Evidence for an Association Between *Chlamydia psittaci* and Ocular Adnexal Lymphomas

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Background: **Ocular adnexal lymphomas may be antigen-driven disorders; however, the source of the putative antigen or antigens is still unknown. Hence, we assessed whether Chlamydiae infection is associated with the development of ocular adnexal lymphomas.** ***Methods:*** **The presence of *Chlamydia psittaci, trachomatis,* and *pneumoniae* DNA was investigated by polymerase chain reaction in 40 ocular adnexal lymphoma samples, 20 nonneoplastic orbital biopsies, 26 reactive lymphadenopathy samples, and peripheral blood mononuclear cells (PBMCs) from 21 lymphoma patients and 38 healthy individuals. Seven patients with chlamydia-positive PBMCs were treated with the antibiotic doxycycline, and objective response was assessed in four patients with measurable lymphoma lesions. Differences in Chlamydiae DNA detection between the case patients and the control subjects were analyzed using the Fisher exact test. All statistical tests were two-sided.** ***Results:*** **Thirty-two of the 40 (80%) ocular adnexal lymphoma samples carried *C. psittaci* DNA, whereas all lymphoma samples were negative for *C. trachomatis* and *C. pneumoniae*. In contrast, none of the 20 nonneoplastic orbital biopsies (0% versus 80%; $P<.001$) and only three of 26 (12%) reactive lymphadenopathy samples (12% versus 80%; $P<.001$) carried the *C. psittaci* DNA. Nine of 21 (43%) patients with chlamydia-positive lymphomas carried *C. psittaci* DNA in their PBMCs, whereas none (0%) of the healthy PBMC donors carried *C. psittaci* DNA in their PBMCs (43% versus 0%; $P<.001$). One month after doxycycline treatment, chlamydial DNA was no longer detectable in the PBMCs of all seven treated patients, and objective response was observed in two of the four evaluable patients.** ***Conclusion:*** **Patients with ocular adnexal lymphoma had a high prevalence of *C. psittaci* infection in both tumor tissue and PBMCs. Persistent *C. psittaci* infection may contribute to the development of these lymphomas, as was also supported by the clinical responses observed in this study with *C. psittaci*–eradicating antibiotic therapy. [J Natl Cancer Inst 2004;96:586–94]**

Several infectious agents have been proposed as risk factors for the development of non-Hodgkin's lymphomas *(1),* and some of these agents have been considered as targets for new therapeutic strategies *(2).* Chlamydiae, which are obligate intracellular bacteria growing in eukaryotic cells, are responsible for a wide spectrum of human diseases *(3)* and have a tendency to cause persistent infections that could play a role in tumor development. Infections by *Chlamydia trachomatis* and *Chlamydia pneumoniae* have been shown to be associated with cervical carcinoma *(4)* and lung cancer *(5),* and there is serologic evidence of an association between chlamydia infection and lymphomas *(6).* In addition, it has also been suggested that the development of cutaneous T-cell lymphoma may be favored by chronic infection by *C. pneumoniae (7).*

Chlamydia psittaci is the etiologic agent of psittacosis, a human lung infection caused by exposure to infected birds *(3).* The occurrence of high rates of chlamydial infection in household cats *(8,9)* and of asymptomatic carriage of *C. psittaci* in cats from breeding catteries *(10)* raises the possibility that human *C. psittaci* infections deriving from pets other than birds may be underdiagnosed. For example, chronic conjunctivitis by *C. psittaci* is a rare condition, but there is some indication that this condition may be more common than previously thought *(11,12).* Hence, a putative association between a persistent chlamydial infection (and chronic antigenic stimulation) and development of ocular adnexal lymphomas (i.e., conjunctiva, lachrymal gland, and orbital soft tissues) represents an attractive issue, laying the basis for new potentially therapeutic strategies for these disorders.

It is noteworthy that ocular adnexal lymphomas share some clinico-pathologic features with gastric lymphomas, for which a pathogenic link with *Helicobacter pylori*–related follicular gastritis has been established *(13).* Indeed, both ocular adnexal and gastric lymphomas are characterized by an indolent course, a large prevalence of marginal zone B-cell histologic type, and a high degree of infiltrating reactive T-cells *(13,14).* Moreover, similar to what was observed in gastric lymphomas, the presence of somatic mutations within immunoglobulin genes in ocular adnexal lymphomas indicates that the development of these

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disorders is probably favored by chronic stimulation provided by unknown antigen or antigens *(15)*.

This study was designed to investigate a possible association between chlamydial infection and lymphomas of the ocular adnexa. We investigated the presence of three Chlamydiae species—*pneumoniae*, *trachomatis*, and *psittaci*—in both tumor tissue and blood samples from case patients with ocular adnexal lymphoma and from control subjects. The possible pathogenic role of *C. psittaci* infection in the development of ocular adnexa lymphomas is discussed, providing a rational background for new therapeutic modalities for the treatment of ocular adnexal lymphomas.

