which has been implicated not only in respiratory diseases but also in chronic inflammatory diseases, including MS, can infect and replicate in neurological cells known to be the equivalent of macrophages.

Cytokine induction in microglial cells by infection

The infection of microglial cells with *C. pneumoniae* induced some cytokines, such as TNF- α , IL-10 and IL-12,

Table 1. Primer sequences used in RT-PCR

Target	Primer sequence
16S rRNA	5'-GGACCTTACCTGGACTTGACATGT-3' (sense) 5'-CCATGCAGCACCTGTGTATCTG-3' (antisense)
β -Actin	5'-GCTATCCAGAAAACCCCTCAA-3' (sense) 5'-CATGTCTCGATCCCAGTAGACGGT-3' (antisense)
TNF- α	5'-AGCCGATGCGTTGTACCTT-3' (sense) 5'-GATAGCAAATCGGCTGACG-3' (antisense)
MMP-2	5'-ACCCTGGGAGAAGGACAAGT-3' (sense) 5'-CGATGCCATCAAAGACAATG-3' (antisense)
MMP-9	5'-ACTACTCTGAAGACTTGCCG-3' (sense) 5'-GGTACAGGAAGAGTACTGCT-3' (antisense)
TIMP-1	5'-CTGGCATCCTCTTGTGCTA-3' (sense) 5'-AGGGATCTCCAGGTGCACAA-3' (antisense)

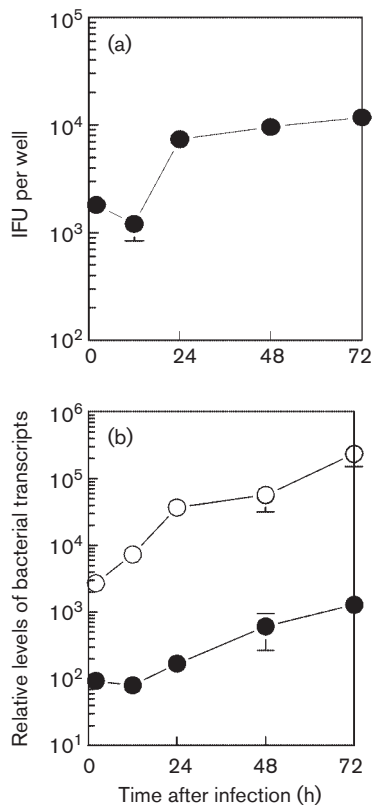


Fig. 1. Growth of *C. pneumoniae* in microglial cells. (a) Kinetics of development of infectious progenies in EOC 20 cells. Lysates of infected cells were prepared at various times after infection and the numbers of infective progenies in the lysates were assessed by IFU assay. Each data point represents the mean number of IFU per well \pm SD for three cultures. Results are representative of three separate experiments. (b) Relative bacterial mRNA expression levels in infected microglial cells. Bacterial mRNA levels were determined by real-time RT-PCR. Data are the mean relative levels of bacterial transcripts \pm SD for three cultures. Data are representative of three separate experiments. \circ , 16S rRNA; \bullet , *omcB*.

but the induction kinetics was dependent on the cytokines tested. The control uninfected EOC 20 cells expressed a minimal level of TNF- α mRNA, but other cytokines tested were not expressed (data not shown). As shown in Fig. 2(a), *C. pneumoniae* infection rapidly induced increased TNF- α mRNA expression, followed by a sharp decrease at the mid to late phase of infection. Treatment of cells with heat-killed bacteria induced TNF- α mRNA, which was detectable 2 h after addition of killed bacteria to the cultures, but the induction kinetics was clearly different to that for infection with viable bacteria. Assessment of TNF- α protein by ELISA in the culture supernatants of the cells infected with bacteria also showed a higher level of TNF- α production during the infection, but addition of heat-killed bacteria induced only a minimum level of the cytokine protein (Fig. 2b). Other inflammatory cytokines, such as IL-10 and IL-12, were also

