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CONCENTRATIONS FOLLOWING PERIODONTAL SCALING AND ROOT
PLANING

A thesis submitted in partial fulfillment of the requirements for the degree of Master of
Science at Virginia Commonwealth University.

by

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Abstract

SERUM ANTI-PHOSPHORYLCHOLINE AND ANTI-CARDIOLIPIN CONCENTRATIONS FOLLOWING PERIODONTAL SCALING AND ROOT PLANING

By Reve W Chaston, DDS

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2006

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Atherosclerosis is an insidious disease with serious morbidity and mortality including ischemic heart disease, stroke, and myocardial infarction. This condition is progressive and can start early in life eventually leading to large plaques and arterial occlusion. Two key components of this process are the immune system and lipids; in particular, LDL which accumulates within the arterial walls and macrophages which recognize and engulf oxidized-LDL (oxLDL) to form foam cells. Knowing that certain antibodies directed against bacterial antigens such as phosphorylcholine (PC) and cardiolipin (CL) show

opsonizing cross-reactivity with oxLDL it can be proposed that there is a link between immune responses to periodontal bacteria and atherosclerosis. The aim of this investigation was to determine whether periodontal bacteria are capable of inducing serum antibodies potentially involved in cardiovascular diseases; specifically, IgG anti-PC, IgG anti-CL, and IgM anti-CL. To test this, 17 subjects with chronic periodontitis received scaling and root planing in conjunction with blood sample analysis to determine if periodontal instrumentation resulted in changes in these serum antibodies. If plaque bacteria are responsible for an immune response then serum levels of these antibodies should decrease following periodontal therapy. We found that serum levels of IgG anti-PC, IgG anti-CL, and IgM anti-CL decreased following periodontal scaling and root planing but the change was significant only for IgG anti-PC (P 0.045). Serum levels of IgM anti-CL approached significance (P 0.054). The results support the hypothesis that the immune response to periodontal bacterial microflora contributes to serum concentrations of antiphospholipid antibodies.

INTRODUCTION

Heart disease is the leading cause of death in North America and results in more than 6 million deaths each year.¹ Cardiovascular mortality rates account for 29% of all deaths despite efforts aimed at controlling conventional risk factors. This condition is progressive often starting early in life and eventually leads to large arterial plaques and occlusion.²⁻⁴ It has been established that smoking, hypertension, hyperlipidemia, diabetes mellitus, age and family history are risk factors for atheroma formation⁵⁻⁸ possibly through endothelial cell injury.^{4,9,10} This injury leads to increased endothelial permeability to monocytes which migrate into the intima where they engulf lipids and become foam cells. Over many years, this initial lesion of foam cells can evolve into an atheroma which could potentially rupture and result in thrombus formation. Because the immune system is a key component of this process, atherosclerosis is regarded by many as an inflammatory disease.¹¹

Likewise, periodontitis is an inflammatory condition of the oral cavity which affects the dental supporting tissues. It has been suggested that severe generalized periodontal disease is found in 8% to 13% of the world's adult population.^{12,13} The prevalence is lower in children and young adults with an estimated 2% to 5% of North Americans between the ages of 11 and 25 being affected.¹⁴ Like atherosclerosis,

periodontitis is progressive, resulting in destruction of the supporting connective tissue, and alveolar bone loss.^{15,16} This process is the result of the bacteria-host interaction.¹⁷ Endotoxin release by subgingival bacteria causes activation of junctional epithelial and vascular endothelial cells which release inflammatory mediators such as Il-8, Il-1 α , PGE₂, and TNF α . Leukocyte and monocyte recruitment to the site is followed by macrophage activation and the further release of these cytokines. Continuation of the inflammatory state occurs as T-cells, B-cells, and plasma cells become more prominent and antibody production begins. The eventual destruction of periodontal connective tissue and bone arises in part due to osteoclast activation by factors such as Il-1, TNF- α , and MMP. If attachment loss continues the end result of the disease is tooth loss.

