DETECTION OF *HELICOBACTER PYLORI* AND *CHLAMYDIA PNEUMONIAE* GENES IN PRIMARY ORBITAL LYMPHOMA

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ABSTRACT

Purpose: Primary orbital non-Hodgkin's lymphoma is a mucosa-associated lymphoid tissue (MALT)-type extranodal marginal zone lymphoma. Chronic antigen stimulation is implicated as a causative agent in the development of some mature B-cell proliferations; for example, there are associations involving *Helicobacter pylori* with gastric or conjunctival MALT lymphoma and *Chlamydia psittaci* with ocular adnexal lymphoma. We examined the molecular signatures of *H pylori* and *Chlamydia* in eight orbital lymphomas**.**

Methods: Polymerase chain reaction (PCR) was performed on DNA extracted from microdissected lymphoma cells. *H pylori* was detected with the urease B and vac/m2 primers. A multiplex touchdown enzyme time-release PCR assay designed to simultaneously detect *Chlamydia trachomatis, Chlamydia pneumoniae,* and *C psittaci* was performed. Authenticity of the PCR-amplified products was verified by Southern blot hybridization.

Results: *H pylori* DNA was detected in an orbital lymphoma of a French patient who had positive serum *H pylori* titer. *C pneumoniae*, but neither *C psittaci* nor *C trachomatis*, DNA was detected in another orbital lymphoma of a Chinese patient from Hong Kong. *H pylori*, *C pneumoniae*, and *C psittaci* genes were not found in the other six orbital lymphomas.

Conclusion: *H pylori* or *C pneumoniae* genomic fingerprints were detected in two of seven primary orbital MALT lymphomas. These findings provide evidence for a possible involvement of particular infectious microorganisms such as *H pylori* and *Chlamydia* in primary orbital lymphoma. These different microorganisms may play similar roles in the etiology of orbital MALT lymphomas from different geographic regions. Antibiotic therapy could be considered for orbital MALT lymphomas associated with positive infection.

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INTRODUCTION

Orbital non-Hodgkin's lymphoma is an extranodal lymphoid malignancy that constitutes approximately 10% of all orbital neoplasms in the United States, 24% of all orbital neoplasms in Japan, and 8% of all extranodal lymphomas.[1-3] The majority of orbital non-Hodgkin's lymphomas are extranodal marginal zone B-cell lymphomas of mucosa-associated lymphoid tissue (MALT) type, a distinct clinicopathologic entity in the World Health Organization (WHO) classification of malignant lymphomas.[4] The majority of MALT lymphomas occur in the gastrointestinal organs, but they may affect virtually any organ, including the ocular adnexa.[5-7] MALT lymphomas often remain localized to their sites of origin for many years, and therefore they have an indolent clinical course.[8] Histologically, MALT lymphomas are characterized by a heterogeneous lymphoid cell proliferation consisting of centrocyte-like, monocytoid, and plasmacytoid cells with occasional mitoses in the marginal zone surrounding reactive lymphoid follicles.[9,10] Another characteristic feature is secondary infiltration of the germinal centers by neoplastic marginal zone B-cells.

The pathogenesis of MALT lymphoma is unknown, though chronic antigen stimulation has been implicated as a causative agent in the development of mature B-cell lymphoproliferative processes.[11-16] Several infectious agents have been proposed as risk factors for the development of MALT lymphomas, and some of these agents have been the targets for therapy with encouraging results.[17] *Helicobacter pylori* is responsible for gastric and duodenal ulcers, gastric carcinoma, and MALT lymphoma.[18-20] *H pylori* is reported to infect 72% to 98% of patients with gastric MALT lymphoma.[21] Eradication of *H pylori* alone induces regression of gastric MALT lymphoma in 70% to 80% of cases.[22] *Chlamydia pneumoniae* has also been associated with malignant lymphomas, as evidenced by a seroepidemiologic study of non-Hodgkin's lymphoma in Finland[23] and an immunohistochemical, ultrastructural, and cell cultural analysis of cutaneous T-cell lymphoma (Sézary syndrome) in the United States.[24]

Recently, infectious agents have also been reported in association with ocular adnexal lymphomas. *H pylori* genes have been detected in four of five conjunctival MALT lymphomas,[16] and conjunctival MALT lymphoma occurring in the setting of adult inclusion conjunctivits infected by *C. trachomatis* has been described in a young adult.[25] In Italy, *Chlamydia psittaci* infection has been found in 32 (80%) of 40 MALT lymphomas of the orbit (15/21, 71%), lacrimal gland (11/11, 100%), and conjunctiva (6/8, 75%).[14] This study examines molecular signatures of *H pylori* and *Chlamydia* in seven orbital MALT lymphomas and one orbital metastatic T-cell lymphoma.