METHODS

Study Participants

Adults with histologically proven non-Hodgkin's lymphomas of the ocular adnexa (i.e., lachrymal gland, conjunctiva, and orbital soft tissue) diagnosed at the San Raffaele H Scientific Institute (Milan, Italy) from 1988 to 2002 were included in this study. Data regarding age; sex; performance status [according to the Eastern Cooperative Oncology Group criteria *(16)*]; concomitant morbidity; history of neoplastic, infective, allergic, and ocular diseases; risk factors for *C. psittaci* infection (e.g., prolonged exposition with household animals and workers of breeding catteries); tumor stage; treatment regimes; and relapse and survival rates were collected. This study conformed to the tenets of the Declaration of Helsinki, and all patients provided oral informed consent.

Tissue and Peripheral Blood Mononuclear Cell (PBMC) Samples

Chlamydiae DNA detection was performed in formalin-fixed, paraffin-embedded 10-μm-thick sections obtained from ocular adnexa lymphoma lesions. Histologic material was available for molecular analysis in 40 patients. Tissue samples from 20 patients who had been referred to the Department of Ophthalmology (San Raffaele H Scientific Institute) for nonneoplastic orbital biopsies (e.g., ectropion, entropion, palpebral ptosis) and from 26 patients who had reactive lymphadenopathies were used as control subjects. The control subjects had similar sociodemographic characteristics (median age = 64 years [range = 44–90 years] and male/female ratio = 0.6 for ophthalmologic control subjects; and median age = 58 years [range = 19–76 years] and male/female ratio = 0.8 for the reactive lymphadenopathy case patients) to those of the lymphoma case patients. Histologic specimens of ocular adnexal lymphomas, nonneoplastic orbital biopsies, and reactive lymphadenopathies were collected and processed during the same period of time and in the same laboratory.

Chlamydiae DNA detection was also investigated in PBMCs from 21 of the lymphoma patients in our series. The time from lymphoma diagnosis to PBMC collection ranged from 1 to 152 months (median = 12 months). PBMC samples were collected at diagnosis or within 12 months of diagnosis in 11 patients and at random in the other 10 lymphoma patients. In three patients, PBMC collection was concomitant with a relapse of the disease. PBMC samples from 38 healthy donors were used as control samples. Similar to the tissue sample groups described above, the control subjects (healthy donors) had similar sociodemographic characteristics (median age = 48 years [range = 23–62 years] and male/female ratio = 0.8) to the case patients (median age = 61 years [range = 34–74 years] and male/female ratio = 0.62).

DNA Extraction and Purification Procedures

Two to three 5-μm sections were cut from formalin-fixed, paraffin-embedded samples from lymphoma and control tissue. The sections were deparaffinized with two xylene washes (10 minutes each) and rehydrated through decreasing concentrations of ethanol (100%, 96%, 80%, 50%, and distilled water) at room temperature. After rehydration, tissue sections were then placed in 100 μL of digestion buffer (150 mM NaCl, 50 mM TRIS [pH 7.5], 50 mM EDTA, and 1% Triton X100) containing 2 mg/mL proteinase K to digest at 50°C for 3 days. The proteinase K–digested mixture was then purified by silica gel spin cartridge (Talent S.R.L, Trieste, Italy) according to the manufacturer's instructions.

DNA extraction from PBMC samples was carried out following a standard phenol/chloroform extraction technique after proteinase K digestion. DNA from the PBMC samples was extracted 1 year later than was the DNA from the lymphoma tissue samples, and DNA purification from lymphoma and PBMC samples was carried out in two different institutions: Centro di Riferimento Oncologico, Aviano, Italy, and San Raffaele H Scientific Institute, Milan, Italy, respectively.

Detection of Chlamydiae DNA

A multiplex touchdown, enzyme time-release polymerase chain reaction (PCR) assay, designed to simultaneously detect *C. trachomatis*, *pneumoniae*, and *psittaci* DNA at bacterial loads lower than 1 infection-forming unit was performed according to a previously published protocol *(17)*. Briefly, 10 μL of template DNA was added to a PCR tube containing 40 μL of PCR mixture overlaid with 1 drop of mineral oil. Blank reactions filled with 50 μL of PCR mixture were interspersed every three to four samples to monitor possible contamination of PCR reagents by Chlamydiae DNA and to rule out any false-positive results. DNA equivalent to less than 1 infection-forming unit/PCR tube of *C. trachomatis* L2, *C. pneumoniae* TW-183, and *C. psittaci* ORNI (a gift from Dr. Adam Meijer, Research Laboratory for Infectious Disease, National Institute of Public Health and the Environment, Bilthoven, The Netherlands) were included as positive controls. Amplified DNA was from the end of the 16S rRNA gene and the beginning of the 16S-23S spacer region in the ribosomal genes *(17)*. The primer pairs used specifically for *C. trachomatis* and *C. pneumoniae* were located entirely in the 16S rRNA gene; for *C. psittaci*, one primer was located in the 16S rRNA gene and one primer was located in the 16S-23S spacer region.

PCR products were analyzed by electrophoresis in 2% agarose gels with ethidium bromide staining. DNA fragment size was quantified by image analysis (Image Station 440CF, NEN Life Science Products, Boston, MA). Three amplifications were performed per tissue and PBMC sample, both for case patients and for control subjects. Case-patient samples were considered positive for chlamydial infection when Chlamydiae DNA was amplified in at least two independent experiments.

