Is there a role for *Chlamydia pneumoniae* infection in systemic lupus erythematosus and in the associated atherosclerotic cardiovascular disease?

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ABSTRACT

Objective. *To search for molecular evidence of Chlamydial infection in systemic lupus erythematosus (SLE) subjects and to assess if there is an association of this infectious agent with coronary artery calcification (CAC), a marker of total atherosclerotic burden.*

Methods. *28 SLE subjects had blood samples drawn and DNA extracted from peripheral blood mononuclear cells (PBMC) and an electron beam computed tomography (EBCT) scan. Polymerase chain reaction (PCR) analysis was performed for* Chlamydia trachomatis *16srRNA and major outer membrane protein (MOMP) and for* C. pneumoniae *16srRNA, MOMP, as well as nested PCR for MOMP.*

Results. *Four of 28 subjects (14.2%) had evidence of* C. pneumoniae *nucleic acid in PBMC. The 16srRNA primers detected* C. pneumoniae *in one patient (3.57%) and the nested PCR MOMP primers in 3 subjects (10.71%). None were positive for* Chlamydia trachomatis. *Two of the 4 subjects with* C. pneumoniae *DNA had abnormal EBCT scans and 2 /11 (18.3%) subjects with abnormal EBCT were positive for* C. pneumoniae. *There were significant associations of* C. pneumoniae *DNA with smoking (OR =3) and corticosteroid use. The odds ratio for subjects with abnormal CAC and detectable* C. pneumoniae *was 1.67.*

Conclusion. *This pilot study demonstrates for the first time that* C. pneumoniae *DNA can be identified in the PBMC of some SLE subjects and there may be an association with CAC. Smoking may be an additional risk factor for infection in this population. Determination of pathogenicity of this organism in atherosclerotic coronary vascular disease in SLE will require further study.*

Introduction

Systemic Lupus Erythematosus (SLE) is a chronic inflammatory autoimmune disease, the origins of which are likely multifactorial. Infection has been studied extensively as a possible trigger and as a major cause of morbidity and mortality in SLE patients. SLE itself, or the resultant immunosuppression from therapy, may make patients susceptible to infections. Although Chlamydial infection has been associated with other autoimmune diseases, little data exists investigating it as an associated factor in SLE.

Not only has Chlamydia received attention as a trigger for autoimmunity, but it also has been demonstrated to be associated with atherosclerosis. *Chlamydia pneumoniae* (*C. pneumoniae*) has been detected in atherosclerotic tissue (1) and peripheral blood momonuclear cells (PBMC) of patients with atherosclerotic cardiovascular disease (ASCVD) (2). Our pilot study presents evidence of Chlamydial infection in the PBMC of SLE patients who are participating in an ongoing study of the risk of premature ASCVD.

Patients and methods

Patients

28 SLE subjects who fulfilled the American College of Rheumatology criteria for SLE were recruited from the rheumatology clinic at the University of Pennsylvania (3). Questionnaires and chart reviews were completed after informed consent was obtained. Subjects were scheduled for electron beam computed tomography (EBCT) to measure coronary artery calcification (CAC) and had blood collected for blood chemistries and PCR analysis for *C. pneumoniae* and *C. trachomatis*.

Laboratory studies

Blood samples were collected for high-sensitivity C-reactive protein (hs-CRP), sedimentation rates (ESR), and fasting lipid levels. One 5 ml sample of EDTA anti-coagulated blood was collected and promptly processed for the detection of Chlamydia DNA by polymerase chain reaction (PCR).

Blood mononuclear cell preparation: Blood collected in EDTA tubes was centrifuged for 20 minutes; then plasma was transferred to be stored at -72°c for serologies. Blood cells were mixed with Roswell Park Memorial Institute Solution at 1:1 ratio and separated using a Ficoll-Histopaque density gradient (Sigma, USA) by centrifuging at 1500 rpm for 20 minutes. The mononuclear cell layer was collected, washed

twice with RPMI and transferred to a sterile Eppendorf tube and stored at –72ºC for DNAextraction.

DNA extraction: Blood mononuclear cells were mixed with hyaluronidase enzymatic digestion buffer, proteinase K and incubated overnight at 50ºC, then extracted with phenol-chloroform and precipitated in cold ethanol. Distilled water was added depending on the amount of pellet.

