BACTERIAL PATHOGENICITY

Influence of iron restriction on Chlamydia pneumoniae and C. trachomatis

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Iron is an essential metabolite for pathogenic bacteria, and the specificity exhibited by bacteria for host-iron chelates may be correlated with host and tissue tropism. The effect of iron restriction on Chlamydia pneumoniae and C. trachomatis was studied by use of the iron-chelating compound deferoxamine. Growth of C. pneumoniae was inhibited much more than that of C. trachomatis and the effect of iron restriction largely depended on the cell line used for propagation. This might reflect differences in tissue tropism of the two chlamydial species. As iron levels are usually higher in men than in women, this might also be connected with the higher prevalence rate of C. pneumoniae antibodies in males, observed in all populations studied so far.

Introduction

Chlamydia pneumoniae is an important cause of respiratory tract infections [1], whereas another member of the same genus, C. trachomatis, is the most frequent cause of bacterial infections of the genital tract [2]. An association between C. pneumoniae and atherosclerosis has been demonstrated in a serological case control study [3] and confirmed by demonstration of the organism in atherosclerotic plaques by PCR, immunocytochemistry, electron microscopy and isolation of C. pneumoniae in cell culture [4]. However, negative results have also been reported [5] and the role of C. pneumoniae in the development or progression of atherosclerosis has yet to be determined.

World-wide, the prevalence of antibodies to C. pneumoniae in adults is high, reaching values of ≥50% [1, 6]. In all studies performed so far, men had a significantly higher sero-prevalence rate than women [1], a difference which has not yet been satisfactorily explained, but which fits well with data on atherosclerosis. Recent studies have noted an association between increased serum ferritin and dietary iron intake and acute myocardial infarction [7–9]. Iron is an essential metabolite for pathogenic bacteria, which have to compete within the host for iron (e.g., by the induction of virulence-related genes). Iron restriction can lead to the expression of specific outer-membrane protein receptors for iron binding. These proteins frequently induce a specific immune response in infected individuals. Furthermore, the specificity exhibited by bacteria for host-iron chelates may be correlated with host and tissue tropism [10].

The present study examined the influence of iron restriction by the intracellular iron-chelating compound deferoxamine (DFA) [11] on the propagation of C. pneumoniae and C. trachomatis in different cell lines and on the expression of different chlamydial proteins.

Materials and methods

The chlamydial strains C. pneumoniae (AR-39) and C. trachomatis (serovar E) from the Washington Research Foundation (WRF), Seattle, WA, USA were propagated in the following cell lines: HL cells [12] (human epithelial cells of unknown origin, WRF); HEP-2 cells (human laryngeal carcinoma cells) [13] (Flow Laboratories, Meckenheim, Germany); Buffalo Green monkey kidney (BGM) cells [14]; and baby hamster kidney (BHK) cells (Department of Virology, University of Freiburg) [15]. The cell lines were grown to confluent monolayers on coverslips in plastic vials for 24 h and were then infected with 100 μl of a suspension of the chlamydial strains containing 10^2–10^5 inclusion forming units (ifu)/ml as described previously [16]. Various concentrations of deferoxamine (DFA; Sigma Chemicals, Deisenhofen, Germany) were added to test for its influence on cell viability.

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Toxic effects occurred at DFA concentrations >500 μM but no direct toxic effects nor such effects on subculture of the cells were found for DFA concentrations up to 500 μM. Therefore, DFA was added at final concentrations of 0, 12, 25, 50, 100, 200 and 500 μM immediately after infection with the chlamydial strain. After centrifugation, supernates were replaced by 1 ml of CMGA medium (Eagle’s Minimal Essential Medium with fetal calf serum 10%, antibiotics and cycloheximide 1 μg/ml, containing DFA at the respective concentrations). The vials were incubated at 35°C in air with CO₂ 5% for 3–6 days for culture of C. pneumoniae and for 2–6 days for C. trachomatis. Infection was assessed by staining chlamydial inclusions in methanol-fixed host cells with a genus-specific, fluorescein-conjugated monoclonal antibody (MAB) (Pathfinder, Kallestad Pasteur, Freiburg, Germany). All experiments were performed in triplicate and ifu counts were calculated as the means of three titrations. Multivariate statistical analysis was performed on the results.