A vital component of the etiology of periodontitis is the lipopolysaccharide present on the outer membrane of Gram-negative periodontal bacteria. This molecule has 3 major component parts; the external O-antigen, the core, and the lipid A which is embedded within the lipid portion of the outer membrane¹⁸ and is responsible for the endotoxin properties. Minor lipopolysaccharide antigenic components have also been identified. Phosphorylcholine (PC) is one such molecule. It is attached to cell wall polysaccharide and lipoteichoic acid^{19,20} and has been identified in over 30% of the supragingival and subgingival flora including *Streptococcus oralis*, *Streptococcus sanguis*, *Actinomyces israelii*, *Actinomyces naeslundii*, *Fusobacterium nucleatum*, *Haemophilus aphrophilus*, and *Actinobacillus actinomycetemcomitans*.^{21,22} The exact function of PC has been difficult to determine but it is possible that certain bacteria may utilize it to gain access to endothelial cells²³ or to the circulation.²⁴⁻²⁶ In Gram-positive pneumococcal organisms

there are surface proteins that require binding to PC residues of teichoic acid (TA) and Lipoteichoic acid (LTA) for proper function.^{27,28} Cell wall lytic enzymes of these bacteria depend on the conversion of TA and LTA associated PC from an inactive to an active form.²⁹ Bacterial adherence, colonization, and invasion are also reliant upon surface PC. It has been shown that *Streptococcus pneumoniae* and *Actinobacillus actinomycetemcomitans* invasion of endothelial cells is dependent on the interaction between surface PC and endothelial surface receptors for platelet activating factor.^{23,25}

Since PC is a component of the LPS motif of many bacteria it potentially plays a role in prompting a host immune response. Lymphocyte responsiveness studies have shown that PC influences polyclonal B-cell differentiation and activation.^{30,31} Additionally there is host production of IgG and IgM antibodies directed against PC which can assist monocyte recognition and phagocytosis of the assaulting pathogen. PC positive strains of *Actinobacillus actinomycetemcomitans* and *Streptococcus pneumoniae* become opsonized by anti-PC IgG inducing PMN respiratory bursts.³² Signs of such host-periodontal pathogen interplay are not only detected locally but also systemically. In fact, Schenkein²¹ demonstrated the ability of periodontal pathogens to produce a systemic response to PC by showing that serum levels of antibodies directed toward PC (Anti-PC IgG) were higher in patients with attachment loss than in those without. Further, it was found that both PC-bearing strains of oral bacteria and oxidized low-density lipoproteins (oxLDL) reacted with anti-PC IgG from human serum.³³ This suggests that antibody produced against certain periodontal bacteria will also react with PC-bearing oxLDL³⁴ and therefore magnify the uptake of this lipid by foam cells and further the progression of atherosclerosis.

Similar to anti-PC is the involvement of the anti-cardiolipin (anti-CL) antibody in the upregulation of macrophage phagocytosis of oxLDL in the pathogenesis of atherosclerosis. This heightened uptake of oxLDL by macrophages occurs through an interaction between the anti-CL antibody and a glycoprotein called β 2GPI. It has been demonstrated that serum β 2GPI binds specifically to available oxLDL via special ligands and that the resulting complex is subsequently targeted by anti- β 2GPI antibodies such as anti-CL.^{35,36} The binding of anti-CL to the oxLDL/ β 2GPI complex keys macrophage uptake of the oxLDL and foam cell formation. The atherosclerotic disease process is therefore accelerated as these foam cells eventually give rise to arterial plaque formation.

Cardiolipin is a mitochondrial phospholipid found in mammalian tissues and eukaryotic organisms and is also produced by some prokaryotic bacteria. Its location in the inner mitochondrial membrane suggests an integral role in normal electron transport and energy metabolism.³⁷ Also, periodontal bacteria including *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* have been found to possess a peptide sequence similar to one on the β 2GPI molecule.^{38,39,40} As such, periodontal infection could stimulate antibody production that is cross-reactive with the β 2GPI molecule. In addition, there is evidence that β 2GPI by itself is immunogenic³⁸ possibly compounding these responses.