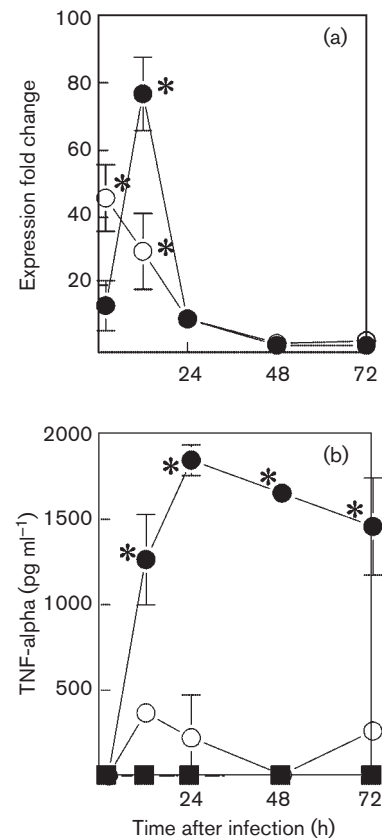


Fig. 2. TNF- α in *C. pneumoniae*-infected microglial cells. (a) Expression of TNF- α mRNA in infected EOC 20 cells at various times after infection. The mRNA levels were determined by real-time RT-PCR. Data are the mean of expression fold changes \pm SD for three cultures. Data are representative of three separate experiments. (b) Production of TNF- α protein in the supernatants from bacteria-infected microglial cells. The production of TNF- α protein in the supernatants was measured by ELISA. Data are the mean \pm SD for three experiments. \blacksquare , No-infection control; \bullet , infected with viable bacteria; \circ , infected with heat-killed bacteria. *, $P < 0.05$ (significantly different from the values obtained with the no-infection control group).

induced by infection as assessed by PCR. As shown in Fig. 3, the induction of IL-12 p40 mRNAs by infection was slow but obvious at 72 h after infection. IL-10 mRNA was induced in the early phase of infection and detectable 12 h after infection, followed by a rapid decrease similar to the TNF- α mRNA induction.

Selective induction of MMP-9 mRNA by infection

MMPs are known to be important factors in regulating the blood-brain barrier and permitting humoral and cellular factors from the systemic circulation to enter the CNS (Rosenberg, 2002b). In addition, MMPs may be important in the disruption of the myelin sheath and axons during the acute inflammatory phase of MS (Leppert *et al.*, 2001;

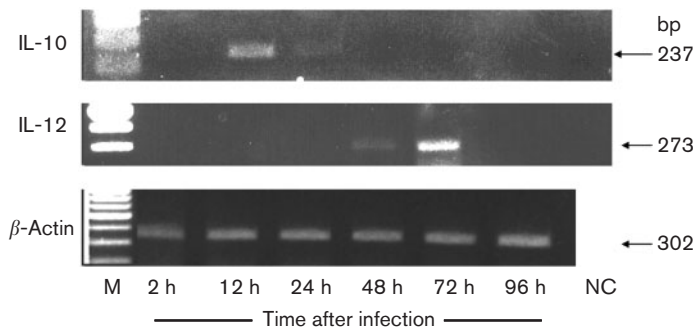


Fig. 3. Expression of IL-10, IL-12 p40 and β -actin mRNA in infected microglial cells. A representative photograph of a gel of the PCR products is shown. M, Molecular marker; NC, negative control for PCR.

Rosenberg, 2002a), which is thought to be associated with *C. pneumoniae* in some cases. Therefore, in further studies mRNAs for MMPs induced in microglial cells by *C. pneumoniae* infection were assessed. As shown in Fig. 4, infection of microglial cells with *C. pneumoniae* induced MMP-9 mRNA at 24 h after infection but not MMP-2 and TIMP-1, although both were inducible in the cells by treatment with a non-specific stimulator PMA during the infection for up to 72 h. Thus infection of the microglia with *C. pneumoniae* selectively induced MMP-9, an important MMP implicated in neurological diseases, including MS (Rosenberg, 2002b). However, other MMPs, such as MMP-2, and TIMP-1, which plays a critical role in controlling MMP activity in tissues, were not altered. Control studies showed that heat-killed *C. pneumoniae* induced minimal, if any, alterations in the level of the MMPs, suggesting that the stimulatory effect was not due merely to active stimulation by some bacterial products such as chlamydial lipopolysaccharide (data not shown).