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METHODS

STUDY SUBJECTS

The protocols were approved by the institutional review boards of the National Eye Institute (NEI) and respective Universities in Hong Kong, Japan, Switzerland, and France. The procedures used in this research conformed to the tenets of the Declaration of Helsinki. Each participant included in this study signed an informed consent form. Eight adult patients (four male and four female) with orbital masses or ocular lesions involving the orbit underwent biopsies in four countries. Their brief clinical histories are listed in the Table. The orbital biopsied specimens of these eight patients were processed for routine pathology in local hospitals (four in Japan, two in France, one in Hong Kong,[26] and one in Switzerland). In seven cases the diagnosis was extranodal marginal zone lymphoma of MALT type; in one case the diagnosis was a central nervous system (CNS) T-cell lymphoma metastasized to the eye and orbit (this case also developed a choroidal MALT lymphoma). Two paraffin slides of each case were then sent to the NEI for molecular analyses.

Microdissection

The orbital MALT lymphoma cells on the slides were carefully microdissected as previously described.[16,27] Briefly, the buffered formalin-fixed paraffin sections stained with hematoxylin and eosin were deparaffinized. Lymphoma cells were selected by visualization under a light microscope and microdissected using a 30-gauge needle or by laser capture microscopy, PixCell II (Arcturus, Mountain View, California).

Detection of *Immunoglobulin Heavy Chain* Gene Rearrangements

The microdissected cells were immediately placed in proteinase K-enriched DNA extraction buffer. Polymerase chain reaction (PCR) amplification was performed using *IgH* FR3A gene (sense: 5′- ACA CGG CYS TGT ATT ACT GT -3′, and antisense 5′- GGA TGG TAC CAA GCT TTG AGG AGA CGG TGA CCA -3′) for the detection of B cell monoclonality.

Detection of *T-cell Receptor-gamma (TCR-γ)* Gene Rearrangements

DNA was extracted from the microdissected cells and subjected to PCR amplification using specific primers for the *TCR-γ* gene, (sense: 5′- AGG GAT GTG TTG GAA TCA GG -3′, and antisense: 5′- CGT CGA CAA CAA GTG TTG TTC CAC -3′). The sense primers used for *IgH* and *TCR-γ* genes were ^{32}P-labeled. The amplified DNA was analyzed on 16% polyacrylamide gel and visualized by autoradiography.

Molecular Detection of *H pylori* Gene

DNA isolated from the microdissected cells underwent PCR amplification using *H pylori* gene-specific primers of HPU54 and HPU18 from the *urease B* gene.[28] Primers HPU54 (5′- TGG GAT TAG CGA GTA TGT -3′) and HPU18 (5′- CCC ATT TGA CTC AAT G -3′) amplified a 132 bp product from the *urease B* gene (nt 1971 to 2102). Electrophoresis on a 15% polyacrylamide ethidium bromide–stained gel was employed for separation and visualization of the PCR products with radioisotope-labeled primers.

The m2 region of *H pylori vacA* gene *(vacA m2)* was amplified by nested PCR as described by Koehler and colleages.[29] The primer sets for the first PCR were m2F1, 5′- TTT GGA GC(C/T) CCA GGA AAC ATT G -3′ and m2R1, 5′- C(C/T)A CAC GCC CAT CTT GGA CAA -3′, and for the second PCR were ^{32}P-labeled m2F2, 5′- ACC CTA AA(C/T) AGC AAC GCA AGC -3′ and m2R2, 5′- GAC AAA AAG ATT CAT CGT GCC TT -3′. One μL DNA template from the first PCR was used for the second amplification. The 101 bp product of the nested PCR amplification was visualized by polyacrylamide gel electrophoresis and autoradiography with radioisotope-labeled primers.