PCR: Polymerase chain reaction (PCR) was performed using 2 ml DNA with primer sequences for *Chlamydia tra - chomatis* (*C. trachomatis*) 16srRNA and MOMP and *C. pneumoniae* 16 srRNA and MOMP, as well as nested PCR for MOMP as published in detail (4,5). In addition, extensive positive and negative controls were available from our previous studies with *C. pneumoni - ae* and *C. trachomatis* and studies on other bacterial DNAs (6,7). Amplification products were detected by performing gel electrophoresis on 2% agarose gel that contained ethidium bromide. The PCR process was carried out under conditions imposing strict contamination precautions.

EBCT: Using a GE/Imatron C-150 EBCT scanner a CAC score based on the calcific area, average density, and the number of plaques (Agatson score) was calculated for each patient (8). An Agatson score of zero was considered a normal scan, and an abnormal scan was one which had an Agatson score greater than zero. Subjects were also given a percentile score which represented their score compared to age and sex-matched scores (e.g. between the 50th and 75th%, between the 75th and 90th%, and > 90th%) (9).

Statistical analysis
All continuous data are expressed as the mean±SD. P values for paired samples of the SLE with and without evidence for *C.pneumoniae* were analyzed using the Marginal Homogeneity test for matched pairs. P values less than 0.05 were considered significant. Odds ratios were calculated for the SLE subjects with and without evidence of *C. pneumoniae* in relation to smoking status and CAC.

Results
The 28 SLE subjects (25 women and 3 men) had a mean age of 42.9 ± 15.8 years. Based on EBCT results, 11/28 subjects (39.3%) had abnormal calcium scores.

Evidence of *C. pneumoniae* DNA was detected in 4/28 subjects (14.3%). *C. trachomatis* DNA was not found. In one patient, DNA was detected by the 16-sr RNA primers and 3 were positive using nested MOMP primers. Data regarding these four subjects are shown in Table I.

A smoking history was defined as ei-ther the past or present use of tobacco. Hypertension was considered present based on either a history of physician-diagnosed hypertension, use of an anti-hypertension medication (unless an angiotensin converting enzyme inhibitor is being used for proteinuria alone), or by a measured mean systolic blood pressure 140, or mean diastolic blood pressure 90 on more than one occasion. High cholesterol was defined as a fasting level >240 mg/dl. A family history of heart disease was identified from self-report of MI, sudden cardiac death, or a revascularization procedure occurring in a first-degree male relative before the age of 55 and/or in female relatives before the age of 65.

Three of the 4 patients with *C. pneumo - niae* DNAhad a history of smoking and hypertension, 2 had diabetes, and only 1 of the 4 either had a history of myocardial infarction or hypercholesterolemia. Of the 11 SLE subjects who had an abnormal calcium score, 2/11 (18.3%) had *C. pneumoniae* DNA positive PBMC. These 2 subjects were older than the 2 subjects who did not have coronary calcification, and their Agatson scores were in the 75th-90th% for historical age and gender-matched controls. Hs-CRP was elevated in 2 of the 4 PCR-positive subjects, neither of whom had an abnormal Agatson score. Table II demonstrates the association of the variables and abnormal CAC be-

Table I. Conventional risk factor, SLE activity, drugs and laboratory variables in 4 SLE subjects positive for Chlamydia DNAby PCR in blood mononuclear cells.

| Pt. no. | Sex and race | Age | *C. pneumoniae* primer | Medications | | Conventional risk factor | | | | | | Laboratory | |
				Cortico-steroid*	DMARD	Smoking	DM	HTN	High cho-lesterol	FHx	HxMI	hs CRP (normal range: 0 - 2.5)	Abnormal calcium score
1	Female	80	Nested	-	-	+	-	-	-	-	-	0.72	+ (75-90th%)
2	Female	37	Nested MOMP	+	+	-	-	+	+	-	-	5.46	-
3	Male	66	Nested MOMP	+	+	+	+	+	-	-	-	2.18	+ (75-90th%)
4	Female	42	16srRNA	-	-	+	+	+	-	-	+	2.61	-

DMARD: Disease-modifying antirheumatic drug; DM: diabetes mellitus; HTN: hypertension; FHx: family history of heart disease; HxMI: history of myocardial infarction.
*Corticosteroid: present or history.

Table II. Association of variable parameters between subjects with SLE with and without Chlamydial nucleic acid.

