High prevalence of *Chlamydophila psittaci* subclinical infection in Italian patients with Sjögren's syndrome and parotid gland marginal zone B-cell lymphoma of MALT-type

M. Fabris[1-3], R. Dolcetti[4], E. Pasini[4], L. Quartuccio[1,3], E. Pontarini[1,5], S. Salvin[1,3], M. Ponzoni[6], C.A. Scott[7], S. De Vita[1,3]

[1]Clinic of Rheumatology and [2]Institute of Clinical Pathology, University Hospital of Udine, and [3]Department of Biological and Medical Sciences, University of Udine, Udine, Italy; [4]Cancer Bio-Immunotherapy Unit, Department of Medical Oncology, Centro di Riferimento Oncologico, IRCCS - National Cancer Institute, Aviano (PN), Italy; [5]Unit of Clinical and Experimental Immunology, Istituto Clinico Humanitas, Rozzano (Milan), Italy; [6]Pathology and Unit of Lymphoid Malignancies, Department of Oncology, San Raffaele Scientific Institute, Milan, Italy; [7]Institute of Pathology, University of Udine, Udine, Italy.

Abstract

Objective

To assess Chlamydophila psittaci (Cp) subclinical infection in patients with Sjögren's syndrome (SS).

Methods

Seventy-four SS patients (55.4 ±13.4 yrs; 94.6% females) were studied. Among them, 18 had salivary gland mucosa-associated lymphoid tissue (MALT) B-cell lymphoma, 20 myoepithelial sialoadenitis (MESA), and 36 no lymphoproliferative disorders (LPD). The presence of Cp DNA was assessed in peripheral blood of all patients by specific PCR protocols. Paired salivary gland samples were also investigated whenever available (34 cases), including lymphomatous and non-lymphomatous samples, as well as major and minor salivary gland tissues. As controls, 225 blood donors were analysed in the peripheral blood.

Results

Overall, Cp DNA was detected in 11/74 (14.9%) SS patients vs. 1/225 (0.4%) controls ($p<0.0001$). Cp was detected at higher frequency in MALT lymphoma patients (6/18, 33.3%), as compared with MESA (3/20, 15%) or patients without LPD (2/36, 5.6%), (MALT lymphomas vs. others: $p=0.02$). A similar Cp prevalence was observed in blood vs. salivary gland tissues, however with a higher frequency in the major than in the minor salivary glands (5/18, 27.8%, vs. 1/17, 5.9%, $p=0.18$). Cp-positive patients were all rheumatoid factor positive (11/11, 100% vs. 40/63, 63.5% Cp-negative; $p=0.014$), while no difference was noticed for anti-SSA/SSB positivity.

Conclusion

In the light of accepted models of MALT B-cell lymphomagenesis and considering previous data implicating Cp infection in ocular adnexa MALT lymphoma, our results suggest that Cp infection could be involved also in a fraction of patients with SS developing lymphoma. The potential therapeutic implications of these findings appear worthwhile.

Key words

Sjögren's syndrome, MALT lymphoma, infection, Chlamydophila psittaci, lymphomagenesis

*Martina Fabris, MD**
*Riccardo Dolcetti, MD**
Elisa Pasini, BS
Luca Quartuccio, MD, PhD
Elena Pontarini, BS
Sara Salvin, MD
Maurilio Ponzoni, MD
Cathryn Anne Scott, MD, PhD
Salvatore De Vita, MD, Prof.

**These authors made an equal contribution to this paper.*

Please address correspondence to:
Martina Fabris, MD,
Institute of Clinical Pathology and Clinic of Rheumatology,
University Hospital of Udine,
Piazzale Santa Maria Misericordia 3,
3100 Udine, Italy.
E-mail: fabris.martina@aoud.sanita.fvg.it

Received on April 22, 2013; accepted in revised form on July 1, 2013.

Funding: this study was supported by grants from the Associazione Italiana per la Ricerca sul Cancro (to RD, contract 10301) and by Regione Friuli Venezia Giulia, Italy (LR26.05.07/art.23). Elisa Pasini is a FIRC fellow.

Competing interests: none declared.

