Cytokine gene expression in the lungs of BALB/c mice during primary and secondary intranasal infection with *Mycoplasma pneumoniae*

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Cytokine gene expression was determined *in vivo* in the lungs and spleens of *Mycoplasma pneumoniae*-infected BALB/c mice by means of qualitative and semiquantitative PCR-mediated mRNA amplification. During the acute phase of both primary and secondary infections, cytokines commonly associated with innate resistance, TNFα, IFNγ, IL-1β and IL-6, were expressed. In contrast, early expression of the genes for IL-2 and IL-2 receptor was detected only during reinfection. Expression was greater in the lungs than in the spleen, attesting to the rapid accumulation of lymphocytes at the infected site. Interestingly, IL-2 mRNA expression declined rapidly and was no longer detectable after 24 h, whereas IL-10 mRNA levels rose sharply during the same period. During reinfection, mRNAs for TNFα and IL-6 were 10-fold and for IFNγ about 50-fold higher than during primary challenge. The results suggest that the pathogenesis of *M. pneumoniae* diseases may be associated with elevated expression of proinflammatory cytokines.

**Keywords**: *Mycoplasma pneumoniae*, pathogenicity, cytokines, mRNA, gene expression

INTRODUCTION

*Mycoplasma pneumoniae* is a well-established human pathogen causing respiratory diseases and an atypical pneumonia. The host--*M. pneumoniae* interaction is characterized by: (i) a prolonged incubation period of between 10 d and several weeks; (ii) slowly progressing clinical signs of fever and a nonproductive, so-called dry cough; (iii) a slowly increasing specific antibody response with high antibody titres first seen in the late acute and convalescent phase (Kenny et al., 1990); and (iv) in contrast to other bacterial diseases of the respiratory tract, an extension of the convalescence phase for a few weeks up to several months (Jacobs, 1991). Patients do not develop protective antibodies after a first natural *M. pneumoniae* infection (Razin & Jacobs, 1992). Moreover, reinfections with *M. pneumoniae* tend to chronicity and result in more severe clinical signs (Barile, 1984). Pathological immune reactions, i.e. exacerbation of the cellular response with lympho-histiocyte infiltrations of lung tissues, induction of autoantibodies and a loss of delayed-type hypersensitivity reaction to tuberculin, are adverse events in the course of prolonged disease (Jacobs et al., 1988; Jacobs et al., 1990; Lind et al., 1992; Rollins et al., 1986; Tsunekawa et al., 1987).

The regulation of protective and inflammatory phenomena at the site of infection is thought to involve the release of soluble mediators that attract, focus and activate other cells of myelomonocytic origin. To promote understanding of the interaction of *M. pneumoniae* with the host's immune response during the acute phase of primary and secondary intranasal challenge, this study focused on the role of *in vivo* cytokine gene expression in the lungs early after infection.

Data on cytokine production in *M. pneumoniae* infection are scarce. Most investigators have determined cytokine levels in human peripheral blood mononuclear cells stimulated, *in vitro*, by *M. pneumoniae* antigen (Kita et al., 1992; Nakayama et al., 1992). These results may, however, not adequately reflect the local immune response in infected tissues. We have therefore adapted the exquisitely sensitive technique of PCR-mediated mRNA amplification (Ehlers & Smith, 1991) to determine, for the first time in *in vivo* experiments, cytokine mRNA levels in the lungs during the acute phase of *M. pneumoniae* infections in
an established animal model (Pietsch & Jacobs, 1993; Street & Mosmann, 1991).

METHODS

Bacterial strain and animals. M. pneumoniae (strain FH) was grown in Hayflick modified Eagle's medium in Roux bottles for 48 h at 37 °C (Hayflick, 1965). Fresh, glass-adherent mycoplasmas were harvested with a cell-scrapper, washed in phosphate-buffered saline (PBS; 0.14 M NaCl, 0.01 M sodium phosphate, pH 7.2), and re-suspended in PBS to OD,0 0.15 (approx. 10^9 c.f.u. ml^-1). Mycoplasma-free BALB/c mice (3-4 months old; Center for Laboratory Animals, Hannover, Germany) were infected intranasally under anesthesia as described recently (Pietsch & Jacobs, 1992). The inoculum for primary infection was (per mouse) 2x10^6 c.f.u. and for reinfections 5x10^6 c.f.u. in 0.05 ml PBS. Reinfection was carried out 20 d after primary infection.