Variable	SLE with Chlamydia (n = 4)	SLE without Chlamydia (n = 24)	p value
Age (mean ± SD) years	44.00 ± 19.27	42.12 ± 15.99	0.45
Smoking (n - %)	3/4 (75%)	12/24 (50%)	0.001 (OR = 3.00)
Cholesterol (mg/dl ±SD)	173.5 ± 77.03	178.08 ± 33.05	0.38
hsCRP(mg/l)	2.92 ± 2.00	19.9 ± 50.31	0.48
Corticosteroid usage (n - %)	4/4 (100%)	(16/24) 75%	0.014
*DMARD (n - %)	2/4 (50%)	15/24 (62.5%)	0.15
Abnormal CAC	2/4 (50%)	9/24 (37.5%)	0.15 (OR = 1.67)

*DMARD: Disease-modifying antirheumatic drug; OR: odds ratio.

tween subjects with and without evidence of Chlamydial nucleic acid. There did not appear to be any correlation with age, cholesterol level, hsCRP, or use of disease modifying anti-rheumatic drugs (DMARDs) between the "DNA+/CAC abnormal" and the "DNA+/CAC normal" subjects.

Corticosteroid use (p=0.001) and smoking (p=0.014) were significantly associated with the presence of *C. pneumoniae*. The odds ratio (OR) for smoking in subjects with versus those without evidence of Chlamydia was 3.0. All 4 subjects with evidence of Chlamydia were smokers. The OR for abnormal CAC was 1.67, thus suggesting a correlation between CAC and PCR evidence of *C. pneumoniae*.

Discussion

Chlamydia pneumoniae has been proposed as a contributor to ASCVD. Circulating monocytes may serve as potential vehicles of the vascular dissemination of *C. pneumoniae* to the atherosclerotic tissue, as has been evidenced by PBMC PCR (10). An inflammatory response can be initiated by this pathogen and it has been shown to have the potential to induce monocyte adhesion to endothelial cells, promote a pro-coagable state, and stimulate inflammatory cytokines, thus potentially enhancing the atherosclerotic process (11-13). Investigations have reported that the prevalence of *C. pneumoniae* is greater in atherosclerotic tissue as compared to normal coronary arteries (1).

Hu *et al.* have demonstrated that a hypercholesterolemic state was necessary to induce atherogenesis in the presence of a Chlamydial infection. Interestingly, the same pattern of hypercholesterolemia (high total cholesterol, high triglycerides, and low HDL) which is associated with chronic Chlamydial infection (14) is the dyslipidemic pattern seen in premenopausal SLE patients (15). Larger studies will need to be completed to examine if there is an association of Chlamydial infection and lipid profiles in SLE.

Atherosclerosis is presently accepted as an inflammatory disease and hs-CRP, a marker of inflammation, is associated with an increased risk of cardiac events (16). In our study, two of the PCR-positive patients had elevated hs-CRP; the implication of this is unclear with the number of patients in this small cohort. Interestingly, the two subjects with elevated CRP levels had normal EBCT scans.

Two of the four subjects with positive PCR had required immunosuppressive therapy and corticosteroids. Immunosuppression may be a predisposing cause for initial or persistent Chlamydial infection and thus introduce another possible factor for accelerated atherosclerosis.

Our pilot results demonstrated *C. pneumoniae* in PBMC of SLE subjects. In addition, we demonstrate for the first time an association with subclinical evidence of atherosclerosis via coronary calcification. We found 14.28% (4/28) of this study group positive for *C. pneumoniae*. The nested MOMP primers detected 3 of the positive tests and the 16sr RNA detected the other positive result. This supports the concept that frequency or prevalence depends on the

numbers and types of primers in each study. *C. pneumoniae* was found in subjects both with normal and abnormal coronary calcium scores. The exact implications of any correlations must await clarification from larger studies. Clearly, *C. pneumoniae* nucleic acids can be found in SLE, but until there is a better standardization of methods, questions will continue to be raised. We recently reported the findings of a large study in which many differences emerged in the interpretation of the results on identical specimens between laboratories (17).

Chlamydia may have a role in the process of accelerated atherogenesis in SLE. There are many covariates which may act synergistically, such as dyslipidemia, hyperhomocysteinemia, immunosuppression, inflammation, traditional risk factors, and *C. pneumoniae* infection. Our data raise many questions. When is the right time to search for this organism? Is there any association between the onset of disease and this infection? Does Chlamydial PCR positivity antedate or follow the onset of SLE or atherosclerosis? Do patients on immunosuppressive therapy have a higher incidence of Chlamydial DNA in either the PBMCs or atherosclerotic tissue? Larger studies with sufficient power need to be completed to support this provocative data and begin to address the question of whether infectious etiologies may represent yet another factor predisposing SLE patients to accelerated atherosclerosis.