Introduction

Sjögren's syndrome (SS) is a chronic autoimmune disease of unknown etiology that affects mostly middle-aged women and is characterised by lymphocytic infiltration and proliferation of lachrymal and salivary glands causing mucous dryness (sicca symptoms) (1). In addition to glandular manifestations, some SS patients display extra-glandular or systemic clinical features such as arthritis, fatigue, cutaneous vasculitis, cryoglubulinaemia, and lymphoma, mostly salivary gland marginal zone B-cell lymphoma of the mucosa-associated lymphoid tissue (MALT)-type, with a 5% incidence of the overall SS patient population (2-4). Lymphocytes are central to the pathophysiology of SS, which is associated with variable degree of lymphocytic infiltration of the affected organs and the accumulation of B cells in exocrine glands under the form of benign aggregates (3). In line with this, most B-cell lymphomas in SS are indolent forms, which may over time transform and progress into aggressive life threatening malignancies, such as diffuse large B-cell lymphomas (4-6). Viral and bacterial infections inducing chronic site-specific inflammation have been significantly associated with MALT lymphomas arising in extra-nodal sites, such as *H pylori, C jejuni*, *B burgdorferi* and Hepatitis C virus (7).

Recently, the association between *Chlamydophila psittaci* (Cp) subclinical infection and marginal zone B-cell lymphoma of the ocular adnexa (OAML) has been disclosed and additionally supported by several studies (8-11). Interestingly, a very recent report suggested an association between Cp infection and both MALT lymphoma of the salivary glands and autoimmune precursor lesions (12).

In the present study, we evaluated the prevalence of Cp infection in a large cohort of Italian patients with SS, some of whom had SS-associated MALT lymphoma.

Material and methods

Patients

Seventy-four Italian SS patients diagnosed between 1999 and 2012 (mean age 55.4±13.4 yrs; mean disease duration 6.3±4.3; 94.6% females; 77% anti-SSA-positive, 63.5% SSB-positive, 68.9% rheumatoid factor positive) were enrolled in the study. All patients fulfilled the European-American classification criteria for SS (13). Within such cohort, patients with either salivary gland B-cell MALT NHL (n=18), or parotid myoepithelial sialoadenitis (MESA), as shown by tissue biopsy (n=20), or no lymphoproliferative disorder (LPD) associated with SS (n=36), were identified (14). Twenty-two patients had type II or type III serum cryoglobulins, all showing a cryoglobulinaemic syndrome: 10 of these patients presented MALT NHL and 8 were associated with MESA. All patients were negative for HCV and HBV chronic infection.

The presence of Cp DNA was assessed in the peripheral blood mononuclear cells (PBMC) of all patients. Furthermore, salivary gland tissue samples were also investigated whenever available, and included tissue biopsies of the major salivary glands from 17 patients, tissue biopsies of minor salivary glands from other 16 patients, and both the parotid biopsy and minor salivary glands biopsies from one patient. A written informed consent, according to the Declaration of Helsinki 1975/83, was obtained for all the enrolled subjects and the study was approved by the Institutional Review Board. Two hundred and twenty-five (127 males, 98 females; mean age 44.4±7, range 18–70 yrs) blood donors resident in the north of Italy were considered as controls, as previously published (15), since this series comprise enough females to cover adequately also the present cohort of SS, even if males were predominant.

Detection of Cp DNA by PCR analysis

The detection of Cp was done by previously described methods (16). In particular, the DNA was extracted from PBMC in all patients using an automated system (Maxwell 16, Promega), and from biopsies, both fresh-frozen and formalin-fixed, paraffin-embedded tissues, using the QIAamp DNA Mini Kit (Qiagen). The presence of Cp

