Chlamyphila pneumoniae changes iron homeostasis in infected tissues

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Abstract

Many bacteria, including Chlamyphila pneumoniae (C. pneumoniae), are dependent on iron (Fe) for their growth. However, it is not known whether bacterial infections affect gastrointestinal uptake and uptake of trace elements in infected tissues. A human C. pneumoniae strain adapted to C57BL/6J mice was used to study hepcidin gene expression in the liver and divalent metal transporter 1 (DMT1) content in the liver and intestine and whether Fe is concomitantly changed in serum, liver, and intestine. The copper/zinc (Cu/Zn) ratio in the serum was used as a marker for infection. Bacterial DNA, mRNA, and hepcidin were measured by real-time PCR, DMT1 by Western blot, and trace elements by ICP-MS on days 2, 5, and 8 of the infection. C. pneumoniae DNA was found in the liver on all days but the number of viable bacteria peaked on day 8. Hepcidin expression increased on days 2 and 5, whereas DMT1 content in the liver increased on day 8. Fe decreased in serum, increased in the liver but was not changed in the intestine during the disease. In the serum, the Cu/Zn ratio peaked on day 5. The peak of viable bacteria in the liver was associated with increased DMT1 and Fe contents and increased hepcidin expression, but this did not affect intestinal Fe uptake. Thus, growth of C. pneumoniae in tissues parallels a redistribution of Fe to those tissues resulting in a changed body homeostasis of Fe.

Keywords: Chlamyphila pneumoniae; Chlamydia pneumoniae; DMT1; Hepcidin; Iron; Liver

Introduction

Chlamyphila pneumoniae (C. pneumoniae) is a common respiratory pathogen, with approximately 50% of the population being seropositive by the age of 20 (Grayston, 2000). These slowly growing intracellular bacteria have biphasic life cycles, alternating between a metabolically active intracellular and a metabolically inactive infectious phase (Kuo et al., 1995). It is noteworthy that the life cycle of C. pneumoniae sometimes seems to be interrupted, resulting in a persistent intracellular infection in which the bacteria are insensitive to antibiotics (Grayston, 2000). C. pneumoniae has been suggested to be involved in the pathogenesis of atherosclerosis as a stimulator of the chronic inflammation in the atherosclerotic plaque (Grayston, 2000; Leinonen and Saikku, 2002). This belief is further supported by the findings of viable and metabolically active bacteria in aortic tissue from patients undergoing coronary artery by-pass grafting (Nystrom-Rosander et al., 2006). However, the factors...
controlling what form of *C. pneumoniae* is present/predominant in an infected tissue are not known.

Essential trace elements are micronutrients crucial for host defence (Pekarek and Engelhardt, 1981; Beisel, 2004; Ilbäck and Friman, 2007), including the development of inflammation (Milanino et al., 1993; Nystrom-Rosander et al., 2003). The normal host response to infection (the acute-phase response) includes increased synthesis of metal-binding proteins and a concomitant flux of trace elements between blood and tissues (Beisel, 2004). Consistent responses in trace elements in generalised infections are a decrease in plasma levels of iron (Fe) and zinc (Zn) and an increase in copper (Cu) (Beisel, 2004; Ilbäck and Friman, 2007). Proteins implicated in the homeostasis of essential metals, including acute-phase proteins, serve as carriers for essential trace elements, such as Fe-binding ferritin (Beisel, 2004), divalent metal transporter 1 (DMT1) (Vyoral and Petrak, 2005), and the Cu-binding ceruloplasmin (Little and Canonico, 1981). Consequently, during infection, there is a flux of trace elements between blood and other tissues, including tissues involved in the disease.

