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The Prevalence of *Chlamydophila pneumoniae* in the Blood Samples of Patients with Primary Cutaneous Lymphomas

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Abstract

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

Aim of the study: The aim of the study was to analyze the frequency of *C. pneumoniae* DNA presence in blood samples of cutaneous T-cell and B-cell lymphomas (CTCL, CBCL) cases.

Material and Methods: Using Q-PCR method we analyzed the presence of *C. pneumoniae* DNA in the blood samples obtained from 57 patients with CTCL (55-MF/Sézary Syndrome (SS), 1-primary cutaneous anaplastic large cell lymphoma (CD30+) and 1-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: *C. pneumoniae* DNA was identified in 13 of 55 cases in MF / SS group (23, 6%), in one 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. The frequency of *C. pneumoniae* DNA occurrence in MF patients group was strongly associated with the progression of the disease; rs = 0.756; p=0.0123 for groups IA→IVB, also for MF + SS patients divided by stages, the presence of *C. pneumoniae* was noted more frequently in advanced (III + IV) stages than in early (I-II) stages (p=0.0139). There are no differences in the main age of MF/SS patients with and without infection.

Conclusion: Our results indicated that the presence of *C. pneumoniae* DNA in the blood cells is frequent event in late stages of MF/SS and may be explained by Th2 shift and suppression of immune system during the course of the disease.

Keywords: Mycosis fungicides; Cutaneous T-cell lymphoma; *Chlamydia pneumoniae*

Abbreviations: ATCC: American Type Culture Collection; bFGF: Basic Fibroblast Growth Factor; *C. pneumoniae*: *Chlamydophila pneumoniae*; *C. trachomatis*: *Chlamydia trachomatis*; CBCL: Cutaneous B-cell Lymphoma; CD: **Cluster** of Differentiation; CTCL: Cutaneous T-cell Lymphoma; CXCL-10: C-X-C Motif Chemokine Ligand 10; DNA: Deoxyribonucleic Acid; *E. coli*: *Escherichia coli*; EDTA: Ethylene-Diamineteraacetic Acid; HELA line: Henrietta Lacks Cell Line; IFN-γ: Interferon Gamma; IL- Interleukin; kDa: Kilodalton; MHC-I: Major Histocompatibility Complex Class I; MF: Mycosis fungoides; NK: Natural Killer; ompA: Outer Membrane Protein A; PBMC: Peripheral Blood Mononuclear Cells (PBMC) ; PCR: Polymerase Chain Reaction; pUC19: Plasmid Cloning Vector from University of California 19; RFLP:

Restriction Fragments Length Polymorphism; RNA: Ribonucleic Acid; SAF: Sézary Cell Activation Factor; SS: Sezary Syndrome; Th: T helper; TNFa: Tumor Necrosis Factor Alpha

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Introduction

Antigen stimulation by pathogens such as bacteria's and viruses has been considered as possible predisposing factor to uncontrolled cell proliferation and development of lymphoid and other tissue neoplastic. 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's disease and *Chlamydia trachomatis* infection may associate with rectal and cervical cancer. Although several an etiologies have been postulated for Mycosis fungicides (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] *C. pneumoniae* is a common intracellular microorganism. 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 age 20 [4-10]. But 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] *C. pneumoniae* can infect, reside and replicate in various cells types including smooth muscle cells, fibroblasts, endothelial cells, bronchial epithelial cells, keratinocytes as well as various immune cells such as macrophages, lymphocytes and natural killers cells (NK) [19,20] It induces the increased release of pro-inflammatory mediators including tumor necrosis factor alpha (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 [22,23]. The role of *C. pneumoniae* in the aetiology of CTCL is controversial. It has been suspected that a localized bacterial infection increases local production of inflammatory cytokines including interferon gamma (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. SAF was originally defined as an inducer of functional interleukin-2 receptors. It is postulated that 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 [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 It was also confirmed that SAF determinant is not of eukaryotic origin and is associated with *C. pneumoniae* bacteria SAF is a protein of approximately 30 kDa, resembling the *C. pneumoniae* T cell activation factor originally described by Halme et al. [26] 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 a therapy.

Materials and Methods

Study and control groups

In this study 60 patients with skin lymphomas (48 from Poland-30 man and 18 women, main age 60.7 ± 13.5 and 12 from Finland-8 man and 4 woman, main age 61.9 ± 21.6) were included, of whom 57 patients were diagnosed with primary cutaneous T-cell lymphomas (CTCL) and 3 patients with B-cell lymphoma according to WHO criteria. The control groups consisted of 40 patients with psoriasis (22 man and 18 women, main age 49.3 ± 14.3), 40 with atopic dermatitis (21 man and 19 women, main age 13.8 ± 7.7) and 40 healthy individuals (21 men, 19 women, 10 from Finland and 30 from Poland, main age 39 ± 15, 0). From the 57 CTCL patients 55 have Mycosis fungoides or Sézary syndrome, 1 patient had primary cutaneous anaplastic large cell lymphoma CD (30+), and 1 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 from 6 MF/SS patients skin biopsies were obtained.