To confirm that the influence of DFA was due to iron restriction, cell culture was performed as described above, but cell monolayers were inoculated with chlamydial strains in the presence of DFA (0, 200 or 500 μM) and various concentrations of ferric chloride (FeCl₃; 0, 20 or 100 μM) or iron-saturated transferrin (0, 0.6 or 6 mg/ml; Sigma).

The influence of iron restriction on the expression of chlamydial proteins was studied after SDS-PAGE of chlamydial elementary bodies grown with and without DFA and then transferred to PVDF membranes as described previously [17]. Protein profiles were examined after Coomassie Blue staining, and immunoblotting were performed with an anti-p54 MAb, established in this laboratory against an isolated 54-kDa protein of C. pneumoniae [17], and with a polyclonal anti-C. pneumoniae antibody positive patient serum (micro-immunofluorescence (MIF) test IgG titre of 512 to C. pneumoniae) and a polyclonal anti-C. trachomatis antibody positive patient serum (MIF titre 128).

### Results

The influence of different DFA concentrations on the number of ifu is shown in Table 1. For C. pneumoniae, the number of ifu decreased significantly at DFA concentrations of 100 μM and higher when grown on HL or HEp-2 cells, whereas growth of this chlamydia species was not significantly decreased with DFA concentrations up to 200 μM on BHK cells and up to 250 μM on BGM cells. In addition to the influence on the number of ifu, the appearance of C. pneumoniae ifu was also different under iron restriction, leading to considerably smaller inclusions (Fig. 1a and b). For C. trachomatis, higher DFA concentrations were necessary to observe similar effects in HEp-2 and HL cells (200 μM for HEp-2, 250 μM for HL) and no significant decrease in number was seen in BGM and BHK cells with DFA concentrations up to 500 μM. When DFA and iron (FeCl₃ or iron-saturated transferrin) were added, the DFA effects were reversible, as shown for HL cells and FeCl₃ (Table 2). The effects of DFA on C. pneumoniae and C. trachomatis in the other cell lines were also completely reversed by the addition of FeCl₃ (data not shown).

In immunoblots of C. pneumoniae with polyclonal antisera, bands at 98, 76, 60, 54, 46, 43 and 39.5 kDa were detected under conditions of iron restriction (200 μM of DFA) and in controls. However, the distribution of bands of the anti-p54 MAB and of the polyclonal antisera with the 54-kDa band of C. pneumoniae was consid-

| Table 1. Influence of different concentrations of DFA on the number of ifu induced in four cell lines (BGM, BHK, HEp-2, HL) by C. pneumoniae AR-39 and C. trachomatis serovar E |
|---------------------------------|--------|--------|--------|--------|--------|
| Chlamydia species | Cell line | 0      | 50     | 100    | 200    | 250    |
| C. pneumoniae | BGM | 40.0 SD 6.0 | 41.3 SD 5.0 | 42.8 SD 2.2 | 38.5 SD 3.0 | 38.0 SD 2.9 | 31.6 SD 4.3 |
| C. trachomatis | BGM | 45.8 SD 10.0 | 48.0 SD 3.9 | 42.2 SD 7.6 | 44.9 SD 7.2 | 44.9 SD 6.9 | 40.6 SD 4.0 |
| C. pneumoniae | BHK | 45.1 SD 11.6 | 42.7 SD 8.6 | 41.5 SD 5.7 | 42.5 SD 5.6 | 34.2 SD 5.4 | 29.2 SD 4.0 |
| C. trachomatis | BHK | 31.8 SD 10.5 | 33.1 SD 4.5 | 34.9 SD 8.3 | 31.4 SD 8.6 | 28.5 SD 9.4 | 26.8 SD 5.6 |
| C. pneumoniae | HEp-2 | 49.9 SD 4.9 | 44.1 SD 2.9 | 31.8 SD 2.0 | 31.7 SD 5.7 | 27.9 SD 6.3 | 18.3 SD 3.7 |
| C. trachomatis | HEp-2 | 63.4 SD 3.5 | 59.5 SD 5.6 | 54.9 SD 3.9 | 33.5 SD 3.9 | 26.9 SD 3.1 | — |
| C. pneumoniae | HL | 61.3 SD 4.5 | 52.0 SD 11.7 | 46.7 SD 3.0 | 43.0 SD 4.7 | 39.1 SD 1.8 | 42.4 SD 5.4 |
| C. trachomatis | HL | 86.0 SD 8.3 | 88.1 SD 7.1 | 80.1 SD 9.1 | 69.9 SD 10.4 | 64.5 SD 8.5 | 48.5 SD 4.8 |

