Abstract. Objective: Investigation of chronic infections with Chlamydophila pneumoniae.

Methods: BALB/c mice were repeatedly infected with C. pneumoniae and tested during a 1-year period. Production of histamine, IFN-γ, IL-6 and antibodies was monitored by ELISA. Live bacteria were cultured and DNA was detected by PCR. Cellular immunity was tested by ELISPOT.

Results: After re-infections, culture positivity and persistence of DNA in lungs and blood were shorter. Detection of DNA at late time points indicated persistent infection in a few mice. Histamine was produced after primary and re-infections, and the level correlated with the number of viable bacteria in lung. IFN-γ, IL-6 levels, IgG2/IgG1 ratio, IgA titres, and level of chlamydial heat-shock protein antibodies were higher after re-infections. IgM antibodies were demonstrated even after re-infections. High number of IFN-γ-producing splenocytes was observed after the third inoculation.

Conclusion: These results promote an understanding of the patho- and immune mechanisms after C. pneumoniae re-infections.

Key words: C. pneumoniae, Re-infections, Histamine, Immune response

Introduction

Chlamydia pneumoniae is known to cause a large spectrum of symptoms in the respiratory tract, ranging from an asymptomatic „carriage” state to severe pneumonia and has recently emerged as an important pathogen associated with atherosclerosis and asthma [1–3]. Particularly, most exacerbations of asthma requiring emergency room visits or hospitalization seem to be associated with acute bacterial and viral infection of the respiratory tract rather than exposure to allergens themselves. C. pneumoniae, among other pathogens, may play a role in the development of asthma exacerbations [4, 5], but the mechanism(s) is not clear. Other investigators, however, indicated an association between an acute infection with Mycoplasma pneumoniae, but not with C. pneumoniae in patients hospitalized for acute exacerbations of bronchial asthma [6]. The prevalence of antibodies to C. pneumoniae increases from the age of 5 to 50% by the age of 20, and slowly continues to increase among adults, indicating that re-infections are common [1]. C. pneumoniae also has a tendency to cause persistent infection [7]. Chronic/persistent infection of non-respiratory sites by C. pneumoniae requires evasion from the respiratory tract via the bloodstream. C. pneumoniae DNA has been detected in human atherosclerotic plaques and in human peripheral blood mononuclear cells (PBMCs) [8, 9]. The frequency and the time course of the appearance of C. pneumoniae DNA in the circulation after primary and repeated infections have not been described. Histidine decarboxylase (HDC), an essential enzyme of histamine production, has been shown to be induced in the mouse lung after a primary infection with C. pneumoniae [10]. However, the production of histamine, a mediator of inflammation and allergic reactions, has never been investigated after primary or challenge infections with C. pneumoniae. IFN-γ and IL-6 are cytokines involved in the regulation of innate and adaptive immunity, but their production and role following C. pneumoniae re-infections are not clear. In our earlier study expression of HDC correlated with that of IL-6 after a single C. pneumoniae infection of mice [10]. However, the production of histamine, a mediator of inflammation and allergic reactions, has never been investigated after primary or challenge infections with C. pneumoniae. IFN-γ and IL-6 are cytokines involved in the regulation of innate and adaptive immunity, but their production and role following C. pneumoniae re-infections are not clear. In our earlier study expression of HDC correlated with that of IL-6 after a single C. pneumoniae infection of mice [10]. However, the production of histamine, a mediator of inflammation and allergic reactions, has never been investigated after primary or challenge infections with C. pneumoniae. IFN-γ and IL-6 are cytokines involved in the regulation of innate and adaptive immunity, but their production and role following C. pneumoniae re-infections are not clear.
These considerations led us to hypothesize that \textit{C. pneumoniae} infection or re-infection of the mouse respiratory tract will induce local histamine release. We also addressed the question whether an association exists between the histamine level in the lung and the presence of viable bacterium or the copy number of bacterial DNA in the lung and blood after primary and re-infections. In addition, we evaluated the development of \textit{C. pneumoniae} antibody and cell mediated immune responses in relation with bacterial replication and cytokine production during re-infections.

Materials and methods

Animal model

Six-8-week-old female BALB/c mice were inoculated intranasally one, two or three times under mild anaesthesia (pentobarbital sodium) with 7.5x10⁵ inclusion forming units (IFU) of \textit{C. pneumoniae} (TW183, ATCC) in a volume of 30 µl or with the same volume of a mock preparation (McCoy cell preparation). Mice receiving a single inoculation were anesthetized and sacrificed, and specimens were taken 1 or 2 days or 1, 2, 4, 8, 10, 16, 18, 24, 36 or 49 weeks after the first inoculation. One group of mice received a second inoculation at week 8, and specimens were taken 1 and 2 days and 1, 2, 4, 8, 20, 28 and 33 weeks later. Three to seven \textit{C. pneumoniae}-inoculated mice were sacrificed at one time point, except at weeks 36 and 49, when only one or two mice, respectively, were sacrificed from the group inoculated only once. Two of the mock-inoculated mice were sacrificed at one time point, except at weeks 20 and 33, when only one mouse was sacrificed from the group that received three inoculations.