Systemic Fe content is primarily regulated by Fe uptake from the intestinal lumen and not at the level of excretion. The circulating peptide hormone hepcidin, with antimicrobial properties (Krause et al., 2000), is involved in the regulation of body Fe homeostasis (Ganz and Nemeth, 2006), including intestinal absorption, Fe-recycling in macrophages, and Fe mobilisation in hepatocytes (Nicolas et al., 2001; Park et al., 2001; Pigeon et al., 2001). During inflammation and infection as well as during Fe overload, hepcidin expression increases, leading to reduced intestinal absorption of Fe (Ganz and Nemeth, 2006). The uptake of Fe in the intestine is mediated by DMT1 (also known as Nramp2), a metal transporter expressed in many tissues in the body (Gunshin et al., 1997). In the intestine, the transmembrane protein DMT1 is localised on the enterocytes and transports Fe from the intestinal lumen into the enterocytes (Donovan and Andrews, 2004). DMT1 expression is tightly regulated by the body Fe status and decreased Fe levels up-regulate DMT1 expression in the duodenum (Gunshin et al., 1997). It is possible that DMT1 expression in the intestine is also affected by hepcidin levels because the decreased hepcidin levels, occurring during iron restriction diet, correlate to increased DMT1 expression (Frazer et al., 2002). Moreover, in cell culture experiments, hepcidin decreases DMT1 expression (Yamaji et al., 2004).

Essential trace elements are not only important for the host immune defence but also for the growth and virulence of many microorganisms (Shankar and Prasad, 1998; Weinberg, 1999; Freidank et al., 2001; Krenn et al., 2005). Many bacteria, including *C. pneumoniae*, need Fe for their growth (Freidank et al., 2001), and excessive Fe in specific tissues has been shown to promote bacterial growth in those tissues (Lounis et al., 2001). In cell cultures of *C. pneumoniae*, Fe restriction has been observed to generate persistent *C. pneumoniae* infection (Al-Younes et al., 2001; Mukhopadhyay et al., 2006). We have recently shown that *C. pneumoniae* infection in mice increased Fe levels in infected tissues (i.e. the liver and the heart) that may be pivotal for bacterial growth (Edvinsson et al., 2008). Moreover, a few studies indicate that infections can alter the gastrointestinal uptake of non-essential and essential elements (e.g. cadmium, Cd) and Fe (Glynn et al., 1998; Ciacci et al., 2004; Ilbäck et al., 2008). Thus, there is ample reason to suspect that some of the sequential changes occurring in blood trace element levels and in target organs of infection reflect a changed gastrointestinal absorption during the course of the infection.

The aim of this study was to investigate whether infection-induced Fe fluxes between serum, liver, and intestine are associated with changes in bacterial replication and hepcidin gene expression in the liver, as well as DMT1 protein expression in the liver and intestine. To accomplish this, an infection model with a human *C. pneumoniae* strain adapted to mice was used. The Cu/Zn ratio in serum was used as a marker of established infection.

**Materials and methods**

**Mice**

Adult female C57BL/6 mice were purchased from Charles River (Copenhagen, Denmark) and maintained at the Animal Department, Biomedical Centre, Uppsala, Sweden. Mice were housed behind strict hygienic barrier cages (TouchSLIMLine, Tecniplast, Scanbur BK A/S, Denmark) at 20±1 °C and a relative humidity of 50±2%. Water and regular chow diet were supplied ad libitum. The animal experiments described in this paper took into account all ethical aspects of the welfare of animals following the recommendations in ‘Guide for the Care and Use of Laboratory Animals’ of the Swedish National Board for Laboratory Animals (CFN). The study was approved (C146/6) by the local Research Ethics Committee for Experimental Use at the Faculty of Medicine, Uppsala University.

**Experimental design**

In this experiment, adult female mice with a mean weight of 24.5 g on day 0 were used. The clinical isolate G-954 of *C. pneumoniae* was propagated in Hep-2 cells and stored in a sucrose-phosphate-glutamate (SPG) solution at −70 °C. Twenty-four mice were divided into 4 groups with each group containing 6 mice. On day 0, 3
groups of mice were infected with \(5 \times 10^8\) ifu in 30\(\mu\)l SPG. The remaining group of mice was sham inoculated with 30\(\mu\)l SPG to serve as a control group. Mice were sedated using Fluothane (Astra Läkemedel, Södertälje, Sweden) and 30\(\mu\)l of the inoculum or SPG was administered intranasally. Control mice and infected mice were kept in separate cages.

Six infected mice were sacrificed on each of the days 2, 5, and 8. On each of these days, sham-inoculated mice \(n = 2\) were concomitantly sacrificed to serve as a healthy control group (total \(n = 6\)). Body weight and rectal temperature were recorded on each day during the study.

