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

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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 x 10⁶ cells per well) were dispersed in 6-well culture plates and incubated for

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**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.
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 μg RNA was performed with avian myeloblastosis virus RT (Promega) with random primers in a commercial reaction mixture (20 μl) (Reverse Transcription System; Promega). The resulting cDNAs (2 μl) were then subjected to either conventional or real-time PCR with primers specific for Chlamydia 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 Chlamydia 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.

**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,
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 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 minimal level of the cytokine protein (Fig. 2b). Other inflammatory cytokines, such as IL-10 and IL-12, were also 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; Rosenberg, 2002b).
Rosenberg, 2002a), which is thought to be associated with \textit{C. pneumoniae} in some cases. Therefore, in further studies mRNAs for MMPs induced in microglial cells by \textit{C. pneumoniae} infection were assessed. As shown in Fig. 4, infection of microglial cells with \textit{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 \textit{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 \textit{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 \textit{C. pneumoniae} infection in microglial cells revealed in this study is consistent with previous findings that \textit{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-\alpha 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).
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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REFERENCES


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±SD for three experiments. *, P<0.05 (significantly different from the values obtained with the non-IFN-treated C. pneumoniae infection group). **, P<0.005 (significantly different from the value obtained with the non-IFN-treated control group).