There are other explanations for the possible association between anti-CL and cardiovascular disease. For example, this molecule has also been implicated in arterial and venous thrombosis. It has been suggested that a significant proportion of thrombotic episodes can be attributed to this and other anti-phospholipid antibodies since

approximately 50% of stroke patients under age 50⁴¹, and up to 20% of idiopathic DVT patients⁴² are positive for these types of antibodies. β 2GPI is a natural anticoagulant⁴³ and under normal conditions attaches to vessel wall endothelium preventing thrombus formation. When anti-CL are targeted against β 2GPI, patients are at an increased risk of developing venous/arteriole thrombosis through disruption of coagulation homeostasis. Such events are common in patients with a condition known as antiphospholipid syndrome which is known for its high incidence of arterial and/or venous thrombosis, recurrent fetal loss, and neurologic disorders which commonly develop. It should be noted however that although anti-CL antibodies are seen in 2 to 4% of the general population, clinical manifestations are not always found.

Increasing evidence over the past 10 years suggests a link between periodontal disease and atherosclerosis. An analysis of the literature devoted to this topic was done by Scannapieco.⁴⁴ Using strict inclusion criteria, 31 studies were selected for review. Four case control studies reported a positive association between indicators of poor oral health and atherosclerosis while 1 showed no association. Eleven of 15 cross-sectional studies report a modest association between periodontitis and cardiovascular disease while 4 show an association with stroke and 1 with peripheral vascular disease. He concluded that periodontitis appears to be associated with atherosclerosis induced diseases.

The aim of this investigation was to determine whether SRP would lead to a reduction in serum antibodies potentially involved in cardiovascular diseases; specifically, IgG anti-PC, IgG anti-CL, and IgM anti-CL. If plaque bacteria are responsible for these

systemic responses then serum levels of these antibodies should decrease following periodontal therapy.

METHODS

Study Population

Seventeen patients presenting for treatment of generalized chronic periodontitis were chosen to participate in this study. The patients were required to be between the ages of 35 and 60, have a minimum of 5 teeth per quadrant, and a minimum of 7 teeth with ≥ 5 mm of attachment loss. Attempts were made to recruit a racially balanced population of both males and females. Exclusion criteria included AIDS and other auto-immune disorders, immunosuppressant medications, hepatitis or other infectious diseases, pregnancy, disabling cardiac or coronary disease, and donation of 450 MLS (one unit) of blood within 8 weeks prior to the study. All subjects signed informed consent forms acknowledging their willingness to participate in the study. This study was approved by the Institute Review Board of Virginia Commonwealth University.

Patients received full mouth scaling and root planing performed over 2 separate appointments spaced 2 weeks apart. A blood sample was drawn from each patient prior to scaling and root planing at the first and second appointment and again at a third visit that was 8 weeks following the second appointment. Collected blood samples were processed for serum and stored at -20°C . At the first and third visit a clinical examination was performed to measure probing depth (PD), attachment loss (AL), bleeding index (BI), gingival index (GI), and plaque index (PI).

ELISA for anti-PC IgG

Measurement of anti-PC concentrations in serum was carried out using a modification of the method previously described.⁴⁵ Immulon 4 HBX ELISA (Dynatech) plates were coated with 0.1 ml of PC-BSA diluted to 1.25 µg of PC/ml in 0.005 M phosphate buffer, pH 7.5, and incubated at room temperature, with shaking, for 15 min. Plates were washed two times, with shaking, in a solution containing 0.01 M phosphate buffer, 0.14 M NaCl, and 0.01% Tween 20 (Fisher) (PBS-Tween). Next, 0.1 ml of sample diluted in PBS-Tween was loaded into the coated wells and incubated at room temperature, with shaking, for 30 min. Following another two washes with PBS-Tween, 0.1 ml of peroxidase-conjugated, affinity-purified, rabbit anti-human IgG (Jackson ImmunoResearch) diluted 1/10,000 in PBS-Tween was added and incubated for 30 min, with shaking, at room temperature. The plates were then washed twice in PBS-Tween as above and then washed twice in dH₂O (to remove residual Tween). Then, 0.1 ml of 3,3',5,5'-tetramethyl-benzidine (100 µg/ml; CalBiochem)-0.006% H₂O₂ in 0.1 M acetate buffer, pH 6.0, was added and incubated at room temperature, with shaking, for 30 min, and 25 µl of 2.5 M H₂SO₄ was then added to stop the reaction and initiate the final color change. Plates were read at A₄₅₀ on a Molecular Devices Vmax enzyme-linked immunosorbent assay (ELISA) reader. Serum anti-PC levels from the patients were then compared to a standard sample for interpolation.