Tissue sampling and tissue preparation

Mice from each group were anaesthetised on days 2, 5, and 8 using Fluothane. The thoracic cavity was opened, and blood was collected in heparinised tubes using heart puncture with a sterile syringe. Liver and intestines were excised and tissue samples therefrom and remaining whole blood by centrifugation. Serum and liver on day 8 was used for immunohistological detection of DMT1. Serum was separated from the blood and liver and gene expression of hepcidin in the liver in samples from the days 2, 5, and 8. Protein was extracted from the intestine and liver for Western blot analysis of DMT1 from samples from days 5 and 8. A tissue sample from the liver on day 8 was used for immunohistological detection of DMT1. Serum was separated from the remaining whole blood by centrifugation. Serum and tissue samples from days 2, 5, and 8 of the liver and intestine were stored at \(-20^\circ\)C until used for determination of Fe. In addition, Cu and Zn were measured in the serum.

DNA extraction for determination of \(C. pneumoniae\)

DNA was extracted from tissue samples and from 200\(\mu\)l blood with the QiaAmp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. DNA was eluted in 50\(\mu\)l buffer AE and then diluted 1:10 in double-distilled water and stored at \(4^\circ\)C until further analysis was performed, i.e. within a few days. In every step of extraction, a negative (no template) control was processed in the same way as the samples. DNA concentration was measured for each sample by NanoDrop ND-1000 (NanoDrop Technologies Inc., Wilmington, DE, USA).

RNA extraction for determination of hepcidin and viable \(C. pneumoniae\) in the liver

Total RNA was extracted from 50 to 100\(\)mg of liver with the Trizol reagent (Invitrogen) according to the manufacturer’s instructions. Frozen liver was homogenised with 2\(\)ml cooled Trizol reagent in a Potter-Elvehjem homogeniser kept on ice. Before homogenisation, homogenisers and pistils were treated with 0.5\(\)M NaOH over night and then rinsed thoroughly with nuclease-free water and dried at \(60^\circ\)C. Quantification of RNA was performed according to the RNA-specific Ribogreen protocol with DNase I (Molecular Probes, Leiden, The Netherlands). RNA integrity was confirmed by electrophoresis of 5\(\mu\)g of total RNA per lane on a formaldehyde/agarose gel followed by visualisation of the 28S and 18S ribosomal RNA bands. Purity of RNA was determined by 260/280 nm ratios, and samples with ratios below 1.80 were not used in the present study.

Reverse transcription PCR (RT-PCR)

cDNA for determination of viable \(C. pneumoniae\) was synthesised from liver RNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) with random hexamers according to instructions from the manufacturer. In each reaction, 250\(\)ng RNA was used. Before RT-PCR, each sample was treated with Amplification grade DNase I (Invitrogen) according to the manufacturer’s protocol.

Real-time PCR for \(C. pneumoniae\)

DNA and cDNA samples were subjected to real-time PCR, amplifying a fragment of the \(C. pneumoniae\) \(ompA\) gene (Kuoppa et al., 2002) as previously described (Nystrom-Rosander et al., 2006).

Hepcidin gene expression

Mouse-specific amplification primers to hepc1 coding for the protein hepcidin were designed to generate a 198 bp RT-PCR-product. Primer sequences were 5\'-AGA AAG CAG GGC AGA CAT TG-3' (forward) and 5\'-GGG GAG GGC AGG AAT AAA TA-3' (reverse). Hepcidin gene expression was measured by real-time RT-PCR on a Rotorgene, RG 3000 (Corbett Research, Mortlake, Australia) with the use of the QuantiTect One-Tube RT-PCR kit with SYBR-Green (Qiagen) according to the instructions from the manufacturer. The RT-PCR mix consisted of 25\(\mu\)l with 1\(\mu\)l enzyme mix, 0.2 mM dNTPs, 5 mM DTT, 4 U RNase-inhibitor, 1 U of heat-labile uracil-DNA-glycosylase, 400\(\)nM primers, and 250\(\)ng total RNA from the liver. Samples were incubated on ice for 15 min before the following RT-PCR conditions: 55°C, 30 min; 95°C, 15 min; 30 cycles of 94°C, 1 min; 55°C, 1 min; and 68°C, 45 s each. After the last cycle, a 7-min extension interval at 68°C was inserted, immediately followed by a melting curve. Melt curve analysis and quantitative gene
expression analysis were carried out by applying Rotor- gene software.