References

1. MAASS M, BARTELS C, ENGEL PM, MAMAT U, SIEVERS HH: Endovascular presence of viable *Chlamydia pneumoniae* is a common phenomenon in coronary artery disease. *J Am Coll Cardiol* 1998; 31: 827-32.
2. BOMAN J, SODERBERG S, FORSBERG J *et al.*: High prevalence of *Chlamydia pneumoniae* DNA in peripheral blood mononuclear cells in patients with cardiovascular disease and in middle-aged blood donors. *J Infect Dis* 1998; 178: 274-7.
3. HOCHBERG MC: Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1997; 40: 1725.
4. GAYDOS CA, QUINN TC, EIDEN JJ: Identification of *Chlamydia pneumoniae* by DNA amplification of the 16S rRNA gene. *J Clin Microbiol* 1992; 30: 796-800.
5. GAYDOS C, FOWLER C, GILL V, EIDEN J,

QUINN T: Detection of *Chlamydia pneumo - niae* by polymerase chain reaction-enzyme immunoassay in an immunocompromised population. *Clin Infect Dis* 1993; 17: 718-723.

6. SCHUMACHER HR JR, GERARD HC, ARA-YSSI TK *et al*.: Lower prevalence of Chlamydia pneumoniae DNA compared with *Chlamydia trachomatis* DNAin synovial tissue of arthritis patients. *Arthritis Rheum* 1999; 42: 1889-93.

7. GERARD HC, WANG Z, WANG GF *et al*.: Chromosomal DNAfrom a variety of bacterial species is present in synovial tissue from patients with various forms of arthritis. *Arthritis Rheum* 2001; 44: 1689-97.

8. AGATSON AS, JANOWITZ WR, HILDNER FJ *et al*.: Quantification of coronary artery calcium using ultrafast computed tomography. *J Am Coll Cardiol* 1990; 15: 827-32.

9. HOFF JA CE, KRAINIK AJ, DAVIGLUS M, RICH S, KONDOA GT: Age and gender dis-tributions of coronary artery calcium detect-ed by electron beam tomography in 35,246 adults. *Am J Cardiol* 2001; 87: 1335-9.

10. BYRNE GI, KALAYOGLU MV: *Chlamydia pneumoniae* and atherosclerosis: links to the disease process. *Am Heart J* 1999; 138 (5, Pt. 2): S488-90.

11. CAMPBELL LA, ROSENFELD M, KUO CC: The role of *Chlamydia pneumoniae* in ather-osclerosis – Recent evidence from animal models. *Trends Microbiol* 2000; 8: 255-7; discussion 257.

12. KALAYOGLU MV, PERKINS BN, BYRNE GI: *Chlamydia pneumoniae*-infected monocytes exhibit increased adherence to human aortic endothelial cells. *Microbes Infect* 2001; 3: 963-9.

13. DECHEND R, MAASS M, GIEFFERS J *et al*.: *Chlamydia pneumoniae* infection of vascular smooth muscle and endothelial cells acti-vates NF-kappaB and induces tissue factor and PAI-1 expression: a potential link to ac-celerated arteriosclerosis. *Circulation* 1999; 100: 1369-73.

14. HU H, PIERCE GN, ZHONG G: The athero-genic effects of chlamydia are dependent on serum cholesterol and specific to *Chlamydia pneumoniae*. *J Clin Invest* 1999; 103: 747-53.

15. FORMIGA F, MECO JF, PINTO X, JACOB J, MOGA I, PUJOL R: Lipid and lipoprotein levels in premenopausal systemic lupus ery-thematosus patients. *Lupus* 2001; 10: 359-63.

16. RIDKER PM, CUSHMAN M, STAMPFER MJ, TRACY RP, HENNEKENS CH: Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. *N Engl J Med* 1997; 336: 973-9.

17. BOMAN J, HOGLAND J, ALLARD A, BLASI F *et al*.: *Chlamydia pneumoniae* PCR methods developed in-house: A survey of intra- and inter-laboratory variation of sensitivity and specificity among 12 laboratories. 2003: 389-92.