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Presence of Chlamydophila pneumoniae DNA in blood cells is a frequent event in patients with the late stage of primary cutaneous lymphomas and with atopic dermatitis

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Abstract

Introduction: Microbial infection and associated super antigens have been implicated in the pathogenesis of cutaneous T-cell lymphoma (CTCL), and many patients die from complicating bacterial infections. It has been postulated that Chlamydophila pneumoniae (C. pneumoniae) infection may be involved in the pathogenesis of Mycosis fungoides (MF) but published data are limited and controversial.

Aim: To analyze the frequency of (C. pneumoniae) DNA presence in blood samples of lymphoma cases.

Material and methods: Using Q-PCR method we analyzed the presence of DNA in the blood samples obtained from 57 patients with CTCL (55 – mycosis fungoides (MF)/Sézary syndrome (SS), one primary cutaneous anaplastic large cell lymphoma (CD30+) and one NKT cell lymphoma) and 3 patients with cutaneous B-cell lymphomas, and 120 individuals from control groups (40 patients with psoriasis, 40 patients with atopic dermatitis and 40 healthy controls).

Results: Chlamydophila pneumoniae DNA was identified in 13 of 55 cases in the MF/SS group (23.6%), in 1 patient with CD30+ large cell lymphoma and in 1 of 3 patients with B-cell lymphomas, and 12 individuals from control groups (40 patients with psoriasis, 40 patients with atopic dermatitis and 40 healthy controls). Presence of C. pneumoniae DNA in MF patients was strongly associated with disease progression; \( r_s = 0.756; p = 0.0123 \) for groups IA \( \rightarrow \) IVB, and was noted more frequently in advanced (III + IV) stages than in early (I–II) stages \( (p = 0.0139) \). There are no differences in the mean age of MF/SS patients with and without infection.

Conclusions: The presence of C. pneumoniae DNA in the blood cells is a frequent event in late stages of MF/SS and may be explained by Th2 shift and suppression of the immune system during the course of the disease.

Key words: Chlamydophila pneumoniae, late mycosis fungoides/Sézary syndrome stages, polymerase chain reaction.

Introduction

Antigen stimulation by pathogens such as bacteria and viruses has been considered a possible predisposing factor to uncontrolled cell proliferation and development of lymphoid and other neoplastic tissue. Helicobacter pylori precedes the development of gastric B-cell lymphoma, mycoplasma-like organisms and hepatitis C virus have been suggested to be associated with Hodgkin disease and Chlamydia trachomatis infection may associate with rectal and cervical cancer. Although several etiologies have been postulated for mycosis fungoides (MF) and Sézary syndrome (SS), their causes remain unknown. Based on the limited experimental and clinical data, it has been speculated that chronic local antigen stimulation by Staphylococcus aureus as well as Chlamydia pneumoniae (C. pneumoniae) may play a role in the pathogenesis of cutaneous T-cell lymphoma (CTCL) [1–3]. Chlamydo-
Presence of Chlamyphila pneumoniae DNA in blood cells is a frequent event in patients with the late stage of primary cutaneous lymphomas and with atopic dermatitis.

**Material and methods**

**Study and control groups**

In this study 60 patients with skin lymphomas (48 from Poland – 30 men and 18 women, mean age: 60.7 ±13.5 and 12 from Finland – 8 men and 4 woman, mean age: 61.9 ±21.6) were included, of whom 57 patients were diagnosed with primary CTCL and 3 patients with primary cutaneous B-cell lymphomas (one follicle-center and two marginal zone lymphomas) according to WHO criteria. The control groups consisted of 40 patients with psoriasis (22 men and 18 women, mean age: 49.3 ±14.3), 40 with atopic dermatitis (21 men and 19 women, mean age: 13.8 ±7.7) and 40 healthy individuals (21 men, 19 women, 10 from Finland and 30 from Poland, mean age: 39 ±15.0). From the 57 CTCL patients, 55 have MF or SS, 1 patient had primary cutaneous anaplastic large cell lymphoma CD (30+), and one had NK/T cell lymphoma. From 55 MF/SS patients, 20 were in early clinical stages (IA–IIA) and 35 patients were in advanced stages (IIb–IVb). Additionally skin biopsies were obtained from 6 MF/SS patients.