Table 1. Patient characteristics and correlations among clinico-pathologic variables and the prevalence of *Chlamydia psittaci* DNA*

Variable	Assessable patients (%)	*C. psittaci* DNA		
		Positive (%)	Negative (%)	*P*†
No. of patients	40	32 (80)	8 (20)	
Median age, y (range)	66 (34–89 y)	62 (34–84 y)	77 (49–89 y)	.02
Male/female ratio	0.7	0.5	1	.24
ECOG performance status ≥2	2 (5)	1 (3)	1 (12)	.36
Prior cancer‡	5 (13)	4 (13)	1 (12)	.95
Chronic conjunctivitis§	14 (35)	12 (38)	2 (33)	.68
Prolonged contact with household animals (n = 26)‖	13 (50)	13/24 (54)	0/2 (0)	.24
Site of disease				
Lachrymal gland	11 (28)	11 (34)	0 (0)	.08
Conjunctiva	8 (20)	6 (19)	2 (25)	.64
Orbital soft tissue	21 (52)	15 (47)	6 (75)	.24
Histology¶				
Marginal zone lymphoma	24 (60)	21 (66)	3 (38)	.23
Diffuse large B-cell lymphoma	5 (13)	3 (9)	2 (25)	.26
Other lymphoma categories	11 (28)	8 (25)	3 (38)	.66
Stage of disease#				
I	26 (65)	19 (59)	7 (88)	
IV	14 (35)	13 (41)	1 (12)	.22
Other extranodal sites	9 (23)	8 (25)	1 (13)	.65
Systemic symptoms	1 (3)	1 (3)	0 (0)	1.00
High LDH serum level**	1 (3)	1 (3)	0 (0)	1.00

*ECOG = Eastern Cooperative Oncology Group *(16)*. LDH = lactate dehydrogenase.

†*P* values were determined using Fisher exact test.

‡Prior cancers were breast (n = 2), ovarian (n = 1), colon (n = 1), and prostate cancer (n = 1).

§Data on chronic conjunctivitis were available for 37 patients (31 with Chlamydia-positive lymphomas and six with Chlamydia-negative lymphomas).

‖Twenty-six patients (24 with Chlamydia-positive lymphomas and two with Chlamydia-negative lymphomas) were interviewed about prolonged contact with household animals.

¶Histology type was defined according to the World Health Organization Classification of Lymphoid Neoplasms *(37)*. All lymphomas shared B-cell phenotype. Other lymphoma categories included follicular lymphoma (n = 1), small lymphocytic lymphoma (n = 4), and unclassified (n = 6). Some unclassifiable lymphomas could be marginal zone lymphomas in which only the small centrocytoid cell component is evident, whereas lymphoepithelial lesions and reactive germinal centers were not detectable.

#Stage of disease was defined according to the Ann Arbor staging system *(38)*. Complementary therapy after surgical biopsy varied according to the stage of disease.

**High is defined as serum levels of lactate dehydrogenase that were higher than 425 U/L (upper limit of normal range at the San Raffaele H. Scientific Institute).

To further rule out the possibility of a false-positive result, all the molecular procedures were repeated twice for both positive and negative case-patient samples by starting from new pathologic material from the same patients and confirming the results obtained in the first round of experiments. Specificity of the amplified fragments was confirmed by direct sequencing of both sense and antisense strands of purified PCR products using an ABI PRISM 310 Genetic Analyzer (Perkin Elmer, Foster City, CA). Sequence specificity was assessed by BLAST search (http://www.ncbi.nlm.nih.gov/blast), and heterogeneity of Chlamydiae sequences across the different samples was investigated by aligning them with the MultAlin software [http://prodes.toulouse.inra.fr/multalin/multalin.html *(18)*]. In lymphoma samples that were negative to time-release PCR, amplification of β-globin was carried out as previously described *(19)*.

Immunocytochemical Techniques

Formalin-fixed, paraffin-embedded 4-μm thick tissue sections of chlamydia-positive lymphomas from seven case patients were immunostained by the Avidin Biotin-Complex method *(20)*, with a monoclonal antibody specific for chlamydia lipopolysaccharide (antibody was included in the Imagen Chlamydia test, working dilution 1:150, DAKO, Glostrup, Denmark). Antigen retrieval was performed with 0.01 M citrate buffer [pH 8] and with six cycles of 5 minutes each at 750 W in a microwave oven. Specificity control experiments were performed using an autoptic lung of a parrot known to be infected by *C. psittaci* (provided by Dr. Alfredo Seijo) as a positive control and by either substituting the primary monoclonal antibodies (monoclonal antibody from the Imagen Chlamydia test) with nonimmune horse serum (100 μL, working dilution 1:20; Vectastain, Burlingame, CA) or using a histologic section obtained from a case of human prostatic nodular hyperplasia as negative controls.

Chlamydia psittaci–Eradicating Antibiotic Therapy

Because of the lack of therapeutic guidelines for treating *C. psittaci*–asymptomatic carriers, antibiotic therapy was proposed to the nine patients who were positive for *C. psittaci* in both their lymphoma and PBMC samples. Two patients refused treatment, and seven patients were treated with doxycycline 100 mg, twice a day, for 3 weeks. The patients who underwent treatment did not receive any concomitant antiblastic, antibiotic, or steroid treatment. The presence of chlamydial DNA in the patients' PBMCs was assessed (using the same molecular approach described above) 1 month after the conclusion of antibiotic treatment.