Southern hybridization was performed using the probe with a sequence of 5′- GAA CCC GCC TTT GAT GAT CAT GTT GGG TTT TAC G -3′. Amplified DNA was transferred from an agarose gel to a nylon membrane. The membrane was prehybridized for 4 hours at 65^{o}C in a 1X hybridized buffer (Life Technologies, Inc, Gaithersburg, Maryland). The ^{32}P-labeled oligonucleotide probe was added to the hybridization buffer at a final concentration of 25 nM, and hybridization was carried out for 14 hours at 5^{o}C below the melting temperature of the probe. Following hybridization, the membrane was washed once for 5 minutes in 2X SSC-0.1% (wt/vol) sodium dodecyl sulfate (SDS), twice for 30 minutes in 0.1X SSC-0.1% SDS at room temperature, and once for 15 minutes in 0.1X SSC-0.1% SDS at the melting temperature of the probe. It was exposed to x-ray film at –70^{o}C for 14 hours, and the film was developed.

Molecular Detection of *C trachomatis, C psittaci,* and *C pneumoniae* Genes

DNA of the lymphoma cells were subjected to three *Chlamydia* genes using a touchdown enzyme time-release PCR assay with ^{32}P-labeled primers.[30] The primer sets specific for the three genes were: CTR70, 5′- GGC GTA TTT GGG CAT CCG AGT AAC G -3′ and CTR71, 5′- TCA AAT CCA GCG GGT ATT AAC CGC CT -3′ for *C trachomatis*; CPN90, 5′-GGT CTC AAC CCC ATC CGT GTC GG -3′ and CPN91, 5′- TGC GGA AAG CTG TAT TTC TAC AGT T -3′ for *C pneumoniae*; CPS100, 5′- CCC AAG GTG AGG CTG ATG AC -3′ and CPS101, 5′- CAA ACC GTC CTA AGA CAG TTA -3′ for *C psittaci.* The expectable PCR product for each gene is 315, 197, and 111 bp, respectively. The cycling times of PCR were 75 seconds at 95°C, followed by 60 cycles of denaturation at 94°C for 45 seconds, annealing beginning at 65°C and ending at 52°C for 45 seconds, and extension at 72°C for 1 minute. The annealing temperature was lowered 1°C every four cycles until it reached 52°C, and this annealing temperature was

maintained until the end of the cycling process. The products of the PCR amplification were visualized by polyacrylamide gel electrophoresis and autoradiography.

RESULTS

The clinical data of the eight cases are summarized in the Table. The most common manifestations were exophthalmos or orbital mass for at least 2 months. Histopathologically, seven biopsies demonstrated classic MALT lymphomas (Figure 1). They were composed of diffuse lymphoid infiltrates; some contained mainly small polymorphic lymphocytes admixed with plasmacytoid and monocytoid cells, and others contained reactive follicles surrounded by small lymphoid cells with irregular nuclei. Follicular colonization and lymphoepithelial lesions were observed in these seven biopsies. The tumors were CD20-positive, indicating B-cell lineage. Only one biopsy was of a T-cell lymphoma, and it was composed of small, polymorphic CD3- and CD4-positive lymphoid cells, indicating T-cell lineage in this case. Molecular analysis detected *IgH* gene rearrangements in the seven B-cell lymphoma cases and *TCR-*γ gene rearrangement in the one T-cell lymphoma case (Figure 2).

TABLE. CLINICAL INFORMATION FOR EIGHT CASES OF ORBITAL LYMPHOMA

CASE NO. COUNTRY	AGE/SEX	PAST HISTORY	SITE	CLINICAL SYMPTOMS	DATE OF BIOPSY DURATION AFTER ONSET
1 Japan	59/M	Unremarkable	R upper orbit	Exophthalmos (4 mm), diplopia, ocular hypertension	June 1996 5 mo
2 Japan	64/F	Diabetes, myocardial infarction	R inferior eye lid	Palpebral conjunctival tumor	May 1997 4 mo
3 Japan	60/M	Myocardial infarction, tuberculosis	L intraconal orbit	Exophthalmos (5 mm)	Feb. 1998 8 mo
4 Japan	62/F	Hypertension	R upper eyelid	Upper eyelid swelling	June 1998 2 mo
5 Hong Kong	47/M	Unremarkable	Bilateral orbit	Proptosis, lid swelling, chemosis	July 2000 16 mo
6 France	58/M	*H pylori* + gastric MALT	Bilateral conjunctiva R orbital involvement	Conjunctival masses, irritation	November 2001 17 mo
7 France	91/F	Diabetes, hypertension	L upper orbit L lid	Exophthalmos, ptosis	May 2002 5 yr
8 Switzerland	50/F	CNS T-cell lymphoma, choroidal MALT lymphoma	L orbit	Diplopia, orbital mass, choroidal mass, OS	July 2003 2 mo