By heart puncture, blood was collected in heparin for PCR analysis, and the serum was separated for the testing of antibody production. The sera that received three inoculations. The DNA from the blood of individual mice was subjected to 4 replicate PCR runs. The DNA in the lungs and spleen was tested in a single and three replicate PCR reactions, respectively. The presence of PCR inhibitors in the samples was excluded by the amplification of mouse \(\beta\)-actin from each sample in a simple PCR. False-positive reactions were highly improbable, since all of the PCR-negative controls (every fifth reaction) and samples from mock-inoculated mice were negative. Samples were considered positive when at least 1 of the nPCR replicates was positive.

Real-time PCR. The LightCycler real-time PCR system (Roche), the LightCycler FastStart DNA Master SYBR Green 1 kit (Roche) and \textit{C. pneumoniae groEL-1} gene-specific nested primer pairs were used. The primer pairs were designed by the DNA Lasergene program (DNASTAR, Inc) and BLAST search: outer primers: F1: 5'-TGA TTA AAA GAG GCC GTC CAG ATT-3' and R1: 5'-CAG GCC AGC ACG GAA CTA CAA CAG C-3', and inner primers: F2: 5'-ACG CAT TCT TGA GTT TCT GAA TT-3' and R2: 5'-AAA GCC GTA AAA GTT GTT GTT GAT-3'. The standard curve was generated by using serial 10-fold dilutions of the amplicon prepared by using the outer primers (2x10⁵-2x10² copies/µl). All reactions were performed in triplicate. The cycling parameters were: denaturation at 95 °C for 10 min; 50 cycles of 95 °C for 2 s, 57 °C for 6 s, 72 °C for 14 s and acquisition at 72 °C. The limit of detectability was 6.7x10² copy numbers/ml. For each sample analyzed, the relative number of \textit{Chlamydia} genome equivalents was established. A lung obtained from a mock-infected mouse and spiked \textit{in vitro} with \textit{C. pneumoniae} at an inoculation dose showed a copy number of 1.2x10⁶/ml.

Histamine, IFN-\(\gamma\) and IL-6 measurements in lungs

Supernatants of the lung homogenates were clarified by centrifugation and were assayed for IFN-\(\gamma\)- and IL-6 contents with the OptEIA ELISA sets (BD Biosciences Pharmingen). The sensitivities of the IFN-\(\gamma\) and IL-6 measurements were 15.6-1000 pg/ml and 125-1000 pg/ml, respectively. A histamine ELISA kit (IBL) was used for the quantitative determination of histamine in the lungs. Clarified supernatants were tested in duplicate according to the manufacturer’s instructions. The lower limit of detection was 2.4 ng/ml.

Detection of antibodies

\textit{C. pneumoniae}-specific antibodies were measured by an in-house developed ELISA test. Concentrated \textit{C. pneumoniae} EBs treated with NP40, and uninfected control McCoy cells treated similarly, served as antigens. Serial serum dilutions were measured using the coated plates, and horseradish-peroxidase (HRP)-conjugated secondary antibodies (\(\alpha\)-mouse IgG-HRP, ICN; \(\alpha\)-mouse IgM-HRP, Sigma; \(\alpha\)-mouse IgA-HRP, Sigma and \(\alpha\)-mouse IgG2a-HRP, Biosource) were used for detection. IgG1 and IgG2a -coated plates (Cappel) were used for determination of the corresponding secondary antibody dilutions that gave quantitatively similar reactivity in the ELISA assay. The titres were determined at dilutions demonstrating an optical density (OD) >0.1 after correction with OD values measured on the control antigen.

\(\text{hsp60}\)-specific antibodies were detected by ELISA, using plates coated with recombinant \text{hsp60} from \text{C. trachomatis} as antigen, kindly provided by MEDAC GmbH (Hamburg, Germany). Sera were tested at a dilution of 1:400. The HRP-conjugated secondary antibody used was that applied in the ELISA with the full \textit{C. pneumoniae} antigen.

Quanti cation of IFN-\(\gamma\) producing cells in spleens

3x10⁶ spleen cells in RPMI medium in 24-well tissue culture plates were stimulated with viable \textit{C. pneumoniae} at a multiplicity of infection of 0.2, or with an equivalent amount of McCoy mock preparation overnight at 37°C in a CO₂ incubator. Ninety-six-well filter plates with a