DNA was analysed by 3 PCR protocols targeting different regions of bacterial genome, including 16S-23S spacer rRNA, OMP-A porin, and Gro-EL chaperonin (hsp-60) (17-19). A multiplex touchdown, enzyme time-release polymerase chain reaction (PCR) assay, designed to simultaneously detect *Chlamydia trachomatis* (Ctra), *Chlamydophila pneumoniae* (Cpneu) and Cp DNA at bacterial loads lower than one infection-forming unit was performed according to a previously published protocol with few modifications. Cp DNA was detected in single PCR assays using different primers: PSIF 5'CGT TGA CTC AAC CTG CAA AG 3'and PSIR 5'CAA CCT AGT CAA ACC GTC CT 3'. The amplified DNA was from the end of the 16S rRNA gene and the beginning of the 16S-23S spacer region in the ribosomal genes; the primer pairs useful specifically for Ctra and Cpneu were entirely comprised within the 16S rRNA gene; for Cp, one primer was located in the 16S rRNA gene and the second primer was located in the 16S-23S spacer region. The omp-A nested PCR and Gro El was done according to published protocols (10, 11, 16). Blank reactions filled with 50 μL of PCR mixture was interspersed every ten samples to monitor possible contamination of PCR reagents by Chlamydia DNA and to rule out any false-positive results. Each assay was performed at least 3 times and the risk of cross contamination was extremely low because the DNA was extracted in Udine and the assays were performed in Aviano by three different technicians in different times. All mix reactions were prepared in a room, while thermal cyclers employed in experiments were located in another laboratory, to avoid cross contamination by amplicons. All employed tips had filter to limit aerosol DNA contamination.

Finally, amplicons were sequenced to confirm the specificity of PCR products. Amplification of the β-globin gene was carried out as a quality control of DNA to avoid any false-negative results.

PCR products were analysed by electrophoresis, and DNA fragment size was quantified by image analysis. The specificity of PCR products was confirmed by sequencing. A careful check of quality and amount of DNA template and constant monitoring of PCR protocol sensitivity were crucial to achieve reliable findings. Reported results correspond to TETR-PCR protocol, the recommended protocol for Cp detection (17), and were confirmed in cases with available material by the other two PCR protocols with a high concordance, consistently with our previous work (15).

Statistical analysis

Statistical analysis was performed using the Instat software (San Diego, CA, USA). The difference between groups in the prevalence of Cp infection were analysed by 2x2 contingency tables using the Fisher's exact test with Woolf's approximation. Odds ratios (OR) were calculated with 95% confidence interval (CI). *P*-values were considered significant if <0.05.

Table I. Prevalence of Cp infection in patients with different salivary gland lymphocytic infiltration, when analysed in PBMC or salivary gland biopsies (SGB).

	Cp in PBMC (total=74 cases)	Cp in SGB (total=34 cases)
Overall	13.5% (10/74)	14.7% (5/34)
NHL of MALT	27.8% (5/18)	36.4% (4/11)
MESA	15% (3/20)	9.1% (1/11)
SS without LPD	5.6% (2/36)	0% (0/12)

Table II. Cp detection in patients where both PBMC and salivary gland biopsies (SGB) were investigated.

Tissue analysis on 34 samples	Cp positive PBMC & SGB	Cp positive only in PBMC	Cp positive only in SGB	Cp negative both PBMC & SGB
Total	11.8% (4/34)	5.9% (2/34)	2.9% (1/34)	79.4% (27/34)
NHL of MALT (n=11)	3	1	1	6
MESA (n=11)	1	1	0	9
SS without LPD (n=12)	0	0	0	12

Results

Overall, Cp DNA was detected in 11/74 (14.9%) SS patients. Considering only the PBMC samples, the prevalence of Cp-positive cases was very significantly higher than in controls (10/74, 13.5% SS patients *vs*. 1/225 healthy controls; OR 35, 95%CI: 4.4–278.7; $p<0.0001$). The overall prevalence of Cp DNA was significantly higher in SS patients with MALT lymphoma (6/18, 33.3%) compared to those without lymphoma (5/56, 8.9%; OR 5.1, 95%CI: 1.3–19.6; $p=0.02$). Patients with MESA showed a higher Cp-prevalence (3/20, 15%) as compared to SS patients without LPD (2/36, 5.6%; $p=0.34$), and they did not differed significantly neither as compared to lymphoma patients ($p=0.26$). The greatest statistical significance was observed comparing Cp prevalence in MALT lymphoma *versus* patients without LPD (OR 8.5, 95%CI: 1.5–47.9; $p=0.01$). Similar results were observed when analysing separately PBMC and salivary tissue samples (Table I).

With particular regard to salivary gland tissues, Cp DNA was detected with higher frequency in the major (5/18, 27.8%, of which 4 parotids and 1 submandibular glands) than in the minor salivary glands (1/17, 5.9% and this case was positive also in the parotid; $p=0.18$).