**DMT1 protein expression**

Tissue samples were thawed on ice and homogenised in 10 volumes of RIPA-lysis buffer as previously described (McKie et al., 2000). Protein aliquots of 80 μg per lane were separated on 10% Tris-Glycine polyacrylamide gels under reducing conditions as described elsewhere (Laemmli, 1970). The separated proteins were electro-blotted onto nitrocellulose membranes (pore size 0.45 μm). Membranes were incubated at 4°C with blocking buffer (5% dry milk dissolved in PBS containing 0.1% Tween 20 (PBS-T)) overnight and then washed 3 × 10 min at room temperature (RT) with PBS-T. Membranes were then hybridised for 1 h at RT with rabbit polyclonal antiserum against DMT1 (generous gift from Dr. Bo Lönnerdal, Department of Nutrition, UCD, USA) diluted 1:25000 in PBS-T. The DMT1 antibodies recognise both DMT1 and the iron responsive element (IRE) containing a splice variant of DMT1-IRE. After 3 × 10 min washes in PBS-T, the membranes were hybridised for 1 h at RT with secondary HRP-conjugated donkey anti-rabbit antibodies (Amersham Pharmacia Biotech) diluted 1:50,000 in PBS-T. Following additional 3 × 10 min washes in PBS-T, DMT1 expression was determined by employing ECL-Advance (GE Healthcare). A ChemiDoc XRS instrument (BioRad) and Quantity-One software (BioRad) were applied to detect and quantify the intensities of the bands. DMT1 expression was normalised to the expression of tubulin. Hybridisations were performed in the same manner as described previously for DMT1 by applying primary tubulin antibodies (ab6161, Abcam) diluted 1:3000 in PBS-T, HRP-conjugated secondary anti-IgGs (ab6734, Abcam) diluted 1:5000 in PBS-T and ECL-Advance (GE Healthcare). Before anti-tubulin hybridisations, the primary DMT1 antibodies were stripped from the membranes by incubation at 60°C for 30 min under gentle rocking in 62.5 mM Tris–HCl, 100 mM 2-mercaptoethanol and 2% SDS dissolved in double-distilled water.

**Immunohistochemistry of DMT1 in the liver**

Frozen livers of controls and *C. pneumoniae*-infected mice (day 8 of infection) were embedded in Tissue-Tek O.C.T. compound (Miles), sectioned (4 μm), and mounted on positively charged slides. Sections were air-dried at RT overnight and then fixed in refrigerated acetone. Endogenous peroxidases were blocked by incubating the slides in 3% H₂O₂ in MeOH for 30 min. DMT1 localisation was then determined according to the following protocol: (i) blocking with 10% goat serum (NGS) for 30 min at RT, (ii) incubation with DMT1 antiserum diluted 1:1000 in 10% NGS for 1 h at RT, (iii) incubation with secondary goat anti-rabbit IgG (Dako) diluted 1:200 in 4% BSA, (iv) incubation with AB-complex (Dako), and (v) detection with 3,3-diaminobenzidin (DAB) (Sigma) according to the instructions of the manufacturers. Counterstaining was performed with hematoxylin. Slides were rinsed with PBS between steps (ii) and (v) using the identical procedure described above and in water following the DAB treatment (step v). Sections treated as above, but without primary DMT1 antiserum, served as negative controls.

**Assessment of trace elements in serum, liver, and intestine**

To determine the elements Fe, Cu, and Zn in the serum and Fe in the liver and intestine, the samples were treated as described earlier (Ilbäck et al., 2003a, b), and the element content was measured by inductively coupled plasma mass-spectrometry (ICP-MS; Perkin-Elmer SCIEX ELAN 6000, Perkin Elmer Corp., Norwalk, CT, USA). For quality control, every fifth sample was checked against reference materials: human whole blood (Batch OK0336) and serum (Batch MIO181) both from Seronorm Trace Elements (Sero AS, Bilingstad, Norway). Furthermore, a Certified Reference Material of Bovine muscle (Community Bureau of Reference, Brussels, Belgium) was used. All reference material measurements for Fe, Cu, and Zn were within 8% variation of the stated value, and the precision of the measurements was within 5% variation.