RNA isolation and cDNA synthesis. Three control (uninfected), primarily infected and reinfected mice were killed at various times from 30 min to 5 d after intranasal inoculation with PBS (control) or M. pneumoniae cells. Lungs and spleens were removed, homogenized immediately in 10 ml lysis buffer (4 M guanidine isothiocyanate (Merck), containing 25 mM sodium citrate (pH 7), 0.5% N-lauroylsarcosine (Sigma) and 100 mM 2-mercaptoethanol (Sigma), and frozen at -70 °C. Total RNA was extracted with phenol/chloroform as described by Chomczynski & Sacchi (1987). RNA precipitates were pelleted at 4 °C with 2-propanol, washed once with 75% ethanol in distilled water containing 0.089 M Tris/HCl, 0.088 M boric acid, 0.002 M EDTA) containing 0.001 % gelatin in a final volume of 25 μl. The reaction mixture was overlaid with a drop of light mineral oil (Sigma), and PCR was performed in a DNA Thermocycler (Perkin-Elmer) for 30 cycles: 30 s denaturation at 94 °C, 45 s annealing at 60 °C, and 1 min extension at 72 °C. In all experiments, control PCRs without cDNA were performed to test for contamination. The reaction product was visualized by electrophoresis using 20 μl reaction mixture at 120 V for 60 min in 2% agarose in half-strength tris/borate/EDTA buffer (single-strength buffer: 0.089 M Tris/HCl, 0.088 M boric acid, 0.002 M EDTA) containing 0.5 μg ethidium bromide ml^-1. One microgram of HinclI-digested pX174 DNA was run in parallel as molecular size markers, providing bands at 1057, 770, 612, 495, 392, 345, 341, 335, 297, 291, 162 and 79 bp. The specificity of the amplified target sequences was validated by their predicted size. PCR-assisted mRNA amplification was repeated at least twice for at least two separately prepared cDNA samples. Data shown are representative of at least three different experiments.

PCR conditions. Cytokine-specific primer pairs (sense and antisense, respectively) were used as follows (sizes of amplified fragments are given in parentheses). β2-Microglobulin: (sense) 5’GGCTCGTCCTGGTGACCCCTAGCTTCT3’ and (antisense) 5’TCTGACGGGTTATGTAGCTTCTA3’ (300 bp); IL-1β: 5’TCAATGGGATGATGAGTATAACCTGTCT3’ and 5’CCCATATTTGGAGAAAGACCGGAT3’ (502 bp); IL-2: 5’ATGTCAGCATGCAGCTGTCTTCCGCA3’ and 5’AGTCAAGATGGTTCTCCAATCAACGCA3’ (320 bp); IL-3: 5’ATGTTCTTCTGCCAGCTCTACCACCA3’ and 5’AGTAACTCATTGTGACCTGCTTCT3’ (351 bp); IL-6: 5’CTGGTTGACACCCAGG-
RESULTS

Kinetic analysis of cytokine gene expression during primary and secondary infection with *M. pneumoniae*

To compare cytokine mRNA expression during primary and secondary infection with *M. pneumoniae*, lungs of BALB/c mice were removed at 30 min, 3 h, 6 h, 24 h, 48 h and 5 d following intranasal infection. PCR amplification showed the presence of TNFα and IL-1β mRNA 30 min after primary and secondary infections and in control mice, whereas IFNγ, IL-6 and GM-CSF (which gave only a faint band) were detected in infected mice only (Fig. 1). The band corresponding to IL-10 mRNA was prominent after 6 h in secondary infections whereas at this time during primary infections it was only weakly expressed. T-cell-specific cytokine mRNA expression, for IL-2 and the IL-2 receptor (IL-2R p55 subunit) was detected 3 h after reinfection but had ceased by 48 h (Fig. 1). T-cell-specific cytokine mRNA expression could not be detected in lungs during the first 5 d of primary infections. In both primary and secondary infection, we were unable to detect IL-3 and IL-4 mRNA expression.