1Statistically significant differences (p<0.05; multivariate analysis).
2Mean number of ifu from three experiments, each run in triplicate; 3Compared to untreated control. SD, standard deviation.
Fig. 1. (a) Immunofluorescence of *C. pneumoniae* TW-183 propagated in HL cells in the absence of DFE (×1200). (b) Immunofluorescence of *C. pneumoniae* TW-183 propagated in HL cells in the presence of 250 μM DFE (×1200).

Table 2. Effect of DFE on *ifi* induced by *C. pneumoniae* AR-39 and *C. trachomatis* serovar E in HL cells is reversible by addition of FeCl₃

<table>
<thead>
<tr>
<th>Chlamydia species</th>
<th>FeCl₃ (μM)</th>
<th>0</th>
<th>100</th>
<th>200</th>
<th>500</th>
</tr>
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<tr>
<td><em>C. pneumoniae</em></td>
<td>0</td>
<td>41</td>
<td>31 (75.6)</td>
<td>29 (70.7)</td>
<td>27 (65.9)</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>26</td>
<td>33 (126.9)</td>
<td>38 (146.2)</td>
<td>35 (134.6)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>33</td>
<td>32 (97.0)</td>
<td>34 (103.0)</td>
<td>41 (124.2)</td>
</tr>
<tr>
<td><em>C. trachomatis</em></td>
<td>0</td>
<td>193</td>
<td>176 (91.2)</td>
<td>155 (80.3)</td>
<td>140 (72.5)</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>76</td>
<td>65 (85.5)</td>
<td>83 (109.2)</td>
<td>92 (121.0)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>63</td>
<td>92 (146.0)</td>
<td>85 (134.9)</td>
<td>100 (158.7)</td>
</tr>
</tbody>
</table>

¹Mean number of ifus from three experiments, each run in triplicate.
²Compared to control (without addition of DFE).
erably weaker in the presence of DFA than in the control (no iron restriction) or when the effect of DFA was reversed by the addition of FeCl₃ (Table 3). In immunoblots of C. trachomatis, a polyclonal anti-C. trachomatis serum reacted with bands at 60, 57, 45, 40, 38 and 29 kDa both in controls with no iron restriction and with C. trachomatis antigen propagated under conditions of iron restriction (200 μM DFA).

Discussion

Cell lines that are used frequently for culture of C. pneumoniae, i.e., HEP-2 and HL cells, were chosen to study the influence of iron restriction by DFA. For comparison, BGM and BHK cell lines were also studied. The effect of increasing concentrations of the iron-chelating compound deferoxamine (DFA) was markedly dependent on the chlamydial species and on the cell line used for propagation. In the case of two human epithelial cell lines used frequently for cultivation of C. pneumoniae (HL and HEP-2 cells), a decrease in the numbers of ifu of C. pneumoniae was observed at lower concentrations of DFA than seen with kidney cell lines (BGM, BHK), which are preferred for culture of C. trachomatis [14, 15].