ELISA for anti-CL IgG and IgM

Measurement of anti-CL concentrations in serum was carried out as directed using kits provided by Pharmacia Corporation (Kalamazoo, MI). Microplate wells were washed one time with 300µl of Wash Buffer for 20 seconds followed by removal of the Wash Buffer. One hundred µl of (cardiolipin IgG or IgM antibody) Calibrators, Controls, and diluted (1:101) patient serum were dispensed into appropriate wells and incubated for 30 minutes followed by removal from the wells. The plates were then washed 3 times with Wash Buffer followed again by liquid removal from the wells. Anti-Human IgG or IgM Conjugate (100 µl) was then added to all wells followed by another 30 minute incubation. Wells were then emptied of liquid and the washing procedure was repeated 3 more times. One hundred µl of Substrate TMB was then placed into all wells and incubated in the dark for 10 minutes followed by the addition of 50 µl of Stop Solution into each well. Absorbance was read at 450 nm on a Molecular Devices Vmax enzyme-linked immunosorbent assay (ELISA) reader. Serum anti-CL levels from the patients were then compared to a standard for interpolation.

Data Analysis

To determine the effect of scaling and root planing on serum levels of IgG anti-PC, IgG anti-CL, and IgM anti-CL repeated measures ANOVA was used to analyze the log of mean measurements recorded for each visit. Log conversion was performed because the outcome data was skewed making it difficult to perform parametric analysis. Multiple regression

analysis was performed to adjust for demographic variables including age, race, sex, smoking status, and visit number. A significance level of 0.05 was used.

RESULTS

Demographic Results and Clinical Parameters

Table 1 provides the patient demographics. Of those that participated in the study, 7 were females and 10 were males. There was a total of 4 African American and 13 Caucasian participants. Smoking status was broken down into three groups; smoker (Y), never smoked (N), and former smoker (F). There were 9 smokers, 4 who had never smoked, and 4 former smokers. Clinical parameters recorded at visits 1 and 3 are reported in Table 2. Differences in PI and GI between visit 1 and 3 reached significance with a *P* value of 0.0009 and 0.0064 respectively.

Serum anti-PC IgG measurements

Serum levels of IgG anti-PC are given in Table 3. At visits 1, 2, and 3 these levels were 69.39 µg/ml, 61.03 µg/ml, and 63.37 µg/ml respectively with a standard error of ±11.81 µg/ml. The difference between these values reached significance (*P* <0.05). Table 4 shows the results of multiple regression analysis of the effects of demographic variables on these serum levels. Visit number reached significance (*P* 0.045) while race, sex, age, and smoking status did not.

Serum anti-CL IgG measurements

Serum levels of IgG anti-CL are also given in Table 3. These levels were 6.11 U/ml, 6.14 U/ml, and 5.57 U/ml at visits 1, 2, and 3 respectively with a standard error of ± 1.30 U/ml. Significance was not reached ($P > 0.05$).

Serum anti-CL IgM measurements

Serum levels of IgM anti-CL can be found in Table 3. These levels were 5.31 U/ml, 4.87 U/ml, and 4.00 U/ml at visits 1, 2, and 3 respectively with a standard error of ± 1.52 U/ml. The difference between these values approached significance with a P value of 0.054. As measured by multiple regression analysis (Table 4), demographic variables did not significantly influence serum levels of IgM anti-CL ($P > 0.05$).