Semiquantitative assessment of cytokine mRNA

To compare the amount of cytokine mRNA in lung tissues during primary and secondary infections, a competitive PCR was used. The cDNAs were serially diluted (10-fold steps) before PCR analysis in the presence of primers and a constant amount of competitor control fragment consisting of 5’- and 3’-primer sequences in a tandem array.

The results enabled semiquantitative comparison of cytokine mRNA expression. After 6 h of primary and secondary infection, there was no difference in the level of expression of β-actin (internal control). However, in lungs of infected mice after both 3 h (data not shown) and 6 h, expression of TNFα and IL-6 mRNA was approximately 10-fold greater, and IFNγ about 50-fold (between 10-fold and 100-fold) greater, than in primarily infected mice (Fig. 2). In primarily infected lungs, TNFα and IL-1β mRNA were elevated about 10-fold in comparison to the non-infected controls (data not shown).

Comparison of cytokine gene expression in lung and spleen

To define differences in local and systemic cytokine expression, lungs and spleens were removed from secondarily infected mice. TNFα, IFNγ and IL-1β mRNA were detected in both lungs and spleens 3 h and 6 h after infection. However, IL-2 and IL-6 mRNAs, which were present at relatively high concentrations in lung tissues, were transcribed to only a minor extent in spleens (Fig. 3; 3 h results not shown). GM-CSF mRNA was only detected in lungs.

DISCUSSION

The fastidious and slow-growing *M. pneumoniae* cells
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Figure 2. Competitive semiquantitative PCR amplification of cytokine mRNA from lungs of primarily and secondarily infected BALB/c mice (6 h after intranasal infection). To compare separate samples quantitatively, PCR was performed on target cDNA serially diluted 10-fold using primers specific for \( \beta \)-actin, TNF\( \alpha \), IFN\( \gamma \) and IL-6 in the presence of a constant amount of competitor control DNA consisting of 5'- and 3'-primer sequences in tandem array. CF, control competitor DNA fragment.

Figure 3. PCR-amplified cytokine mRNA from the lungs and spleens of secondarily infected BALB/c mice (6 h after infection). L, lung; S, spleen.

We have conducted a kinetic and quantitative analysis of cytokine mRNA expression in the lungs of control and immune mice infected with \( M. \) pneumoniea. Five important findings emerge from these studies. (i) During primary infection, transcription of cytokine genes for TNF\( \alpha \), IFN\( \gamma \), IL-1\( \beta \) and IL-6 was observed. This result is in keeping with the interpretation that these cytokines, thought to be involved in innate resistance to bacterial infection, are non-specifically produced early during inflammation (Ehlers et al., 1992; Hirano et al., 1990; Pirmez et al., 1993). (ii) During secondary infection, the expression of granulocyte-macrophage colony stimulating factor, acting in the bone marrow as a growth and differentiation factor, was increased only in the lungs of infected animals. (iii) The expression of TNF\( \alpha \) and IL-1\( \beta \) mRNA was elevated 10-fold in primarily infected mice when compared to control mice. During reinfection the expression of proinflammatory cytokines like TNF\( \alpha \) and IL-6 were 10-fold and for IFN\( \gamma \) about 50-fold higher as early as 6 h after challenge when compared to primary infection. (iv) During secondary infection, mRNA for IL-2, detected as early as 3 h after challenge, was present at higher concentrations in the lungs of infected animals colonize host respiratory epithelium cells. In in vitro experiments, it has been shown that only specifically opsonized \( M. \) pneumoniea cells are phagocytosed by alveolar macrophages (Kist et al., 1982), indicating that \( M. \) pneumoniea may escape non-specific host response mechanisms. We used a high infecting inoculum to mimic the 'post-propagation' phase of \( M. \) pneumoniea infection in humans. It has been documented that especially during secondary infections with \( M. \) pneumoniea an exacerbation of the cellular response may aggravate the disease. Histological examination of affected lungs has revealed peribronchial and perivascular histiolymphocytic infiltraions (Jacobs et al., 1988). It was therefore of interest to compare the acute phase of both primary and secondary infection in order to study the involvement of cytokines in the acute inflammatory process.
than in the spleens, providing direct evidence that lymphocytes infiltrate the site of infection (Pabst, 1992). (v) IL-2 mRNA expression was transient, peaking at 3-6 h post reinfection, and becoming undetectable by 48 h, while, concomitantly, IL-10 mRNA levels rose sharply.