CNS = central nervous system; L = left; R=right; MALT = mucosa-associated lymphoid tissue.

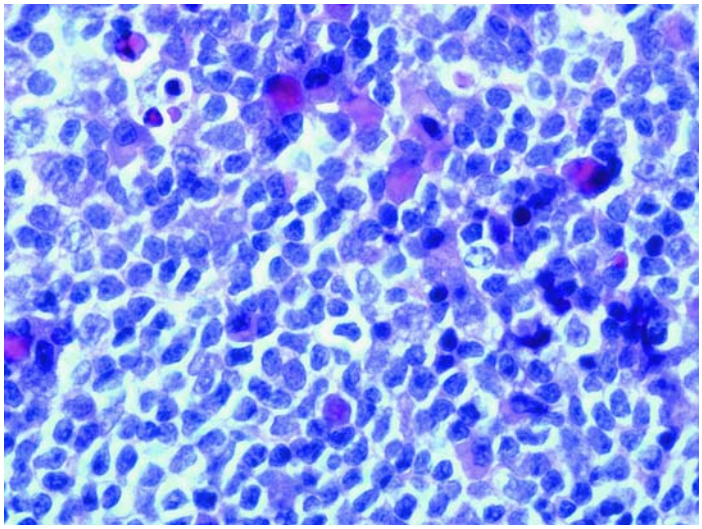

FIGURE 1

An orbital MALT lymphoma is composed of many atypical lymphoid cells and a few plasma cells and is variable in size (hematoxylin and eosin, ×400).

PCR amplification found positive *H pylori* DNA, which was confirmed by Southern blot, in one B-cell MALT lymphoma. This case was a French patient with a positive serum *H pylori* titer and gastric MALT lymphoma confirmed by biopsy. This patient had bilateral conjunctival MALT lymphomas with minimal orbital invasion. Interestingly, only *C pneumoniae* was detected in the Chinese patient from Hong Kong with bilateral orbital lymphomas whose serum titer against *Chlamydia* was below detectable level. PCR amplification did not discover genes of *Chlamydia* or *H pylori* in the other MALT lymphoma cells of the other five patients from Japan and France nor the one T-cell lymphoma from Switzerland (Figure 2). The Epstein Barr virus (EBV) genome was not detected in all eight orbital lymphomas (Figure 2).