Before treatment, four of the seven patients had measurable lymphoma lesions in the orbit or conjunctiva (one at diagnosis and three with relapsed disease), whereas the remaining three patients were lymphoma-free with a follow-up of more than 1

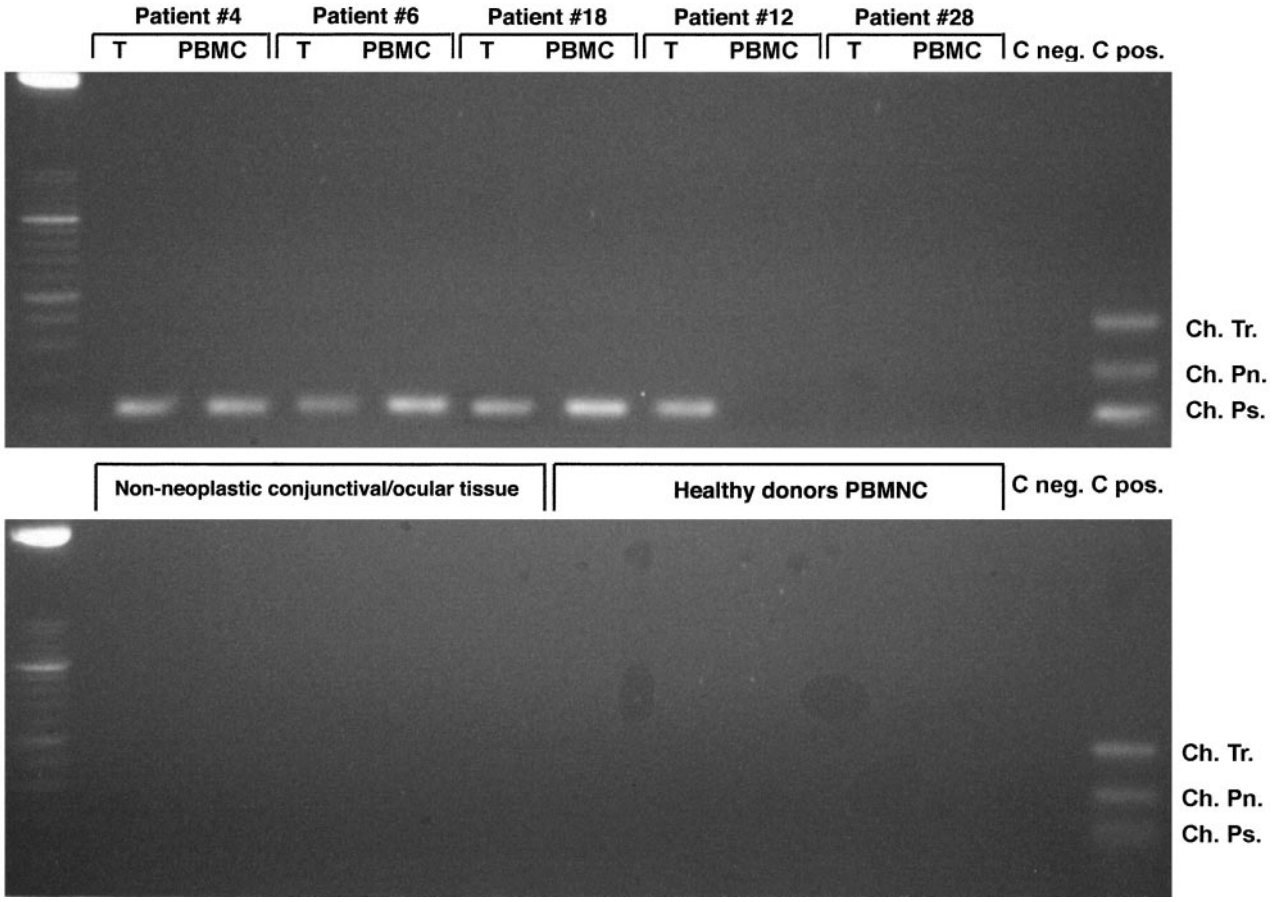

Fig. 1. Amplification of *Chlamydia psittaci* DNA in paired neoplastic tissues (T) and peripheral blood mononuclear cell (PBMC) samples from five patients with ocular adnexal lymphoma (upper panel), in nonneoplastic conjunctival and orbital tissues from control subjects (lower panel; left side), and in PBMC samples from healthy donors (lower panel; right side). C neg. = negative controls; C pos. = positive controls for *Chlamydia trachomatis* (Ch. Tr.), *pneumoniae* (Ch. Pn.), and *psittaci* (Ch. Ps.). The PBMC samples for patients 4 and 6 were collected at diagnosis, whereas the PBMC sample for patient 18 was taken more than 5 years after lymphoma diagnosis, concomitantly with a relapse of the disease.

year. A contrast computed tomography scan of the orbits was performed every 3 months in the four case patients with measurable disease. Objective response (i.e., to antibiotic treatment) was defined according to World Health Organization criteria *(21)*. Briefly, complete remission was defined as the disappearance of all clinical evidence of the disease, partial response was defined as more than a 50% reduction of all measurable lesions, stable disease was defined as regression of any measurable lesion by less than or equal to 50% (minimal response) or no change for the measurable lesions, and progressive disease was defined as appearance of any new lesion or increase in the size of the previously involved sites of at least 25%. The seven patients who underwent treatment and who were eligible for analysis provided signed informed consent.