The cytokine pattern characteristic of primary infection [see (i) above] has also been found in ELISA and bioassays in in vitro experiments with human peripheral blood mononuclear cells after stimulation with M. pneumoniae cells (Kita et al., 1992). In contrast, secondary M. pneumoniae infection is characterized by a distinct cytokine pattern: the presence of IL-2 and IL-2R mRNAs attests to the accumulation and activation of T-cells in the infected tissues as early as 3 h after reinfection. In addition, TNFα and IFNγ mRNA levels are significantly increased. At present, the cellular source for this enhanced expression remains unknown, but IL-2 (from T cells) has been shown to increase production of TNFα and IFNγ in other cells of myelomonocytic origin (Cuturi et al., 1989).

In man, the acute phase of M. pneumoniae infection is characterized by a non-specific polymophonuclear leucocyte-rich exudate in the bronchial lumen (Rollins et al., 1986). This recruitment of myelomonocytic cells correlates well with the characteristic cytokine pattern documented in this study of the early stages of infection in mice. Subsequent to these early events, there is a lymphoplasmonic infiltration of the bronchiolar and perivascular walls (Jacobs et al., 1988; McGee & Taylor-Robinson, 1981; Pietsch & Jacobs, 1993). In hamsters, the cuff formation around the bronchial tract and the small vessels shows a maximum at about 14 d after challenge (Barile et al., 1988; Cimolai et al., 1992). The majority of the lymphocytes that cuff the bronchial tree are T-cells, and thymectomy has been shown to prevent the accumulation of peribronchial lymphocytic infiltrations (McGee & Taylor-Robinson, 1981). It is possible that these lymphocytic infiltrations, reminiscent of a delayed-type hypersensitivity reaction, are due to prolonged TNFα and/or IFNγ stimulation of the vasculature, as has been demonstrated in other disease models of chronic inflammation (Kindler et al., 1989; Mielke et al., 1992). Identification of the cellular and molecular mediators of this aspect of disease awaits the results of ex vivo analyses of the later stages of disease.

It is important to emphasize that our present studies are strictly confined to the acute phase of primary and secondary infections with M. pneumoniae and do not directly address the involvement of cytokines in the immunopathology of prolonged and chronic disease. Nevertheless, there is a clear indication that the immune response during secondary infection is accelerated and distinctly more pronounced in terms of the expression of proinflammatory cytokines. The finding that IL-2 mRNA expression was transient (up to 48 h) is most striking, particularly since the disappearance of IL-2 mRNA is coincident with the appearance of IL-10 mRNA during secondary infection. Bearing in mind the concept of cross-regulatory networks of cytokine-secreting cells, it is tempting to speculate that IL-10 serves to turn off the specific T-cell response (Fiorentino et al., 1991). The cellular source of the IL-10 mRNA detected in these studies is at present unclear, and cellular depletion experiments aimed at defining its origin are in progress.

Future studies will also be directed at (i) identifying the cellular components responsible for expression of the other cytokines produced in response to M. pneumoniae infection and detected in this study; (ii) correlating cytokine patterns with histomorphological findings in order to define the relative contribution of distinct cytokines in inflammatory and protective events during M. pneumoniae infection. In this regard, it will be of particular interest to scrutinize more closely the later phase of infection during which dense lymphocytic infiltrations develop in the peribronchial space. Correlating cytokine patterns with immunopathological findings is a first step in our attempt to develop immunomodulatory strategies aimed at reducing disease-promoting mechanisms whilst leaving protective mechanisms unimpaired.

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REFERENCES


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