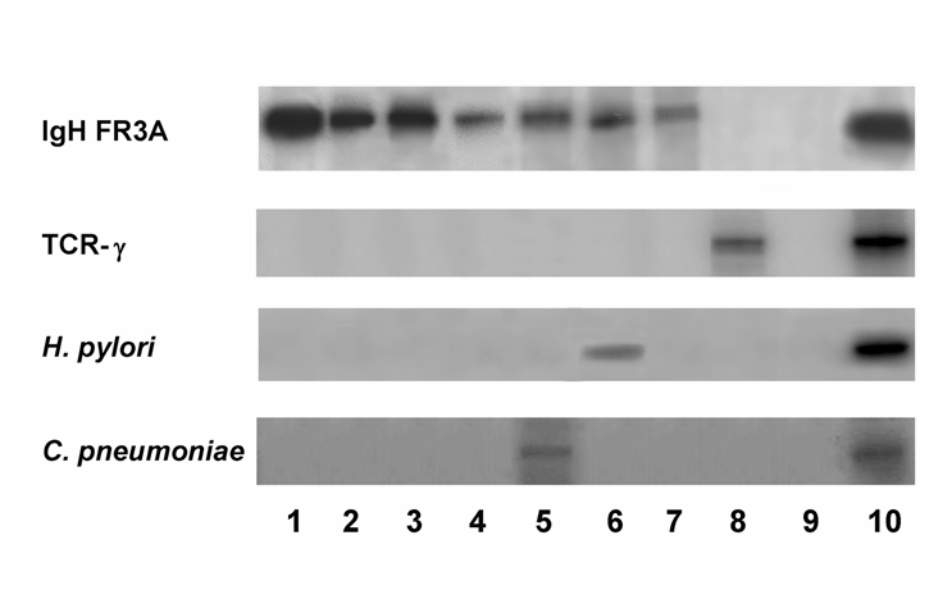

FIGURE 2

The products of polymerase chain reaction amplification in the eight cases of orbital MALT lymphoma demonstrate positive *H pylori* gene in one case and *C pneumoniae* gene in another case. *IgH* gene rearrangements are detected in seven cases and *TCR-γ* gene in one case (lanes 9 and 10 represent negative and positive controls, respectively).

DISCUSSION

This study identifies *H pylori* and *C pneumoniae* molecular signatures in two of eight orbital lymphomas or two of seven primary orbital MALT lymphomas in Europe and Asia. Recently, direct associations between an infectious agent and ocular adnexal lymphoma have been published; *H pylori* DNA was found in 80% of conjunctival MALT lymphomas in the United States and *C psittaci* DNA in 80% of ocular adnexal lymphomas in Italy.[14,16] Despite these intriguing associations, *H pylori* and *C pneumoniae* genes have not been directly isolated and linked to orbital MALT lymphoma.

The low-grade extranodal marginal zone lymphoma often recapitulates the cytomorphologic features of MALT lymphoma.[31] Typically, these lymphomas arise from sites normally devoid of lymphoid tissue, but are preceded by chronic inflammatory, usually autoimmune, responses that result in the accumulation of lymphoid tissue. These accumulated lymphocytes undergo chronic antigenic stimulation and clonal selection, which may lead to antigen independence and development of MALT lymphomas. The most notorious example of this process is gastric MALT lymphoma, which arises from lymphoid tissue acquired as the result of chronic *H pylori* infection in the stomach. Furthermore, *H pylori* is one of the most diverse bacterial species and is naturally competent for genetic transformation.[32] Those *H pylori* species with the *CagA* gene are capable of activating protein kinase cascades and increasing expression of certain proto-oncogenes.[33] The presence and role of *H pylori* molecular fingerprints in orbital MALT lymphoma may well be comparable to that in conjunctival or even gastric MALT lymphoma.[16] The patient with positive results in this study (case 6) also had a diagnosis of gastric MALT lymphoma, and his ocular MALT lymphoma mainly involved the conjunctiva.

A higher prevalence of *C psittaci* infection is reported in Finnish patients with ocular adnexal lymphoma as compared with those in that general population.[34] *C psittaci* DNA has been detected and sequenced in 71% of orbital lymphomas in an Italian study, where a subset of these patients have responded to antibiotic treatment.[14,35] However, other investigators found that "blind" antibiotic treatment of 11 Austrian patients with ocular MALT lymphoma and untested for *C psittaci* infection produced no response after median follow-up of 9 months.[36] Although two independent studies in the United States cannot replicate the involvement of *C psittaci* in ocular adnexal lymphoma, the investigators of the studies suggest that this discrepancy may relate to geographic and continental differences.[37,38] *C pneumoniae* infection has also been hypothesized to be causally associated with different types of malignancies,

including cutaneous T-cell lymphoma.[12] After reviewing six seroepidemiologic studies, Littman and colleagues[39] presented a causal association between this microorganism and lung cancer. Serologic evidence for an association between chronic *C pneumoniae* and lymphoma has been reported significant with an odds ratio (OR) of 7.3 in Finland.[23] Inflammation caused by chronic infection with *C pneumoniae* may be involved in the carcinogenic process; however, this relationship is difficult to further define through serologic data alone.