The selective induction of MMP-9 by *C. pneumoniae* infection in microglial cells revealed in this study is consistent with previous findings that *C. pneumoniae* infection of human peripheral blood monocytes and mouse peritoneal macrophages induced secretion of MMP-9 (Kol *et al.*, 1998; Rupp *et al.*, 2004; Vehmaan-Kreula *et al.*, 2001), and a selective expression of MMPs, including gelatinase B (MMP-9), was evident in the tissue of MS patients (Bar-Or *et al.*, 1999). In addition, TNF- α and MMP are thought to have together a critical role in MS pathophysiology, such as damage of the myelin sheath (Bar-Or *et al.*, 1999).

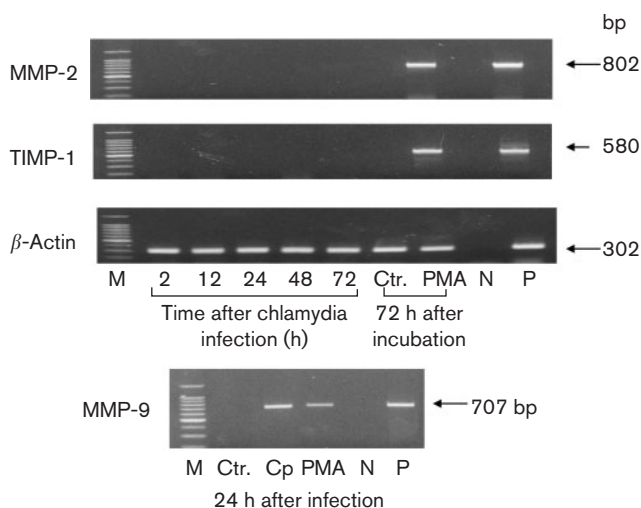


Fig. 4. Expression of MMPs, TIMP-1 and β -actin mRNA in infected cells or stimulated with PMA. The cells were infected with *C. pneumoniae* or treated with PMA (100 ng ml⁻¹) at the indicated time point. A representative photograph of a gel of the PCR products is shown. M, Molecular marker; Ctr., control; N, negative control for PCR; P, positive control for PCR; Cp, *C. pneumoniae*.

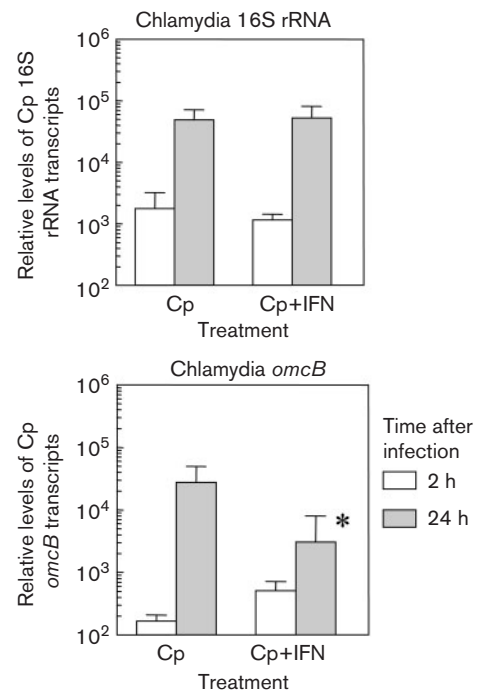


Fig. 5. Effect of IFN- β on *C. pneumoniae* growth in microglial cells determined by production of gene transcripts. Cells were pretreated with IFN- β (IFN) for 24 h, infected with bacteria (Cp) and then incubated further for 2–24 h in the presence of IFN- β . Levels of *C. pneumoniae* mRNA were measured by real-time PCR. Data are the mean \pm SD for three experiments. *, $P < 0.05$ (significantly different from the value obtained with the *C. pneumoniae*-infected control group at the same time point).

Therefore, the marked production of TNF- α and the selective induction of MMP-9 by *C. pneumoniae* infection in the microglia *in vitro* as shown in this study may provide valuable information on the possible involvement of *C. pneumoniae* infection in neurological diseases such as MS (Sriram *et al.*, 1999).