Table 1
Patient Demographics

Characteristic	Mean	SD
Age (years)	45.6	±7.23
Sex		
	n	%
M	10	59
F	7	41
Race		
		0
B	4	24
W	13	76
Smoker		
		0
F	4	24
N	4	24
Y	9	52

Abbreviations:

Sex: M = male, F = female

Race: B = black, W = white

Smoker: F = former; N = never smoked; Y = smoker

Table 2
Clinical Parameters at Visit 1 and Visit 3

Measurement	Visit 1	Visit 3	SE	P Value
PD	3.53	3.26	±0.18	0.1189
AL	3.04	3.00	±0.34	0.8557
PI	1.14	0.71	±0.13	0.0009
GI	1.59	1.23	±0.10	0.0064
BI	0.59	0.49	±0.07	0.1458

Table 3
Serum Levels of IgG anti-PC, IgG anti-CL, IgM anti-CL

IgG anti-PC ($\mu\text{g/ml}$)	
Visit	Mean
1	69.39
2	61.03
3	63.37
	Std Error ± 11.81
	<i>P</i> Value 0.042

IgG anti-CL (U/ml)	
Visit	Mean
1	6.11
2	6.14
3	5.57
	Std Error ± 1.30
	<i>P</i> Value 0.174

IgM anti-CL (U/ml)	
Visit	Mean
1	5.31
2	4.87
3	4.00
	Std Error ± 1.52
	<i>P</i> Value 0.054

Table 4
Multiple Regression Analysis of the Effects of Demographic Variables on Serum Levels of IgG anti-PC, IgG anti-CL, IgM anti-CL

IgG anti-PC	
Variable	<i>P</i> Value
Race	0.732
Sex	0.141
Smoke	0.240
Age	0.681
Visit number	0.045
IgM anti-CL	
Variable	<i>P</i> Value
Race	0.312
Sex	0.901
Smoke	0.702
Age	0.637
Visit number	0.057

Discussion

The relationship between poor dental health and atherosclerosis has become a topic of interest in health care.⁴⁶⁻⁴⁹ Clinical measures of periodontitis have been found to show a positive association with coronary heart disease⁵⁰⁻⁵⁵ and emphasis is now being placed on understanding the mechanism of interaction between periodontal disease and atherosclerosis. As such, this study has focused on the systemic influence of periodontal bacteria by measuring serum levels of anti-PC and anti-CL antibodies before and after SRP. A decrease in these values following such treatment would implicate periodontal bacteria as a source of these factors and help explain the association between oral infection and cardiovascular pathology. It has already been shown that anti-PC directed against periodontal pathogens is cross reactive with oxLDL³³ which could potentially lead to events that culminate in atherosclerotic plaque formation.

As expected, we found a decrease in serum IgG anti-PC from visit 1 to visit 3. The mean serum level prior to initiation of SRP was 69.39 µg/ml. At the 3rd appointment, 2 months following completion of the periodontal therapy, this was reduced to 63.37 µg/ml. The lowest serum level of IgG anti-PC was noted at the second appointment (61.03 µg/ml). The slight increase that occurred between visit 2 and 3 may be attributed to the 8 week period of time between these two visits which may have allowed recolonization of the diseased sites and a subsequent immune response. This could happen if patient plaque control was not adequate following completion of scaling and root planing. Loe found that with inadequate oral hygiene gingivitis can develop within 10-21 days and that this

gingival inflammation corresponds to increased prevalence in periodontal bacteria.⁵⁶ Also, with inadequate home care and periodontal maintenance, pathogens such as spirochetes return to baseline levels by 42 days.⁵⁷

Another explanation for the increase in serum antibody between the second and third appointments is the possibility that each SRP visit had an “immunization” effect due to instrumentation induced bacteremia which resulted in antibody production. In other words the periodontal treatment may have had a dual effect by decreasing IgG serum levels through reduction of PC bearing bacteria but also slightly increasing these levels due to a systemic exposure to the bacteria caused by the hand instrumentation.

The difference in serum levels of IgG anti-PC between the first and third appointment reached significance with a *P* value of 0.042. The effect of SRP on these systemic antibodies was not affected by race, sex, age, or smoking status as demonstrated by multiple regression analysis which failed to show any significant correlation between these variables and the antibody levels.

Also measured in this study were serum anti-CL IgG and IgM levels. Because these molecules are potential players in vascular plaque and thrombosis formation, their link to periodontal bacteria becomes an important issue. Like IgG anti-PC, these two factors experienced an overall decrease between visits 1 and 3 which approached significance for IgM anti-CL (*P* value 0.054) but was not significant for IgG anti-CL. The lack of significance can be explained in part by the small number of subjects that participated in the study. It is likely that increased statistical power with a larger number of patients would have resulted in statistically significant reductions in IgM antibody levels. As with anti-PC,

multiple regression analysis failed to show any significant correlation between race, sex, age, and smoking status and anti-CL levels.