Statistical Analysis

Differences in Chlamydiae DNA detection between case patients and control subjects and associations between Chlamydiae DNA expression and clinico-pathologic variables were analyzed using the Fisher exact test for categorical variables (Statistica 4.0 statistical package for Windows, Statsoft, Tulsa, OK). All statistical tests were two-sided, with an overall statistical significance level of .05.

Results

Prevalence of *Chlamydia psittaci* DNA in Ocular Adnexal Lymphomas

The clinico-pathologic characteristics of the 40 patients who participated in the study are reported in Table 1. *Chlamydia psittaci* DNA was found in lymphoma samples from 32 of the 40 (80%) case patients analyzed, whereas none of the samples carried DNA sequences for either *C. trachomatis* or *C. pneumoniae* (Fig. 1). Sequence analysis of PCR products from 13 case patients positive for *C. psittaci* confirmed the specificity of the amplified fragments (by BLAST search) and showed a discrete degree of sequence heterogeneity among the DNA sequences from the lymphoma case patients (Fig. 2), indicating that these lymphoma samples carried unrelated variants of *C. psittaci* and, most important, ruling out possible sample contamination from a common source. The finding that about half of the *C. psittaci*–positive lymphoma samples were not positive for the DNA of this microorganism in all of the three PCR amplifications performed per sample (data not shown) indicates that the bacterial load of *C. psittaci* was generally low in these tissue samples. Notably, all 20 nonneoplastic conjunctival or orbital biopsy samples were negative for all three of the Chlamydiae

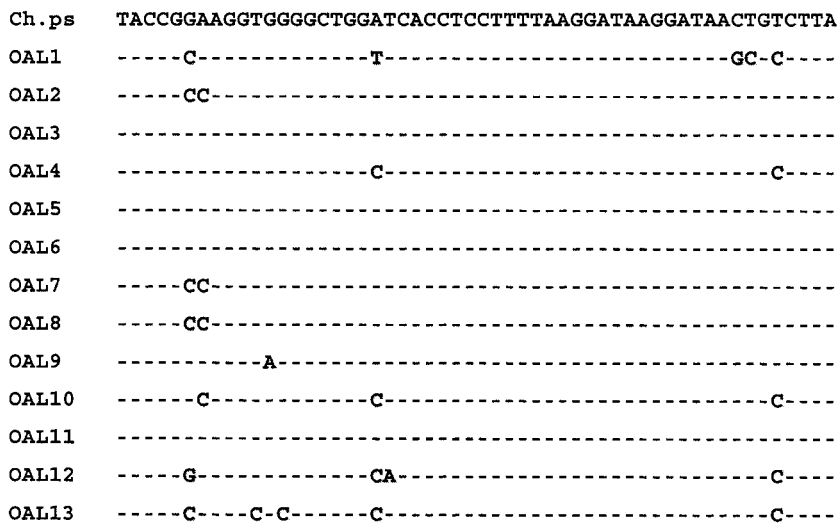

Fig. 2. Sequence alignment of polymerase chain reaction products from case patients with ocular adnexal lymphoma. A dashed line indicates that the sequence of the patient has the same base as the *Chlamydia psittaci* (Ch. ps) sequence. A letter indicates that the sequence of the patient has a different base (given as the new letter) to that of the Ch. ps sequence. All sequences were identified as specific for Ch. ps as shown by BLAST search. OAL = ocular adnexal lymphoma tissue, followed by the case patient number; A = adenosine; T = thymine; C = cytosine; G = guanine.

species investigated, whereas three of the 26 (12%) reactive lymphadenopathy samples were positive for *C. psittaci* DNA. Hence, *C. psittaci* DNA was statistically significantly more common in tissue samples among lymphoma patients than among nonneoplastic biopsy patients (80% versus 0%, respectively; $P<.001$) and reactive lymphadenopathy patients (80% versus 12%; $P<.001$).

Chlamydia psittaci DNA in PBMC Samples from Lymphoma Patients

The presence of Chlamydiae DNA was also assessed in PBMC samples from 21 patients with *C. psittaci*–positive lymphomas to verify whether a systemic chlamydial infection was present at the time of diagnosis and whether the infection persisted over time (Table 2). *Chlamydia psittaci* DNA was detected in PBMC samples from nine (43%) of the 21 patients. Five of the nine *C. psittaci*–positive PBMC samples were collected at diagnosis, thus indicating an underlying systemic *C. psittaci* infection at the onset of the disease. The other four *C. psittaci*–positive PBMC samples were collected more than 5 years after the diagnosis of lymphoma, concomitant with a relapse of the disease in three of the patients. Remarkably, DNA sequences from *C. pneumoniae* and *trachomatis* were not detected in PBMC samples from lymphoma case patients, and none of the 38 PBMC samples from healthy donors was positive for Chlamydiae species DNA (Fig. 1) (43% for the case patients versus 0% for the healthy donors; $P<.001$).