**Material collection and DNA samples**

From all patients and control groups peripheral whole blood samples (EDTA-K2 (Medlab Products, Raszyn, Poland) were collected and stored at −80°C. Additionally from 6 examined patients skin biopsies were collected and stored at −80°C. DNA was extracted using Blood Mini A (A&A Biotechnology, Gdynia, Poland) and 5% of CO2 for 2 days, according to ATCC protocol. Further, DNA was precipitated using common sodium acetate-ethanol technique [1] in a final volume of 20 μl and stored at −20°C for further analyses. DNA concentration and purity were assessed by NanoDrop ND1000 (ThermoScientific, Wilmington, DE, USA).

Chlamyphila pneumoniae strain and cell lines of C. pneumoniae TW183 strain was purchased from American Type Culture Collection (ATCC) (Rockville, MD, USA) and stored at −80°C. In order to propagate C. pneumoniae for PCR optimization, HELA 229 line (ATCC) cells were infected by TW183 lysate in a 24-cell plate, followed by centrifugation (15 min, 400×G) and incubated at 37°C and 5% of CO2 for 2 days, according to ATCC protocol. After incubation, cells were harvested and stored at −80°C. Presence of C. pneumoniae in cells was further confirmed by diagnostic nested-PCR assay (BLIRT-DNA, Gdansk, Poland).

**Quantitative polymerase chain reaction analysis**

In order to create a specific quantitative assay for C. pneumoniae detection we checked (BLAST N database) with active disease and was not found after psoralen and ultraviolet therapy.
15 reference sequences of the *C. pneumoniae* strains and for diversification *C. trachomatis* strains and the ompA gene was chosen as a good molecular target for detection and quantification.

**Optimization and validation of QPCR assay**

We amplified 805 bp fragment of *C. pneumoniae* TW183 strain based on following primers: 5’CCGGCCCTACAATAAGCATTAC and 5’GAGCTTCTGCAGTAAGTGACCA. The sequence was confirmed by RFLP method. After purification, the PCR product was cloned into pUC19 plasmid, followed by propagation in *Escherichia coli* strain, isolation, purification and spectrophotometric quantification of plasmid. This construct was applied as a positive control for the calibration curve. The primers for QPCR assay were designed using VNTI software (Invitrogen, Life Technologies, Carlsbad, CA, USA): 5’AACAAAGTCTCGGACCATCAATTAC, 5’GGCTGAGCAATGCGGATGTTAC. The conditions of PCR reaction: 2× SybrGreen Supermix (Bio-Rad, Hercules, CA, USA), 170 nM of each primer, 4 mM MgCl₂ and ddH₂O were mixed with 2 μl of template DNA to a final volume of 17 μl. QPCR was performed in iCycler

Table 1. Frequency of *Chlamydia pneumoniae* DNA in blood samples from patients with various types of lymphomas, psoriasis, atopic dermatitis and healthy control groups