**Statistical analysis**

Because the experimental design involves 1 control group and 3 infected groups, analysis of variance (ANOVA) was applied. For the primary ANOVA, the Kruskal–Wallis test was used. In the case of rejecting the null hypothesis in the ANOVA, Dunn’s method for multiple comparisons was adopted to establish which of the groups differed significantly. Before combining the control samples, each trace element was compared in the tissues of sham-inoculated animals on days 2, 5, and 8 with a Kruskal–Wallis test, in order to find potential time trend effects in non-infected mice. Data in figures are expressed as follows: the central thick line indicates the mean value, the bottom side of the box the standard deviation. Outliers and extremes are indicated with opened and filled circles, respectively. Asterisks (*) in the figures denote a statistically significant difference (p < 0.05) between non-infected and infected mice.
Results

Clinical response to infection

Mice responded to the infection with clinical signs of disease (including ruffled fur and inactivity) and a concomitant decrease in body temperature and body weight (data not shown). Clinical signs were most pronounced on day 5 and gradually resolved until day 8. For ethical reasons, one mouse had to be sacrificed on day 5.

C. pneumoniae distribution in the blood and the liver

Numbers of mice positive for C. pneumoniae ompA DNA and mRNA in the liver on days 2, 5, and 8 are shown in Fig. 1. Although the number of mice positive for C. pneumoniae DNA was highest on day 8, C. pneumoniae copies per ng DNA peaked on day 5 and then decreased to day 8 (data not shown). C. pneumoniae was detected in the blood of 3 mice on day 2 but not in any mouse on days 5 and 8.

Hepcidin gene expression in the liver

In comparison with control mice, hepcidin gene expression was up-regulated in the liver of the infected mice on day 2 and peaked on day 5 (Fig. 2). On day 8, expression levels were down-regulated by 10-fold as compared with day 5 but still higher in infected mice as compared with controls (Fig. 2). Melt curve analysis of generated real-time RT-PCR products demonstrated identical melting points, indicating a high specificity of designed primer pairs.

DMT1 protein expression in the liver and intestine

DMT1 expression in the livers of infected mice was reduced on day 5 but increased on day 8 as compared with the controls (Fig. 3). In the intestine, no difference in DMT1 expression was observed between infected and control mice (Fig. 3).

DMT1 localisation in the liver on day 8

DMT1 was detected in the form of granular deposits in the cytoplasm of hepatocytes, and the expression appeared to be strongest in hepatocytes surrounding the central vein (Fig. 4a and b). No difference in the localization of DMT1 was observed in the livers of the infected mice (day 8 of infection). No DMT1 staining was observed when primary antibodies were omitted (Fig. 4c).

Trace elements in the serum, liver, and intestine

Results of the Fe measurements in serum, liver, and intestine are shown in Fig. 5. Because of technical problems, serum results were only obtained from 4/6 mice on day 2, from 3/5 mice on day 5 but from all mice

Fig. 1. Numbers of mice positive for Chlamydia pneumoniae DNA and mRNA in the liver on days 2 (n = 6), 5 (n = 5), and 8 (n = 6) post-infection.

Fig. 2. Normalised hepcidin gene expression in the liver in Chlamydia pneumoniae-infected and control mice on days 2, 5, and 8. The central thick line indicates the mean value, the bottom side of the box the standard error subtracted from the mean value, and the upper side the standard error added to the mean value. The whiskers extend out to the range of the standard deviation. Outliers are indicated with open circles.
on day 8. In the serum, Fe decreased to 37% ($p < 0.05$) in the controls on day 8. In contrast, the Fe content in the liver increased to 126% ($p < 0.05$) in the controls on day 8. The intestinal concentration of Fe was not changed by the infection. The Cu/Zn ratio was elevated and peaked on day 5 ($p < 0.05$) (Fig. 6), i.e., when clinical signs of disease and bacterial counts in the liver peaked.