The current study identifies *C pneumoniae*, not *C psittaci*, in an orbital MALT lymphoma of a Chinese patient in Hong Kong (case 5).[26] Seroprevalence of *C pneumoniae* is relatively high in some geographic areas, including Japan, Taiwan, and Hong Kong, ranging from 40% to 90%, limiting the value of this serologic index.[34,40-42] Thus, it is important to emphasize that molecular identification is the current "gold standard" for the diagnosis of *Chlamydia* infection, notably for *C pneumoniae*.[43] Interestingly, the Chinese patient with positive *C pneumoniae* gene and bilateral recurrent orbital MALT lymphoma had a negative serum titer against *Chlamydia*.[26]

Chlamydiae are obligate intracellular bacteria that replicate in a vacuole inside a host cell. Chlamydial infection has been shown to protect against apoptotic stimuli.[44] This is likely important for the ability of chlamydiae to reproduce in human cells. In a recent investigation, *C pneumoniae* was demonstrated to interfere with the host cell's apoptotic machinery via destruction of the proapoptotic BH3-only proteins, Bim/Bod, Puma, and Bad during infection.[45] This interference may protect the host cell against apoptotic stimuli during infection with chlamydiae. It is a plausible speculation that the inhibition of apoptosis is important for the growth of the bacteria resulting in chronic infection. The chronic, persistent chlamydial infection may perhaps lead to the recruitment of clonal specific lymphoid cells and the development and progression of MALT lymphoma.

This study does not detect *H pylori* or *C pneumoniae* in a metastatic T-cell lymphoma into the orbit. T-cell leukemia or lymphoma is well known to be associated with human T-lymphotropic virus type-1 (HTLV-1) infection.[46] EBV is classically associated with Burkitt's lymphoma and undifferentiated nasopharyngeal carcinoma.[47,48] It is also implicated in other malignancies, such as T-cell non-Hodgkin's lymphoma and Hodgkin's disease.[48,49] No EBV DNA is discerned in our eight orbital lymphomas.

As this study has detected infectious microorganism DNAs in two (28.6%) of seven orbital MALT lymphomas, one containing *H pylori* (14.3%) and the other *C pneumoniae* (14.3%), the findings support the possible involvement of certain particular pathogens in MALT lymphoma pathogenesis. Pathogens or antigens attract T cells, which recruit and activate B cells.[50] This process promotes MALT lymphoma growth. The different microbes associated in orbital MALT lymphomas may relate to geographic differences in the incidence of their infections[38]; *H pylori* infection is higher in France, *C psittaci* in Italy, and *C pneumoniae* in Southeast Asia. The differences may also relate to the genotypic and phenotypic differences among the strains of the pathogen.[37] Other disease cofactors, such as host genetics and environments, could potentially play a role as well. Additional surveys for *Chlamydia* and *H pylori* in orbital MALT lymphoma in larger and/or other populations may help to clarify the issue. In light of our findings and those from Italy, along with the disparate clinical responses to eradicating antibiotic therapy in patients with ocular adnexal MALT lymphoma (encouraging in Italy and one center in the United States[35,51,52] but ineffective in Austria and a different center in the United States[36, 37]), it is advisable to search for other pathogens or sources of chronic antigenic stimulation in the orbital MALT lymphomas without recognizable pathogens or antigens.

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PEER DISCUSSION

DR HANS E. GROSSNIKLAUS: Low-grade malignant lymphomas arising from mucosa-associated lymphoid tissue (MALT) were first described in 1984 by Isaccson and Wright.[1] MALT lymphoma is generally isolated to extranodal sites including the gastrointestinal tract, salivary gland, thyroid and ocular region. MALT lymphoma often occurs in the setting of autoimmune disorders or chronic antigen stimulation. In 1991, Wotherspoon and co-authors reported the association of H. pylori gastritis in 110 of 450 patients with gastric MALT lymphoma.[2] Chan and co-workers later identified H. pylori DNA in 5 cases of conjunctival MALT lymphoma.[3] In 2004. Ferreri and coworkers reported that 32 (80%) of 40 Italian patients with ocular adnexal lymphoma harbored *Chlamydia psittaci* DNA.[4] The organism was identified by immunostaining with a monoclonal antibody for Chlamydial lipopolysaccharide and PCR for C. psittaci DNA. Seven of those patients were positive for both C. psittaci in both the lymphoma and peripheral blood mononuclear cells. Those patients were treated with doxycycline 100 mg twice a week for three weeks. Follow-up studies by that group showed complete, partial, and minimal responses to the doxycyline therapy and both H. pylori and C. psittaci DNA by PCR in another group of patients with ocular adnexal MALT. Other groups have empirically treated with antibiotic therapy for Chlamydia and H. pylori in patients with ocular adnexal MALT lymphoma. There appear to be geographical differences among patients with Chlamydia or H. pylori associated with ocular adnexal MALT, as patients in south Florida, the northeastern United States, and Japan failed to show a relationship between C. psittaci infection and ocular adnexal lymphoma.