<table>
<thead>
<tr>
<th>Diagnosis/clinical stage</th>
<th>Frequency of <em>Chlamydia pneumoniae</em> DNA</th>
<th>I vs. II vs. III–IV stages</th>
<th>Early vs. late stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polish patients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 48)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Finnish patients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 12)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Both patient groups</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 60)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MF patients:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IA</td>
<td>0/2</td>
<td>0/3 (0%)</td>
<td>0/16 (0%)</td>
</tr>
<tr>
<td>IB</td>
<td>0/13</td>
<td>0/13 (0%)</td>
<td>12/35 (34.3%)</td>
</tr>
<tr>
<td>II A</td>
<td>1/3</td>
<td>1/4 (25%)</td>
<td>3/17 (17.6%)</td>
</tr>
<tr>
<td>II B</td>
<td>1/11</td>
<td>2/13 (15%)</td>
<td>6/11 (54.5%)</td>
</tr>
<tr>
<td>III</td>
<td>3/6</td>
<td>3/6 (50%)</td>
<td>1/3 (33%)</td>
</tr>
<tr>
<td>IVA</td>
<td>0/0</td>
<td>1/3*</td>
<td>4/10 (40%)</td>
</tr>
<tr>
<td>IVB</td>
<td>2/2</td>
<td>2/2 (100%)</td>
<td></td>
</tr>
<tr>
<td>MF summary</td>
<td></td>
<td>9/44 (20.5%)</td>
<td></td>
</tr>
<tr>
<td>SS patients:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IVA</td>
<td>0/1</td>
<td>0/1</td>
<td></td>
</tr>
<tr>
<td>IVB</td>
<td>4/5</td>
<td>4/5 (40%)</td>
<td></td>
</tr>
<tr>
<td>SS summary</td>
<td></td>
<td>4/11 (36.4%)</td>
<td>(MF vs. SS, NS, p = 0.266)</td>
</tr>
<tr>
<td>All MF/SS patients</td>
<td>11/43 (25.6%)</td>
<td>2/12 (16.7%)</td>
<td>13/55 (23.6%)</td>
</tr>
<tr>
<td>c-ALCL (CD30+)</td>
<td>1/1</td>
<td>0</td>
<td>1/1</td>
</tr>
<tr>
<td>NK/T lymphoma</td>
<td>0/1</td>
<td>0</td>
<td>0/1</td>
</tr>
<tr>
<td>All CTCL patients</td>
<td></td>
<td>14/57 (24.5%)</td>
<td></td>
</tr>
<tr>
<td>Primary cutaneous B-cell lymphomas</td>
<td>1/3</td>
<td>0</td>
<td>1/3 (33%)</td>
</tr>
<tr>
<td>All lymphomas</td>
<td></td>
<td>15/60 (25.0%)</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AD patients</td>
<td></td>
<td>5/40 (12.5%)</td>
<td></td>
</tr>
<tr>
<td>Psoriasis patients</td>
<td></td>
<td>1/40 (2.5%)</td>
<td></td>
</tr>
<tr>
<td>Healthy individuals</td>
<td></td>
<td>0/40 (0%)</td>
<td></td>
</tr>
</tbody>
</table>

Positive results obtained only from skin biopsy material. MF – mycosis fungoides, SS – Sezary syndrome, c-ALCL – cutaneous anaplastic large cell lymphoma, AD – atopic dermatitis.
and fluorescence data were automatically collected and analyzed by iCycler iQ Optical Software ver. 3.0a (Bio-Rad, Hercules, CA, USA). QPCR conditions were: initial denaturation – 95°C/3 min; 40× (95°C/20 s, 65°C/30 s, 72°C/20 s, 77°C/5 s) fluorescence reading step). A calibration curve was performed in triplicates using 6 ten-fold dilutions (10⁻⁴ to 10⁻¹) of pUC19 plasmid DNA containing ompA gene. The slope was = −3.497; efficiency = 93.2% with a correlation coefficient of R² = 0.997. Dynamic melt-curve analysis and agarose-gel electrophoresis were used for all post-PCR reaction tubes to confirm the size of expected amplicon (147 bp). All reactions containing analyzed DNA were performed in duplicates. If the ΔCt between replicates was > 0.3 and/or we found a melting peak with a different melting point > 1°C from the expected one, the reactions were repeated. As a method of quantification we chose the relative quantity method and for histograms we applied ΔCt method, where ΔCt = (mean Ct of analyzed group) – (mean Ct of calibration points).

**Statistical analysis**

All statistical analyses were done using the Statistica 8.0 (StatSoft Inc., Tulsa, OK, USA), Pearson and Fisher’s exact test. For all statistical tests, we used a comparison related significance level of \( p < 0.05 \).