Our serum measurements of IgG anti-CL were 6.11 U/ml, 6.14 U/ml, and 5.57 U/ml at visits 1, 2, and 3 respectively. The corresponding IgM anti-CL values were 5.31 U/ml, 4.87 U/ml, and 4.00 U/ml. The fact that these values are so small may be a reason why they change so little following treatment. Amoros⁵⁸ categorized patients based on IgG anti-CL levels: Patients with low (<20 U/ml) antibody titer and patients with medium to high (≥ 20 U/ml) antibody titer. Of 87 patients, 61 were placed in the low titer group and only 26 in the medium to high group. It can be assumed that significant antibody induction must be present in order to produce a titer ≥ 20 U/ml. The patients in this study were not selected based upon their antibody levels prior the treatment and only about 20% of periodontitis patients have anti-CL levels >15 U/ml. Since a number of our patients lacked severe periodontitis the induction of anti-CL may have been limited whereas greater disease severity may have lead to higher titer levels. It should also be mentioned that auto-immune diseases such as systemic lupus erythematosus have been linked to the presence of serum anti-CL. In our study, by selecting patients that lacked major systemic diseases, we eliminated other etiologies of anti-CL antibodies which may explain why our subjects expressed these low levels. However, such patient criteria are necessary in order to isolate the significance of periodontitis in anti-CL formation.

It is noted that IgG anti-PC showed a more dramatic response to the periodontal treatment than did IgG anti-CL. In fact, IgG anti-CL levels were virtually unchanged during the 2 week period between visits 1 and 2. The difference between these two

antibody responses can be attributed to the fact that IgG anti-PC is an IgG2 response which shows a more rapid decline following antigen removal. Therefore, by the 2nd appointment the IgG anti-PC levels should show greater reduction in serum levels than the IgG anti-CL levels.

The fact that SRP resulted in a decrease in anti-CL antibodies provides evidence that oral bacteria are a source of these antibodies. Even though it has been found that individuals infected with other bacteria and viruses possess these circulating antibodies⁵⁹ there has been minimal evidence prior to this study linking periodontal bacteria to these molecules. For example, *H. pylori*, which is known for its colonization of gastric mucosa, has been found to increase the risk of coronary heart disease.⁶⁰ It has also been found that there is a 33% prevalence of antibodies reactive with β 2GPI in patients with *H. pylori* infections.⁶¹ Other bacterial infections that may trigger the production of these antibodies range from spirochetes infections such as syphilis and Lyme disease to those involving non-periodontal staphylococcal and streptococcal organisms.⁶² The current study now provides some insight into the role periodontal bacteria may play in this immune response.

Both PI and GI showed statistically significant reductions from visit 1 to visit 3 while PD and BI measurements decreased only slightly. Higher values of these clinical parameters at visit one would indicate more disease severity which may have led to higher initial serum levels of the measured antibodies and possible greater reductions in these titers following SRP. The mean PD at visit 1 and visit 3 was 3.53mm and 3.26mm (respectively) while the mean AL measurements was 3.04 mm and 3.00 mm. These numbers represent whole mouth means and therefore include many non-diseased sites

which would not respond to periodontal therapy. This may explain the minimal change in PD and AL that occurred with treatment. The mean PI, GI, and BI at visits 1 and 3 were 1.14 and 0.71, 1.59 and 1.23, and 0.59 and 0.49. These clinical parameters suggest minimal inflammation and plaque. Not only may this explain why there was minimal change in PD and AL following SRP but it also suggests that there may have been a more dramatic change in serum antibody levels had the patients exhibited greater disease severity with corresponding plaque and inflammation.

A possible flaw in the study design could have been excluding the measurement of clinical parameters such as PI, GI, and BI at the second appointment. Because of this it is not possible to determine changes in periodontal health or patient home care at the second SRP appointment. Instead, we are only able to determine how these indices differ between appointments 1 and 3. This makes it difficult to relate the slight increase in serum IgG anti-PC between visits 2 and 3 to patient plaque control.

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