In this study, Chan and coworkers used the polymerase chain reaction (PCR) technique on DNA from laser capture microdissected ocular adnexal lymphoma cells to evaluate for the presence of *H. pylori*, *C. trachomatis*, *C. pneumoniae*, and *C. psittaci*. Their results were positive for *H. pylori* DNA in a French patient who had a positive serum titer for *H. pylori* and *C. pneumoniae* in the orbital lymphoma of a Chinese patient from Hong Kong. Neither *H. pylori* nor *Chlamydia* DNA was detected in an additional 5 orbital lymphomas. These results are in accordance with the work by Ferreri's group and others in that geographic differences appear to be involved with the *Chlamydia* or *H. pylori* ocular adnexal MALT lymphoma association.

I have several questions for Dr. Chan. Do you believe the organism (*Chlamydia* or *H. pylori*) is spread via the systemic circulation, via saliva, or by another mechanism? Are you aware of any group identifying the actual *H. pylori* organism in ocular adnexal lymphoma? Have either of your patients been treated for *Chlamydia* or *H. pylori*? Do you have any insight regarding the geographic differences in this disease? I wish to thank Dr. Chan and her co-authors for this most provocative paper.

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DR ALLAN J. FLACH: With the *C. psittaci* in 80 percent of the Italian families, was there a bird transmitter in the family? In other words, were those pet-induced diseases?

DR VICTOR M. ELNER: Inclusions of lipopolysaccaride (LPS) or DNA in cells may be due to non-specific uptake by the cells. Thus, although LPS or DNA may be prone to be incorporated into proliferating cells, these agents may not induce the lymphoma. How might one exclude this possibility in your studies? On another point, antibiotic treatment may reduce chronic antigenic simulation, resulting in the partial responses. Have any of these patients been re-biopsied to ascertain whether their lymphomas were indeed eradicated, and that the apparent clinical improvement was not solely due to reduction of superimposed inflammatory responses associated with the neoplasm?

DR HUGH R. TAYLOR: People who are studying *Chlamydia pneumoniae* by PCR are very concerned about the artifact induced by deparaffinization of sections. It was not clear in your presentation exactly how you went about the handling of sections and if that might account for false negatives. *C. pneumoniae* has been extensively studied because of a possible link with atherosclerosis in extraocular locations. From a variety of other studies there is now a concept that there actually is a secondary deposition or infection of atheromatous plaques by *C. pneumoniae* so it is a secondary phenomenon not a primary pathogenic. Do you have an idea with the lymphoma whether the Chlamydia are actually colonizing these cells?

DR. CHI-CHAO CHAN: Dr. Grossniklaus asked, "Do you believe the organism (*Chlamydia* or *H. pylori*) is spread via the systemic circulation, via saliva, or by another mechanism?" Aside from iatrogenic transmission of *Helicobacter (H.) pylori* via endoscopy, no definite modes of transmission for *H. pylori* have been identified.[1] However, association studies suggest that there are three other potential routes for the transmission of *H. pylori*: person-to-person transmission (e.g. oral-oral or fecal-oral), waterborne transmission (e.g. contaminated water), zoonotic (e.g. cats and other pets and animals) or vector borne transmission (e.g. flies). Person-to-person transmission is considered to be the most likely route of transmission considering that isolation of *H. pylori* from non-human reservoirs has been inconsistent.[1]

Chlamydial infection is transmitted in a variety of manners. In the case of *Chlamydia trachomatis*, the infection may be spread via sexual contact, vertical transmission from mother to infant through an infected birth canal, water- or secretion borne transmission, and vector borne (e.g. flies). For *Chlamydophila psittaci*, zoonotic transmission occurs. Psittacine birds (of the order Psittaciformes and from which *C. psittaci* derives its name) include common pets such as cockatiels, cockatoos, and lovebirds and are the primary reservoir for *C. psittaci* with human serving as an incidental host via acquisition through the aerosol or fecal-oral routes.[2, 3] *Chlamydophila pneumoniae* is transmitted person-to-person (e.g. oral-oral or aerosol routes).