**Results**

The presence of *C. pneumoniae* DNA was detected in 14 of 57 (24.5%) CTCL patients and in 1 of 3 patients with B-cell lymphomas (primary cutaneous follicle-center B cell lymphoma). In the CTCL group, positive results were found in 13 of 55 (23.6%) MF/SS patients and in one patient with CD30 (+) anaplastic large cell lymphoma. *Chlamydophila pneumoniae* was detected only in 1 of 6 analyzed skin biopsies taken from MF/SS patients (Table 1). From the controls, bacterial DNA was present in the blood samples in 1 of 40 (2.5%) psoriasis patients, in 5 of 40 (12.5%) patients with atopic dermatitis, and in none of 40 healthy individuals. No differences between MF and SS patients were observed (\( p = 0.266 \)). The frequency of *C. pneumoniae* DNA in the MF/SS group correlated with disease progression (\( r_s = 0.756, p = 0.0123 \), Figure 1). None of 16 samples from MF patients with disease (IA or IB) were positive while *C. pneumonia* was detected in 3 of 17 (17.6%) cases in stage IIA or IIB, and in 6 of 11 (54.5%) MF patients in stages III or IV of disease. In SS patients, *C. pneumoniae* DNA was found in 4 of 11 (36.4%) patients with stage IVB. Cumulatively in the MF/SS group, *C. pneumoniae* infection was found in 1 of 20 (5.0%) patients in early clinical stage (IA–IIA) and in 12 of 35 (34.3%) patients in advanced clinical stage (IIB–IVB). The results were statistically significant (\( p = 0.0139 \)). No differences in mean age between MF/SS patients with and without infection were observed (Figure 1).

**Discussion**

Cutaneous T-cell lymphoma is a malignancy of skin-homing Th1 and/or Th2 T cells. A prominent feature of CTCL is immunosuppression, which increases the risk of bacterial and viral infections in patients especially in the advanced stages of disease. The pathophysiology of this immunodeficiency is probably multifactorial. Data from experimental studies suggest that the T-cell repertoire in CTCL patients is significantly contracted [27]. Immunological abnormalities in CTCL are typically associated with depressed ability of peripheral blood cells to produce the Th1 cytokines, IFN-γ and IL-2 as a result of Th2 skewing [28–37]. During CTCL progression, reduced T-cell-mediated cellular immune responses and diminished natural killer cell activity also develop [38, 39]. Wysocka *et al.* have demonstrated a direct relationship between the extent of the pool of circulating malignant T-cells and an impaired immune response [40]. Also the functions of natural killer (NK) cells, including cellular cytotoxicity and production of IFN-γ, become increasingly impaired as the circulating tumor burden increases. The authors showed an inverse correlation between circulating clonal T cells and activation status of both NK cells and CD8 T cells with a diminishing expression of a number of the activation markers CD69 and CD25, as well as decreased intracellular IFN-γ production [40]. The following observations have two main pathophysiological implications. The impaired cellular immune response, which is pivotal for direct antitumor responses, leads to further acceleration of growth of the malignant T-cells population. Another consequence of the decline in cytotoxic T-cell and NK cell functions is impaired activity against opportunistic infectious pathogens. This theory has been supported by the clinical observations [41–43]. It is suggested that the Th2 cytokine pattern may create a permissive environment for *C. pneumoniae* infection. This concept is supported by few experimental studies.
and clinical observations [44]. Referring to our results, *C. pneumoniae* DNA was found in 25% of all patients with CTCL. The frequency of chlamydial infection correlated with the stage of disease. The positive results were seen in 5% and 34% of patients with low and high clinical stages of disease, respectively. This observation may confirm the important role of impaired cellular immunological response in the control of opportunistic infections such as *C. pneumoniae* in CTCL patients. The association between Th1 response impairment and *C. pneumoniae* infection may be also supported by an increased incidence of bacterial DNA in patients with atopic dermatitis (12.5%) (Th2 cytokines pattern) vs. psoriatic patients (2.5%) (Th1 cytokines pattern) and healthy controls (0%) (immunocompetent individuals). *Chlamydia pneumoniae* DNA was detected only in one skin biopsy in our study group, in accordance with the results of German and Italian investigators, who did not detect any bacterial DNA in skin biopsies from MF/SS patients, and confirm that *C. pneumoniae* infection could not be estimated as a primary triggering factor in the pathogenesis of CTCL, in spite of Casselli et al. who had described skin presence of *Chlamydia* spp. (and HHV8) in all recurrences of CD30+CTCL as well as in routine control blood samples [45–47].

**Conclusions**

Our results suggest that *C. pneumoniae* infection is not a primary event in the pathogenesis of CTCL, but may be estimated as a risk factor complicating advanced stages of the disease associated with Th2 shift and deficiency of antibacterial defence mechanisms.

**Acknowledgments**

The study was approved by the local research ethics committee at the Medical University of Gdansk. The study is financed by the Polish Ministry of Science and Higher Education, grant 02-0066/07/253.

**Conflict of interest**

The authors declare no conflict of interest.

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