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

Bogusław Nedoszytko[1], Piotr Wierzbicki[2], Leena Karenko[3], Agata Maciejewska-Radomska[4], Przemysław Stachewicz[2], Monika Zabłotna[1], Jolanta Gleń[1], Liisa Väkevä[3], Roman J. Nowicki[1], Małgorzata Sokołowska-Wojdyło[1]

[1]Department of Dermatology, Venereology and Allergology, Medical University of Gdansk, Gdansk, Poland
[2]Department of Histology, Medical University of Gdansk, Gdansk, Poland
[3]Department of Dermatology and Allergology, University of Helsinki and Helsinki University Central Hospital, Helsinki, Finland
[4]Individual Specialist Medical Practice, Gdynia, Poland

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 lymphoma. The presence of *C. pneumoniae* was confirmed in 1 of 40 psoriatic patients (2.5%), in 5 of 40 patients with atopic dermatitis (12.5%) and in none of 40 healthy individuals. Presence of *C. pneumoniae* DNA in MF patients was strongly associated with disease progression; $rs = 0.756$; $p = 0.0123$ for groups IA → 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 *Chlamydophila pneumoniae* (*C. pneumoniae*) may play a role in the pathogenesis of cutaneous T-cell lymphoma (CTCL) [1–3]. *Chlamydo-*

phila pneumoniae is a common intracellular micro-organism. Seroepidemiological studies indicate that *C. pneumoniae* infection is by far the most common human chlamydial infection in different cohorts, with seropositivity in at least 50% of the general population over the age of 20 [4–10]. But polymerase chain reaction (PCR) studies on asymptomatic healthy adults (more than 1000) had established only 1% of positivity in nasopharyngeal swabs' specimens [11]. In addition to pneumonia, pharyngitis, bronchitis and asthma *C. pneumonia* is also associated with arteriosclerosis, lung cancer, multiple sclerosis and Alzheimer's disease [12–18]. *Chlamydophila pneumoniae* can infect, reside and replicate in various cell types including smooth muscle cells, fibroblasts, endothelial cells, bronchial epithelial cells, keratinocytes as well as various immune cells such as macrophages, lymphocytes and natural killer cells (NK) [19, 20]. It induces the increased release of pro-inflammatory mediators including tumor necrosis factor α (TNF-α), interleukin 6 and 8 (IL-6, IL-8), basic fibroblast growth factor (bFGF) and up regulates adhesion molecules [21]. Recently it has been suggested that *C. pneumoniae* infection may also stimulate the IL-10 production which down regulates the expression of major histocompatibility complex class I (MHC-I), inhibits apoptosis and increases the longevity of the host cell, enhancing the survival of bacteria itself [22–24]. The role of *C. pneumoniae* in the etiology of CTCL is controversial. It has been suspected that a localized bacterial infection increases local production of inflammatory cytokines including interferon γ (IFN-γ) (critical in immunity and immunopathology of chlamydial infection) and CXCL-10, a cytokine chemo attractive for epidermotropic T lymphocytes [24]. Studies on the growth requirements of the abnormal T lymphocytes in MF/SS lead to the identification of a so-called Sézary cell activation factor (SAF) that stimulates the growth of both malignant and non-malignant T cells. Sézary cell activation factor was originally defined as an inducer of functional IL-2 receptors. It is postulated that a combination of SAF and IL-2 stimulates the propagation of oligoclonal T-cell populations from the peripheral blood mononuclear cells (PBMC) of patients with SS, with approximately one third of those cell clones containing the predominant malignant clone [3, 25]. Using a monoclonal antibody inhibitory for SAF activity Abrams *et al.* demonstrated that SAF is present in more than half skin biopsies taken from patients with MF [26]. It was also confirmed that SAF determinant is not of eukaryotic origin and is associated with *C. pneumoniae* bacteria [3]. Sézary cell activation factor is a protein of approximately 30 kDa, resembling the *C. pneumoniae* T cell activation factor originally described by Halme *et al.* [27]. Abrams *et al.* confirmed the presence of *C. pneumoniae* DNA and RNA in the skin by PCR and reverse transcription – PCR and by sequence analysis of the PCR products. The authors showed that *C. pneumoniae* antigen expression was associated

with active disease and was not found after psoralen and ultraviolet therapy.