Sequences of PCR products from lymphoma and paired PBMC samples of four of the nine patients with both *C. psittaci*–positive lymphoma and PBMCs were compared, showing that with the exception of a single nucleotide, the same DNA sequences were present in paired tumor and PBMC samples (Fig. 3). This observation is consistent with a persistent infection by a single bacterial strain per patient.

Table 2. *Chlamydia psittaci* DNA detection in peripheral blood mononuclear cells (PBMCs) from 21 patients with *C. psittaci*-positive lymphomas

Time*	No. of patients†	Lymphoma status, No. of patients	No. of patients with *C. psittaci* DNA in their PBMCs
≤1 y	11	At diagnosis, 11	5
>1 y–≤5 y	4	Remission, 4	0
		Relapse, 0	0
>5 y	6	Remission, 3	1
		Relapse‡, 3	3

*Time interval between non-Hodgkin's lymphoma (NHL) diagnosis and collection of the PBMC samples.

†Eleven of 32 patients with *C. psittaci*–positive lymphoma were not available for PBMC sampling. *C. psittaci* DNA was not found in PBMC samples from two additional case patients with *C. psittaci*–negative lymphoma. In these case patients, the PBMC samples were collected within 1 year from NHL diagnosis.

‡Cases in which PBMC samples were collected concomitantly with a relapse of disease.

Immunocytochemical Studies

In the parrot's lung that carried *C. psittaci* infection, antichlamydial lipopolysaccharide antibodies stained a limited number of scattered interstitial cells with macrophage-like morphology (data not shown). *Chlamydia psittaci*–positive cells displayed a granular and predominantly intracytoplasmatic immunoreactivity. Interestingly, similar findings were observed in four of the seven ocular adnexal lymphoma samples that were positive for *C. psittaci* DNA (Fig. 4). In particular, immunoreactive cells were characterized by macrophage-like morphology with a relatively abundant cytoplasm, a granular reactivity, and an overall cell size larger than the surrounding neoplastic lymphocytes; these immunoreactive cells were not found within blood vessel lumina.

Chlamydia psittaci–Eradicating Antibiotic Therapy

Characteristics of the seven patients treated with *C. psittaci*–eradicating antibiotic treatment are summarized in Table 3. All patients completed the antibiotic treatment, and there were no cases of symptomatic toxicity or changes in biochemical parameters observed. One month after doxycycline treatment was concluded, Chlamydiae DNA was no longer detectable in the PBMC samples of the seven treated patients. Objective response among the four case patients who had measurable lymphoma lesions before treatment was progressive disease, stable disease (6 months follow-up), minimal response (reduction of 45% of the tumor mass; 20+ months follow-up), and complete remission (18+ months follow-up).

Fig. 3. Sequence alignment of polymerase chain reaction products from paired lymphoma (T) and peripheral blood mononuclear cell (PBMC) samples from four case patients with ocular adnexal lymphoma. A dashed line indicates that the sequence of the patient has the same base as the *Chlamydia psittaci* (Ch. ps) sequence; a letter indicates a different base. With the exception of a single nucleotide, DNA sequences from paired lymphoma and PBMC samples were identical in all case patients. OAL = ocular adnexal lymphoma tissue, followed by the case patient number; A = adenosine; T = thymine; C = cytosine; G = guanine.

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Ch.ps      TACCGGAAGGTGGGGCTGGATCACCTCCTTTTAAGGATAAGGATAACTGTCTTAGGACGG

OAL3T      ------------------------------------------------------------
OAL3PBMC   ------------------------------------------------------------

OAL5T      ------------------------------------------------------------
OAL3PBMC   ------------------------------------------------------------

OAL9T      ----------A-------------------------------------------------
OAL9PBMC   ------------------------------------------------------------

OAL11T     ------------------------------------------------------------
OAL11PBMC  ------------------------------------------------------------
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DISCUSSION

This study provides evidence for an association between *C. psittaci* infection and the presence of ocular adnexal lymphoma. *Chlamydia psittaci* DNA was detected in 80% (32/40) of the ocular adnexal lymphoma case patients investigated, as confirmed by sequencing analysis. The slight degree of heterogeneity observed among the DNA sequences of *C. psittaci* derived from different patients indicates that ocular adnexal lymphomas may carry unrelated variants of *C. psittaci*. Because of the high sensitivity of our PCR approach, the high prevalence of *C. psittaci* in this study might merely reflect the occurrence of a subclinical Chlamydiae infection that is widespread among the general population. Nevertheless, this possibility can be ruled out because the nonneoplastic conjunctival and orbital tissues and PBMC samples from healthy donors were all negative for *C. psittaci* DNA and only 12% of reactive lymphadenopathy samples were positive for *C. psittaci* DNA.