For C. trachomatis, higher DFA concentrations were necessary to demonstrate similar effects, and with BGM cells, only a very small effect was observed with extremely high DFA concentrations (500 μM). This is in agreement with a recent study which reported no decrease in the number of ifu of C. trachomatis propagated in polarised human endometrial epithelial cells in the presence of desferal [10]. In the latter study, C. trachomatis inclusions were reported to be morphologically altered under conditions of iron restriction and a similar change was observed for C. pneumoniae in the present study. The DFA effects were reversible by the addition of iron, thus indicating that iron restriction, rather than other possible effects of the chelating compound DFA, was responsible for the effects. Deferoxamine has been shown to reduce the infectivity of a number of pathogens, e.g., Neisseria meningitidis [18], N. gonorrhoeae [19], Legionella pneumophila [20], Trypanosoma cruzi [21], Histoplasma capsulatum [22, 23] and Helicobacter pylori [24], whereas with Yersinia enterocolitica deferoxamine may enhance virulence by supplying iron to the pathogen and by causing immunosuppression of the host [25, 26]. There is little knowledge about the influence of iron restriction on the growth of chlamydia species. In a study published in 1991, no influence of DFA at concentrations up to 30 μM on the propagation of C. psittaci in macrophages activated by γ-interferon was reported. However, higher DFA concentrations could not be tested because of toxic effects on the macrophages [11]. In the present study, the lowest DFA concentration necessary for a significant decrease in ifu counts was 100 μM for C. pneumoniae grown on HEP-2 or HL cells. A study by Raulston [10] reported that the propagation of C. trachomatis serovar E on McCoy and HEC-1B cells was not significantly influenced by a DFA concentration of 100 μM, which is in agreement with the observations with C. trachomatis on all four cell lines tested in the present study. The latter could not detect iron-repressible proteins in C. trachomatis. However, the reaction of the anti-p54 MAb and of the patient serum with the 54-kDa band of C. pneumoniae was considerably weaker than in the control (no iron restriction) or when the effect of DFA was reversed by the addition of FeCl₃, indicating a possible influence of iron restriction on the expression of this protein. A similar effect was shown by Bereswill et al. [24] for a Pfr-protein of H. pylori, which showed a lower intensity in immunoblots under conditions of iron restriction by DFA.

In a paper published recently by Igietseme et al. [27], three potential mechanisms for the cytokine-induced restriction of C. trachomatis growth in transformed human epithelial cells were described. Nitric oxide induction and tryptophan catabolism contributed 20% and 30% of interferon (IFN)-γ-mediated restriction respectively, and iron restriction was the least effective. However, the combination of these three systems accounted for >60% of the inhibition observed, indicating that immune control of chlamydial growth in human epithelial cells may involve multiple mechanisms, including iron deprivation.

A study by Scidmore and Hackstadt with ⁵⁹Fe-loaded transferrin, demonstrated a low level of delivery of the iron isotope to the elementary bodies of C. trachomatis [28]. The genomes of both C. trachomatis and C. pneumoniae lack homologues for bacterial siderophores or siderophore receptors that bind and transport iron, suggesting that chlamydiae obtain iron from host cell pools [29]. The variations observed between the various cell lines in the present study might reflect differences in tissue tropism of the two chlamydial species, which could explain the different kinds of symptoms observed in patients infected with these chlamydia species. Furthermore, iron levels are usually higher in men than in

Table 3. Reactions in immunoblots with C. pneumoniae antigen propagated in HEP-2 cells with CMGA medium without DFA (CMGA), CMGA with 200 μM DFA (DFA), or CMGA with 200 μM DFA and 100 μM FeCl₃ (FeCl₃) with polyclonal anti C. pneumoniae antibody and with MAb anti-p54 (in parentheses)

<table>
<thead>
<tr>
<th>kDa band</th>
<th>CMGA</th>
<th>DFA</th>
<th>FeCl₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>98</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>76</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>60</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>54</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>46</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>39.5 (MOMP)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+, band present in immunoblot; ++, strong band present in immunoblot; MOMP, major outer-membrane protein.
women [30], and this might contribute to the higher prevalence of C. pneumoniae antibodies in adult males observed in all populations studied to date.

References


11. Murray HW, Granger AM, Testeltbaum RF. Gamma interferon-activated human macrophages and Toxoplasma gondii. Chla-