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 or Mini AX Tissue (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's protocols. 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).

Chlamydophila 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 CO_2 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)

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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'CCGGCCTA-CAATAAGCATTTAC and 5'GAGCTTCTGCAGTAAGTGACCA. The sequence was confirmed by RFLP method. After purification, the PCR product was cloned into pUC19 plas-

mid, 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'AACAAAGTCTGCGAC-CATCAATTAC, 5'GGCTGAGCAATGCGGATGTTATCAC. The conditions of PCR reaction: 2× SybrGreen Supermix (Bio-Rad, Hercules, CA, USA), 170 nM of each primer, 4 mM $MgCl_2$ and ddH_2O were mixed with 2 µl of template DNA to a final volume of 17 µl. QPCR was performed in iCycler

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

Diagnosis/clinical stage	Frequency of *Chlamydophila pneumoniae* DNA			I vs. II vs. III–IV stages	Early vs. late stage
	Polish patients (*n* = 48)	Finnish patients (*n* = 12)	Both patient groups (*n* = 60)		
MF patients:					
IA	0/2	0/1	0/3 (0%)	0/16 (0%)	1/20 (5.0%)
IB	0/13	0/0	0/13 (0%)		
IIA	1/3	0/1	1/4 (25%)	3/17 (17.6%)	
IIB	1/11	1/2	2/13 (15%)		12/35 (34.3%) *p* = 0.0139
III	3/6	0/0	3/6 (50%)	6/11 (54.5%)	
IVA	0/0	1/3*	1/3 (33%)		
IVB	2/2	0/0	2/2 (100%)		
MF summary			9/44 (20.5%)		
SS patients:					
IVA	0/1	0/0	0/1		
IVB	4/5	0/5	4/10 (40%)		
SS summary			4/11 (36.4%) (MF vs. SS, NS, *p* = 0.266)		
All MF/SS patients	11/43 (25.6%)	2/12 (16.7%)	13/55 (23.6%)		
c-ALCL (CD30+)	1/1	0	1/1		
NK/T lymphoma	0/1	0	0/1		
All CTCL patients			14/57 (24.5%)		
Primary cutaneous B-cell lymphomas	1/3	0	1/3 (33%)		
All lymphomas			15/60 (25.0%)		
Controls					
AD patients					5/40 (12.5%)
Psoriasis patients					1/40 (2.5%)
Healthy individuals					0/40 (0%)

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^{+8} to 10^{+3}) of pUC19 plasmid DNA containing ompA gene. The slope was = –3.497; efficiency = 93.2% with a correlation coefficient of R^2 = 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 histographs 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 pneumonia* 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 *C. pneumoniae* DNA in the MF/SS group correlated with disease progression ($rs = 0.756$, $p = 0.0123$, 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).
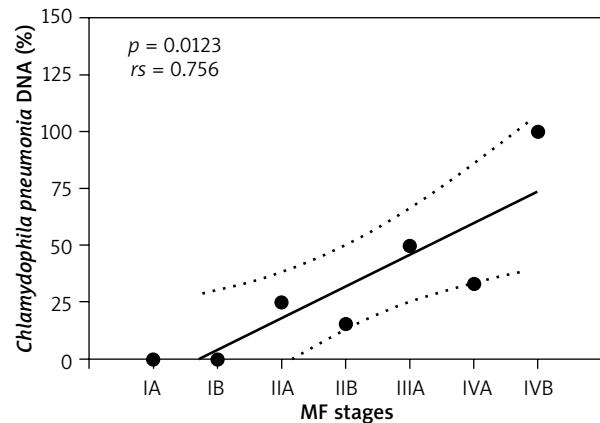

Figure 1. Frequency of *Chlamydophila pneumonia* in mycosis fungoides/Sezary syndrome patients with different clinical stage of disease

Discussion

Cutaneous T-cell lymphoma is a malignancy of skin-homing Th1 and/or Th_2 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

Bogusław Nedoszytko, Piotr Wierzbicki, Leena Karenko, Agata Maciejewska-Radomska, Przemysław Stachewicz, Monika Zabłotna, Jolanta Gleń, Liisa Väkevä, Roman J. Nowicki, Małgorzata Sokołowska-Wojdyło

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). *Chlamydophila 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

Conflict of interest

The authors declare no conflict of interest.

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