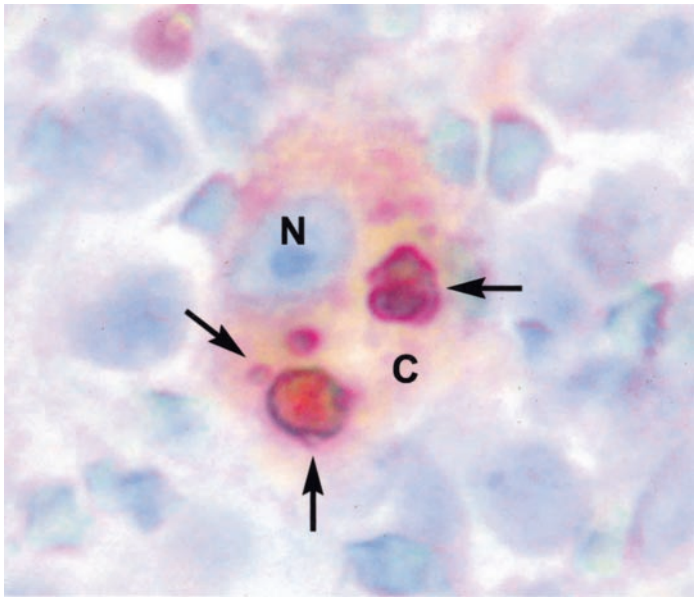

Fig. 4. Immunocytochemical studies in ocular adnexal lymphoma samples. Imagen Chlamydia monoclonal antibody (Dako, Glostrup, Denmark) directed against *Chlamydial lipopolysaccharide* (Avidin Biotin-Complex technique) shows immunoreactivity for chlamydia within the cytoplasm (C) of a macrophage-like cell, displaying a brownish granular staining (arrows). N = nucleus.

The strength of the association between *C. psittaci* infection and ocular adnexal lymphoma is also supported by the statistically significantly higher prevalence of *C. psittaci* infection detected in ocular adnexal lymphoma patients than in the general population *(22)*. Unlike *C. pneumoniae*, which has a broad diffusion worldwide *(3)*, *C. psittaci* infection is a rare condition in European countries *(23)*. Although data for the prevalence of *C. psittaci* DNA detection in blood samples from the general population are lacking, serology data indicate that only 3% of elderly individuals in Northern Europe are *C. psittaci* IgG-seropositive *(22)*. Serology assays, however, are not an adequate method to detect *C. psittaci* infection, because they are severely limited by high cross-reactivity among different Chlamydiae species and between Chlamydiae and other microorganisms *(24)*. In fact, lipopolysaccharide, the antigen used in available serologic tests, is a common molecule found among several microorganisms. Moreover, in some geographic areas, the seroprevalence of *C. pneumoniae* has been reported to range from 40% to 80% *(22)*, limiting the value of serologic screening for *C. psittaci*. Thus, it is important to emphasize that DNA detection by PCR analysis is the current gold standard for chlamydial infection recognition *(25,26)*.

Chlamydial infections have also been associated with neoplastic diseases *(4–6)*. In fact, Chlamydiae species are known to establish persistent infections, to be mitogenic *in vitro (27)*, to induce polyclonal cell proliferations *in vivo (28)*, and to cause resistance to apoptosis in infected cells *(29)*. Nevertheless, any association between *C. psittaci* and cancer has not been convincingly demonstrated thus far. The possibility that *C. psittaci* infections may be underdiagnosed *(8)*, however, highlights the need for research on the potential contribution of this microorganism to tumor development in humans. Indeed, chronic ocular infections by *C. psittaci* could be more common than previously recognized *(9,11,12)*. If this is the case, then the parallelism between the association of gastric lymphomas with *H. pylori*–related chronic gastritis *(13)* and the potential pathogenic link between chronic conjunctivitis related to chlamydial infection and ocular adnexal lymphomas appears to be worthy of analysis. Interestingly, the incidence of ocular adnexal lymphomas is growing *(30)*. Notably, this increase in incidence is substantially higher than that reported for systemic lymphomas *(31)* and does not seem to be biased by demographic or reporting artifacts or by variations in diagnostic criteria *(30)*. Our experience confirms this trend, with a 71% increase in the number of ocular adnexal lymphoma cases diagnosed at our institution (San Raffaele H Scientific Institute) between 1994–1997 and 1998–2001. These

Table 3. Characteristics of patients treated with *Chlamydia psittaci*–eradicating antibiotics*

Patient No.	Sex/ age†	PS†	Histology type	Tumor stage†	Site†	1st line therapy	OR	Lymphoma at time of ATB	ATB OR	OR duration (months)	Status
4	F/71	1	MZL	IV	Lachrymal gland	IFN	PR	Local relapse	CR	18+	Alive 98 mo NED
5	F/74	1	MZL	IV	Orbit	CEOP	CR	Local relapse	PD	—	Alive 185 mo ED
24	M/34	0	MZL	IV	Conjunctiva	RT	CR	Local relapse	SD	6	Alive 148 mo ED
40	M/72	0	SLL	I	Orbit	—	—	At diagnosis	MR‡	20+	Alive 20 mo ED
6	F/56	0	MZL	IV	Lachrymal gland	Ritux	CR	Remission	—	—	Alive 47 mo NED
22	F/74	1	MZL	I	Conjunctiva	Ritux	CR	Remission	—	—	Alive 44 mo NED
35	F/61	0	MZL	IV	Orbit	CHOP + R	CR	Remission	—	—	Alive 35 mo NED

*PS = performance status was determined using Eastern Cooperative Oncology Group criteria *(16)*. OR = objective response at first-line treatment; ATB = antibiotic; ATB OR = objective response of lymphoma at eradicating antibiotics. F = female, M = male; MZL = marginal zone lymphoma, SLL = small lymphocytic lymphoma; IFN = interferon, CEOP = cyclophosphamide, epidoxorubicin, vincristine, and prednisone; RT = radiotherapy, Ritux = rituximab, CHOP = cyclophosphamide, doxorubicin, vincristine, and prednisone; PR = partial response, CR = complete remission, PD = progressive disease, SD = stable disease, MR = minimal response; ED = evident disease, NED = no evident disease.