Discussion

In the present study, infection with *C. pneumoniae* induced a changed Fe homeostasis. High concentrations of *C. pneumoniae* were found in the liver on days 2 and 5, but the number of viable and metabolically active bacteria was most pronounced on day 8. Hepcidin gene expression in the liver was increased on all days with a peak on day 5, whereas the content of DMT1 in the liver decreased on day 5 but increased on day 8. DMT1 in the intestine was not changed on any day. Fe decreased in the serum and was unchanged in the intestine, whereas in the liver, it increased during disease. Thus, the presence of metabolically active and viable bacteria in the liver was associated with the induction of hepcidin, with increased DMT1 expression as well as Fe accumulation in the liver.

![Fig. 3. DMT1 protein expression in the liver and in the intestine on days 5 and 8 in *Chlamydophila pneumoniae*-infected mice as compared with control mice. The central thick line indicates the mean value, the bottom side of the box the standard error subtracted from the mean value, and the upper side the standard error added to the mean value. The whiskers extend out to the range of the standard deviation. Outliers and extremes are indicated with open and filled circles, respectively.](image)

![Fig. 4. Representative DMT1 localisation in the livers of control mice (day 8 post-inoculation). DMT1 was localised in hepatocytes in the form of intracellular granular deposits (arrows; A and B). A stronger DMT1 staining was detected in hepatocytes surrounding the central vein (A and B). No difference in DMT1 localisation was observed in the livers of *Chlamydophila pneumoniae*-infected mice. DMT1 was not detected when primary DMT1 antibodies were omitted (C). Legends: cv: central vein; s: sinusoid; n: nucleus. Magnifications: 400× (A and C) and 1000× (B).](image)
In all infections, an extensive adjustment in host metabolism occurs with the aim to penalise and to destroy the invading microorganisms, repair damaged tissue, and restore tissue function to normal conditions (Ilbäck and Friman, 2007). These infection-induced changes in host metabolism are accompanied by an often extensive and simultaneous flow of trace elements between blood and tissues (Ilbäck et al., 2004). For instance, regardless of infectious aetiology, ceruloplasmin is released from the liver concomitant with an uptake of Zn in the liver, resulting in an increased Cu/Zn ratio in the serum (Funseth et al., 2000; Ilbäck et al., 2003b; Beisel, 2004). The Cu-containing ceruloplasmin is an important acute-phase reactant in generalised infection (Beisel, 2004), and the Cu level in plasma is known to increase in infections that are caused by various microorganisms (Friman et al., 1982; Ilbäck et al., 1983, 2003b; Beisel, 2004). In this study, an expected increase in the serum Cu/Zn ratio was observed, which peaked on day 5 when clinical signs tended to be most severe.

Different techniques have demonstrated the presence of C. pneumoniae in atherosclerotic plaques (Leinonen and Saikku, 2002; Campbell and Kuo, 2004), and C. pneumoniae infection in hyperlipidemic mice has been observed to accelerate the atherosclerotic process (Campbell and Kuo, 2004; de Kruif et al., 2005). Moreover, a hypothesis has been advanced that elevated levels of body Fe is a risk factor per se in the development of atherosclerosis (Sullivan and Weinberg, 1999; Shah and Alam, 2003; Yuan and Li, 2003) but convincing evidence of this has thus far not been shown (Shah and Alam, 2003). Nevertheless, similarly to many other bacteria, C. pneumoniae needs Fe as a growth factor (Schaible and Kaufmann, 2004) and restriction of Fe in cell cultures has been shown to inhibit the growth of C. pneumoniae, causing it to stay in a more persistent form (Al-Younes et al., 2001; Freidank et al., 2001). Thus, it is reasonable to assume that the life cycle and the growth of the intracellular bacterium C. pneumoniae in infected tissues are influenced by the amount of Fe that is available in the host cell. Accordingly, in this study, we found increased Fe levels in the liver that were associated with an increased number of viable C. pneumoniae.