Dr. Grossniklaus asked, "Are you aware of any group identifying the actual *H. pylori* organism in ocular adnexal lymphoma?" I am not aware of any group that has identified the *H. pylori* organism in ocular adnexal lymphoma.

Dr. Grossniklaus asks, "Have either of your patients been treated for *Chlamydia* or *H. pylori*?" The patient from France with *H. pylori* was treated with antibiotics after finding gastric disease. This was about the same time that the conjunctival MALT lymphoma was diagnosed. The patient from Hong Kong was treated with antibiotics after our findings, but was also treated with radiation therapy, so it is difficult to make associations between the disease and antibiotic treatment.

Dr. Grossniklaus asked, "Do you have any insight regarding the geographic differences in this disease?" Geographic locale, age, race, socioeconomic status, and hygiene seem to play roles in the prevalence of *H. pylori*. Most of our information about *H. pylori* infection rates comes from seroprevalence studies. Higher rates of infection tend to occur at a younger age in developing countries compared to developed countries and in regions characterized by lower socioeconomic status and higher density living.[1] Looking at racial differences in the US, we find that whites of non-Hispanic origin have lower prevalence of infection compared to African-Americans or Hispanics.

It is well known that *Chlamydia trachomatis* is sexually transmitted. *Chlamydophila pneumoniae* (previously known as *Chlamydia* TWAR strain) is the most common non-viral intracellular human respiratory pathogen. Antibodies against *C. pneumoniae* become more prevalent after 5 years of age in developed countries whereas in developing countries infection with *C. pneumoniae* often occurs before 5 years of age.[4]

Chlamydophila psittaci may also exhibit geographic differences. With respect to *C. psittaci* DNA and ocular adnexal lymphomas, specifically, Ferreri and colleagues noted that 80% of the Italian patients examined carried *C. psittaci* DNA.[5] More recently, Chanudet and colleagues examined the prevalence of *C. psittaci* DNA in patients with ocular adnexal MALT lymphomas residing in different geographic regions.[6] The prevalence of *C. psittaci* DNA in German ocular adnexal MALT lymphoma patients was higher than that of patients in the East coast of USA, which was higher than patients hailing from the Netherlands. Interestingly, Italy as well as the United Kingdom, and Southern China had lower prevalence rates.[6] France may also have a lower prevalence of this infectious DNA in ocular adnexal lymphoma patients.[7] Thus, geographic variability in *C. psittaci* infection is noted.

In addition, geno- and phenotypically variable strains of *H. pylori* and *Chlamydia* exist and it is probable that certain strains are more prevalent in specific geographic areas than others.

Dr. Flach asked about bird exposure. I am not aware of publications on isolation or detection of *C. psittaci* from the birds in Italy where association of ocular adnexal MALT lymphoma and *C. psittaci* is reported.

Dr. Elner asked if the DNA could be non-specific. While this is possible, the way in which we perform the microdissection and selectively attain only the lymphoma cells makes DNA non-specificity less likely. However, this study did not demonstrate if and how the microorganisms transformed normal B-lymphocytes to MALT lymphoma cells. We did not rebiopsy the tissue, but I am aware that the researchers in Italy rebiopsied the tissue (examination of peripheral blood mononuclear cells in which *C. psittaci* DNA was detected). After they treated with an antibiotic, they found no molecular fingerprinting in white blood cells.[8]

Dr. Taylor asked if there were false negatives and false positives. We used paraffin sections that were fixed in formalin or other chemicals. In this manner, sterilization of a high amount of infectious agents and other contaminants is achieved. We deparaffinize the sections first and then stain them. With microdissection, the occurrence of contamination is considered to be very low.

Dr. Taylor asked about *C. pneumoniae* and atherosclerosis. A recent article mentioned that *C. pneumoniae* is probably in the blood.[9] While it may probably not be the cause of atherosclerosis, *C. pneumoniae* does associate with lymphoma by chronic antigenic stimulation.

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