†Age, performance status, stage, and site of disease at diagnosis. Stage of disease was defined according to the Ann Arbor staging system *(38)*.

‡Minimal response (response of 45%).

epidemiologic trends may be related to unrecognized pathogenic factors, among which infectious agents could be included.

Chronic persistence of infectious agents causes prolonged direct antigen stimulation, which is thought to play an important role in the clonal expansion of some lymphomas (e.g., follicular and marginal zone B-cell lymphomas), as indicated by the presence of somatically hypermutated immunoglobulin genes with a pattern of ongoing mutations *(32)*. The presence of somatic hypermutations in clonally rearranged immunoglobulin heavy-chain variable genes in marginal zone B-cell lymphomas of the ocular adnexa shows a molecular similarity with the normal properties of B cells positively selected by a cognate antigen within the germinal center *(33)*. In particular, the mutation rates observed in immunoglobulin-variable genes of ocular adnexal lymphomas are similar to those reported for gastrointestinal marginal zone lymphomas *(15)*, whose association with chronic antigenic stimulation by *H. pylori* is well established *(13)*. The possibility that ocular adnexal lymphomas arise in the context of chronic inflammation has also been recently suggested *(15);* however, the source of the putative antigen or antigens presumably involved in this process is still unknown. *Chlamydia psittaci* may, indeed, provide such an antigenic stimulus, thus contributing to the pathogenesis of ocular adnexal lymphomas. As reported in Table 1, *C. psittaci* infection was more common among patients with marginal zone B-cell lymphomas and other low-grade lymphomas (88%) than among those with diffuse large B-cell lymphomas (60%), indicating that there may be different pathways leading toward lymphomagenesis, similar to what has been reported for gastric mucosa-associated lymphoid tissue lymphomas *(34)*. However, the fact that diffuse large B-cell lymphomas were more often negative for *C. psittaci* DNA than were marginal zone B-cell lymphomas is consistent with the possibility that a proportion of *C. psittaci*-positive marginal zone lymphomas may evolve toward a more aggressive histotype (i.e., diffuse large B-cell lymphomas), which is no longer responsive to (and dependent on) the antigenic stimulation provided by the microorganism. Of note, 38% of the patients in this study who had *C. psittaci*–positive lymphomas had a prior history of chronic conjunctivitis (Table 1). Nevertheless, a more thorough characterization of the association between ocular adnexal lymphoma and *C. psittaci* is presently limited, most importantly by the facts that sequencing of the *C. psittaci* genome has not been completed and that reagents suitable to specifically investigate the expression of bacterial proteins in lymphoma tissues are lacking. These limitations need to be overcome to identify candidate bacterial peptides to be used in ex vivo functional assays aimed at demonstrating whether *C. psittaci* is able to provide stimuli that promote lymphoma cell growth.

This is the first study, to our knowledge, to demonstrate the presence of *C. psittaci* DNA in PBMCs of humans. In fact, we observed *C. psittaci* DNA in the PBMCs of half of our patients with chlamydia-positive lymphomas at diagnosis. Thus, our data suggest that infection of PBMCs by *C. psittaci* persists over time in a high proportion of case patients. In fact, three of the four chlamydia-positive PBMC samples collected more than 5 years after the lymphoma diagnosis were collected concomitantly with a disease recurrence, further supporting the possible involvement of *C. psittaci* in sustaining long-term lymphoma cell growth. Our findings are similar to recent observations *(35)*, which demonstrated the presence of *C. pneumoniae* DNA in circulating cells of humans, and support the previously reported *(36)* hypothesis that a prolonged *C. psittaci* infection in PBMCs could favor continuous reinfections that could, in turn, chronically trigger antigenic stimulation. Immunohistochemical data from the present study suggest that monocyte/macrophage system components may play a critical role in such a scenario. Although we did not find mononuclear cells immunoreactive for anti-chlamydia antibodies within blood vessel lumina in the ocular adnexal lymphoma samples we studied, available immunohistochemical data do not allow the distinction between these cells being resident or circulating elements.

In conclusion, although only a limited number of lymphoma patients were treated with antibiotics specific for chlamydia infection, our observation of lymphoma regression after *C. psittaci* eradication with doxycycline treatment provides additional evidence for a role of *C. psittaci* infection in the development of ocular adnexal lymphomas. Similar to findings observed in gastric marginal zone lymphomas *(2)*, in which *H. pylori* eradication was followed by an objective response in 70%–80% of case patients, two out of four of our case patients with ocular adnexal lymphoma and measurable disease showed objective regression after antibiotic treatment. These data should, however, be interpreted with caution, given the small number of

treated patients. Nevertheless, the findings deserve to be confirmed in a phase I/II prospective trial to conclusively assess whether *C. psittaci*–eradicating antibiotics may constitute a new therapeutic strategy against ocular adnexal lymphomas.

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