In recent years, interest has been focused on the liver as a site of regulation of whole-body Fe status and on hepcidin as a key regulatory component (Ganz, 2003). In addition to being expressed in hepatocytes, hepcidin is expressed in the heart and pancreas (Ilyin et al., 2003). Hepcidin levels increase in response to infection and inflammation and exert a negative effect on Fe efflux.
from macrophages, hepatocytes, and enterocytes by binding to the Fe transporter Ferroportin 1 (Nemeth et al., 2004). Thus, an increased hepcidin expression and associated increase in intracellular retention of Fe could affect both serum and tissue levels of Fe. Accordingly, in the present study, there was an increased Fe concentration in the liver during C. pneumoniae infection and a progressive decrease of Fe in the serum that were associated with an increased expression of hepcidin in the liver.

Absorption of Fe in the intestine is mediated by DMT1 (Ganz and Nemeth, 2006), and increased hepcidin expression normally causes reduced intestinal absorption of Fe (Ganz and Nemeth, 2006), probably because of a decrease in DMT1 expression (Yamaji et al., 2004). DMT1 expression is tightly regulated by the body Fe status, and decreased Fe levels are known to up-regulate intestinal DMT1 expression (Gunshin et al., 1997). Even though hepcidin expression occurred in the liver on all days of the infection, there was no evidence of down-regulation of DMT1 in the intestine. In the liver, DMT1 expression was decreased on day 5 but then increased on day 8. It is noteworthy that on day 8, the hepcidin expression had turned back and was then only slightly to moderately increased. Thus, there is reason to suspect that most of the sequential changes that occurred in Fe levels in the serum and in the liver reflect an increased microbial activity and that the changed Fe homeostasis (at least in part) involved DMT1-mediated Fe regulation.

Circulating bacteria are taken up by macrophages, such as the Kupffer cells present in the liver. Further, it has been reported that C. pneumoniae replicates in Kupffer cells in a mouse model of liver infection (Marangoni et al., 2006). This finding of C. pneumoniae replication in the liver and the well-known need of Fe for C. pneumoniae growth (Freidank et al., 2001) are in line with the present results of increased Fe levels in the liver in the late phase of the C. pneumoniae infection, i.e. on day 8 and at the time when C. pneumoniae turned into increasing numbers of viable and active bacteria. Moreover, there was an increased expression of DMT1 at that time of an increased activity of C. pneumoniae in the liver. Thus, it would be tempting to suggest that the C. pneumoniae infection due to a need of Fe overcomes the normal regulation of Fe homeostasis, resulting in an increasing concentration of Fe in infected organs. In the present study, DMT1 was observed to be localised in hepatocytes in the form of granular deposits but not in Kupffer cells in both controls and infected animals. This is in agreement with previous investigations which indicated that the role of DMT1 in hepatocytes is to transport transferrin-bound iron from recycling endosomes to the cytoplasm (Fleming et al., 1998). Interestingly, DMT1 expression was strongest in hepatocytes surrounding the central veins, and it may be possible that these cells comprise the main site for recruitment of transferrin-bound iron from the systemic circulation. There are indications that the uptake of Fe in macrophages such as Kupffer cells occurs via Nramp1 (Kuhn et al., 1999; Zwilling et al., 1999). It is thus possible that also Nramp1 expression was increased in the Kupffer cells in the livers of the C. pneumoniae-infected mice on day 8 as compared with controls and that this contributes to the increased accumulation of Fe in the liver in the present study. However, the antibodies used in the present study were directed to a highly conserved region of both the IRE and non-IRE forms of DMT1 (Nramp2) with little sequence homology to Nramp1 (Leong et al., 2003). Hence, we were not able to examine the localization or expression of Nramp1 in the present investigation.

In conclusion, C. pneumoniae infection increased liver hepcidin expression and decreased Fe in the serum and increased Fe in the liver. Moreover, with an increasing number of metabolically active and viable bacteria in the liver, DMT1 expression increased in the liver but was unaffected in the intestine. These results demonstrate that C. pneumoniae infection affects body Fe regulation. However, it remains to be shown if these changes are specifically caused by C. pneumoniae or if they are a consequence of infection-induced changes in host metabolism.

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