Material collection and DNA samples

From all patients and control groups a peripheral whole blood samples (EDTA-K2 (Medlab Products, Raszyn, Poland) was collected and stored at -80°C. Additionally from 6 examined patients skin biopsies was collected and stored at -80°C. DNA was extracted using Blood Mini or Mini AX Tissue (A&A Biotechnology, Gdynia, Poland) (A&A Biotechnology, Gdynia, Poland) according to manufacturer's protocols. Further, DNA was precipitated using common sodium acetate-ethanol technique in a final volume of 20 µl and stored at -20°C for further analyses. DNA concentration and purity was assessed by NanoDrop ND1000 (ThermoScientific, Wilmington, DE, USA).

C. pneumoniae strain and cell lines *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 24-cell plate, followed by centrifugation (15 min, 400 x 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 Gdańsk, Poland).

Quantitative PCR Analysis

In order to create a specific quantitative assay for *C. pneumoniae* detection we checked (BLAST N database) 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.

Optimisation and validation of QPCR assay

We amplified 805 bp fragment of *C. pneumoniae* TW183 strain based on following primers: 5'CCGGCCTACAATAAGCATTTAC and 5'GAGCTTCTGCAGTAAGTGACCA. Sequence was confirmed by RFLP method. After purification, the PCR product was cloned into pUC19 plasmid, followed by propagation in *E. coli* strain, isolation, purification and spectrophotometric quantification of plasmid. This construct was applied as a positive control for calibration curve. The primers for QPCR assay were designed using VNTI software (Invitrogen, Life Technologies, Carlsbad, CA, USA): 5'AACAAAGTCTGCGACCATCAATTAC, 5'GGCTGAGCAATGCGGATGTTATCAC. The conditions of PCR reaction: 2 x 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 and fluorescence data were automatically collected and analyzed by iCycler iQ Optical Sofware ver. 3.0a (Bio-Rad, Hercules, CA, USA). QPCR conditions were: initial denaturation - 95°C/3 min; 40x (95°C/20s, 65°C/30s, 72°C/20s, 77°C /5 s-fluorescence reading step). A calibration curve was performed in triplicates using 6 ten-fold dilutions (10E+8 to 10E+3) of pUC19 plasmid DNA containing ompA gene. The slope was = -3,497; efficiency = 93.2% with a correlation coefficient 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 melting peak with a different melting point >1°C from expected, the reactions were repeated. As a method of quantification we chose relative quantity method and for histographs we applied ΔCt method, where ΔCt= (mean Ct of analyzed group)-(mean Ct of calibration points).

Statistical analyses

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. 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. C. 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 was observed (p = 0.266). The frequency *C. pneumoniae* DNA in MF/SS group correlated with disease progression (rs=0.756, p=0.0123). 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 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

CTCL is a malignancy of skin-homing Th_2 T cells; however the reason for Th_2 bias remains unclear. 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 Th_1 cytokines, interferon gamma and IL-2 as a result of Th_2 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 extend of the pool of circulating malignant T-cells and an impaired immune response [40]. Also the functions of natural killer (NK)

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

Diagnose/Clinical stage	Frequency of Chlamydophila pneumoniae DNA				
	Polish patients (N=48)	Finnish patients (N=12)	Both patients groups (N=60)		
MF patients					
IA	0/2	0/1	0/3 (0%)	0/16 (0%)	1/20 (5%)
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%)		
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					12/35 (34.3%) p=0,0139
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%)		
B-cell lymphoma	1/3	0	1/3 (33%)		
All lymphomas			15/60 (25%)		
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; cALCL: Cutaneous Anaplastic Large Cell Lymphoma; AD: Atopic Dermatitis.

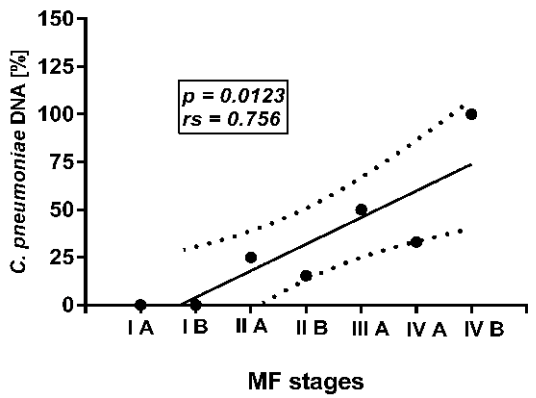

Figure 1 Frequency of Chlamydophila pneumonia in mycosis fungoides/Sezary syndrome patients with different clinical stage of disease.

cells, including cellular cytotoxicity and production of interferon 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 number the activation markers CD69 and CD25, as well as decreased intracellular IFN-γ production. 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 Th_2 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 stated in 5% and 34% of patients in 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 Th_1 response impairment and *C. pneumoniae* infection may be also supported by increased incidence of bacterial DNA in patients with atopic dermatitis (12.5%) (Th_2 cytokines pattern) vs. psoriatic patients (2.5%) (Th_1 cytokines pattern) and healthy controls (0%) (immunocompetent individuals). *C. pneumoniae* DNA was detected only in one skin biopsy in our study group, in accordance with the results of German and Italian investigators, who not detected bacterial in the skin biopsies from MF/SS patients, and confirm that *C. pneumoniae* infection could not be

estimated as a primary event in the pathogenesis of CTCL, in spite of Casselli et al. case describing skin presence of Chlamydia spp (and HHV8) in all recurrences of CD30+CTCL as well as in routine control blood samples [45-47].

In the summary, 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.

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