Modulation of bacterial growth and MMP-9 induction by IFN- β

IFN- β is utilized as a therapeutic treatment agent for MS (Zhang *et al.*, 2002). Therefore, the possibility that IFN- β modulates bacterial growth in microglial cells as well as induction of MMP-9 by infection was assessed. As shown in Fig. 5, treatment of cells with IFN- β did not alter the levels of bacterial 16S rRNA transcripts. However, the levels of *omcB* transcripts were significantly inhibited by the treatment at 24 h after infection. In addition, IFN- β treatment of cells markedly suppressed the induction of MMP-9 by *C. pneumoniae* infection (Fig. 6). However, IFN- β treatment of non-infected control cells also reduced the constitutive expression level of MMP-9.

Even though IFN- β treatment markedly inhibited induction of MMP-9 by *C. pneumoniae* infection in microglial cells, inhibition of bacterial growth in cells by IFN- β was only minimal. That is, chlamydial inclusion formation in microglial cells was not significantly altered by IFN- β treatment (data not shown). *omcB* is a component of the disulfide-linked outer-membrane protein complex that gives

structural stability to elementary bodies (Clarke *et al.*, 1988; Hatch *et al.*, 1986). In contrast, 16S rRNA is expressed throughout the entire developmental stage of bacteria and the expression levels may be parallel with metabolic activity of the organisms (Shaw *et al.*, 2000). Therefore, the high 16S rRNA but low *omcB* expression observed in IFN- β -treated cells may indicate incomplete chlamydial growth. These results may be related to the IFN- γ -induced persistence in cells of *C. pneumoniae* which paralleled up-regulation of bacterial DNA replication but not cell division-related genes (Byrne *et al.*, 2001).

Thus the results obtained in this study demonstrate that *C. pneumoniae* can infect and replicate in microglial cells, the predominant macrophage-like cell in the CNS. Infection of microglial cells with these bacteria resulted in induction of proinflammatory cytokines, similar to induction of cytokines by these bacteria in macrophages (Kol *et al.*, 1998; Redecke *et al.*, 1998). Furthermore, selective induction of MMP-9 by *C. pneumoniae* infection in these cells and inhibition of MMP-9 induction by IFN- β , which is widely utilized in therapy for MS, indicate that this infection model utilizing EOC 20 microglial cells may be a useful model for study of *C. pneumoniae* infection, particularly for neurological diseases associated with this organism.

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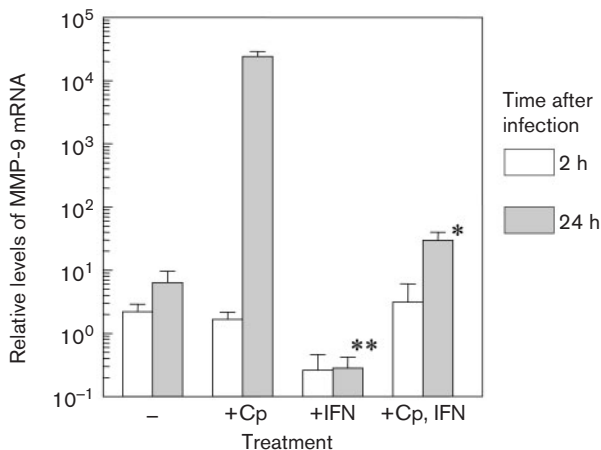


Fig. 6. Inhibitory effect of IFN- β treatment on MMP-9 induction by *C. pneumoniae* infection in microglial cells. Cells were pre-treated with IFN- β (IFN) for 24 h, infected with *C. pneumoniae* (Cp) and then incubated further for 2–24 h in the presence or absence of IFN- β . The level of MMP-9 mRNA was determined by real-time RT-PCR. Data are the mean \pm SD for three experiments. *, $P < 0.05$ (significantly different from the values obtained with the non-IFN-treated *C. pneumoniae* infection group). **, $P < 0.05$ (significantly different from the value obtained with the non-IFN-treated control group).

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