When focusing on the 34 patients studied both in PBMC and in salivary glands (Table II), Cp positive cases were 7 in total, but Cp was detected in both sites in 4/7 (57.1%), only in PBMC in 2/7 (28.6%), and only in the salivary gland biopsy in one patient (Table II).

Of note, rheumatoid factor was positive in all Cp positive cases (11/11, 100%), *i.e*. significantly more frequently than in Cp-negative patients (40/63, 63.5%), (OR 13.4, 95%CI: 0.8–237.1;

p=0.014). By contrast, the presence of anti-SSA/SSB autoantibodies did not differ significantly between the two groups (63.6% in Cp-positive *vs*. 79.4% in Cp-negative; *p*=0.26).

Cp-positive did not differ significantly from Cp-negative patients with regard to sex, age, SS time from diagnosis, follow-up and previous therapies (data not shown). We retrospectively searched for any possible occupational exposure. Only in one case (a man with MALT lymphoma) we may account for this; the patient was an employer in a phone company, involved in the maintenance of telephone lines: in particular, during his work, he used frequently check lines stations contaminated by bird droppings.

Finally, Cpneu DNA was detected in 3/74 (4.1%) of patients, 2/3 were also Cp positive. All Cpneu positive DNA were extracted from PBMC, no salivary glands resulted Cpneu positive. No Cpneu DNA positive samples were found in controls. Finally, all patients and controls were negative for CTra DNA in all the study samples.

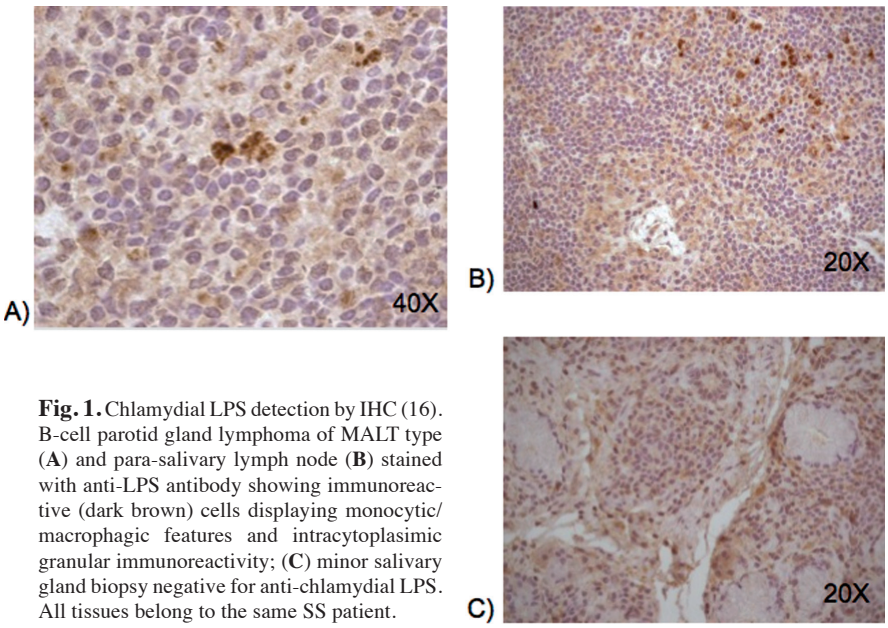

Fig. 1. Chlamydial LPS detection by IHC (16). B-cell parotid gland lymphoma of MALT type (**A**) and para-salivary lymph node (**B**) stained with anti-LPS antibody showing immunoreactive (dark brown) cells displaying monocytic/macrophagic features and intracytoplasimic granular immunoreactivity; (**C**) minor salivary gland biopsy negative for anti-chlamydial LPS. All tissues belong to the same SS patient.

Discussion

In the present study, performed in a series of Italian SS patients of Caucasian origin, a possible link between Cp infection and SS was discovered, with a stronger association in patients with lymphoma. Cp infection was demonstrated in one third of SS cases with salivary gland MALT lymphoma versus about 5% of SS without an associated LPD. When analysing prelymphomatous stages (MESA), Cp infection was found in 15% of cases. Cp infection has been consistently linked to MALT lymphomas of the ocular adnexa (8-11). Recently, one paper investigated the association of Cp infection with non gastro-intestinal extranodal MALT lymphomas and precursor lesions, including also a small series of tissue samples from SS patients, however detailed information about diagnosis and classification was lacking (12). Compared to our results, the prevalence of Cp infection was higher in SS with precursor lesions and lower in salivary gland MALT lymphomas.

The definition of the prelymphomatous stages of B cell lymphoproliferation in SS represent a complex issue, where the integration of clinical, pathologic, and molecular data is needed (20). In the present series, molecular studies showed a clonal B cell proliferation in two out of three Cp-positive patients with MESA (data not shown), suggesting a significant association between SS-associated atypical lymphoproliferation and Cp infection.

The best-characterised infection model for lymphomagenesis involving sustained stimulation of the immune system linked to malignant transformation is the H. pylori-associated MALT lymphoma (21). Infection may contribute to lymphomagenesis by promoting favourable conditions for lymphocyte transformation, such an increased proliferation or decreased apoptosis of lymphoid cells.

Another model of infection-related autoimmunity and lymphoproliferation is represented by HCV-related cryoglobulinemia, also showing an increased frequency of MALT involvement (22). According to the H. pylori and HCV models, the pathogen would represent a chronic source of antigens, causing a sustained local inflammation, giving rise to lymphoid clones that still remain dependent upon the stimulation by the infectious agent (23). The present evidence of a significant association between Cp infection and SS-related lymphomas may be therefore relevant to design a new therapeutic option to prevent a frank lymphoma development at least in a subgroup of SS patients.

An additional important issue from this study, is that all the Cp-positive SS-patients were positive for the RF, but not for anti-SSA/SSB autoantibodies. This result appears in line with our recently published data showing a link between RF and lymphoma in a large series of SS patients in a multicentre study (24). Experimental data highlighting why one definite infection may predispose to the preferential expansion of RF-positive B-cell clones has been produced for what concerns HCV infection: in an animal model, the immune response developed against viral antigens may also be autoreactive, with RF specificity. In this model, a biologic link was therefore demonstrated between infection, autoimmunity, and lymphoproliferation (23).

There are some limitations in this study, including the number of cases investigated, the mono-centric nature, and the fact that Cp infection was searched both in PBMC and in MALT affected tissue only in a fraction of cases. With particular regard to the last issue, it is interesting to note that in ocular lymphomas a possible discrepancy in Cp positivity in PBMC *versus* tissue samples from the same patient was also observed (10, 16).

Cp is the causative agent of psittacosis and represents the most important animal chlamydiosis of zoonotic character (25), but recent surveys showed that Cp can be found in non-avian domestic animals and wild animals as well (26, 27). Since the persistence or the load of the pathogen may differ in different tissues, also after antibiotic treatment, the requirement of larger studies where different tissues are investigated needs to be stressed. Of note, Cp infection prevalence, as detected by currently available techniques, may differ in major or minor salivary glands, a higher prevalence being detected in the latter in this study.

In one Cp-positive SS patient with MALT lymphoma, minor salivary glands and iuxta-parotideal and jugular lymph nodes resulted Cp-negative by PCR, while the parotid lymphomatous tissue and the PBMC were strongly Cp-positive (data not shown). However, when investigated by immunehistochemistry (IHC), not only the parotid lymphomatous tissue but also the lymph nodes were strongly positive for chlamydial lypopolysaccarides (LPS), while the minor salivary glands were again negative (Fig. 1). This case highlights the relevance of the site and the technical approach employed to investigate Cp infection. Both the PBMC and multiple tissues samples should be studied with a panel of different laboratory approaches, in our opinion (molecular analysis, IHC, serology). Previous antibiotic treatments given for different purposes should be also taken into account, if possible, since they may also affect the results.

Conclusion

Despite the discussed limitations, the present results confirm a possible role of Cp infection at least in a subgroup of SS-related lymphomagenesis of MALT, in line with current models of MALT lymphomagenesis (21-23) and consistent findings on Cp infection in ocular adnexa MALT B-cell lymphomas (8-11). Future controlled clinical trials focusing on Cp investigation and eradication in SS appear now worthwhile.

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