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Endotoxemia and the host systemic response during experimental gingivitis

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Abstract

Aim—To assess endotoxemia episodes and subsequent changes in serum inflammatory biomarkers using the experimental gingivitis model

Materials and Methods—Data from 50 healthy black and white adult males and females were compared for serum concentrations of endotoxin, and serum biomarkers [neutrophil oxidative activity, interleukin (IL)-1 β , IL-6, IL-8, C-reactive protein, and fibrinogen] at baseline, at 3 weeks of experimental gingivitis, and after 2 weeks of recovery. Means were compared using repeated measures ANOVA.

Results—Endotoxemia was reported in 56% of the serum samples at three weeks of induced gingivitis. At two weeks of recovery, endotoxin levels decreased to levels similar to those reported at baseline. Neutrophil oxidative activity increased significantly following three weeks of gingivitis versus baseline ($p < 0.05$). In the endotoxin-negative group this increase was associated with the black subjects whereas in the endotoxin-positive group change in neutrophil activity was driven by the female subpopulation. Serum cytokines, CRP, and fibrinogen levels did not change during the study.

Conclusions—Experimental gingivitis was associated with endotoxemia and hyperactivity of circulating neutrophils, but not with changes in systemic levels of cytokines and acute phase proteins. This may be attributed to the mild nature and the short duration of the induced gingivitis.

Keywords

dental plaque; experimental gingivitis; inflammatory response; endotoxin; neutrophils; cytokines; acute phase proteins

Introduction

There is wide-spread interest in studying the systemic impact of oral infection. Gingivitis is universal, reversible, and rapidly inducible. Thus, it provides an appropriate model for investigation of the host response to oral infection. It can be induced experimentally by the “Experimental Gingivitis Model” (EGM) (Löe et al. 1965).

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Conflict of interest

The authors declare that they have no conflicts of interest related to this study.

Gingivitis is associated with thinning of the sulcular epithelium and dilation of the gingival vasculature (Zoellner et al. 2002), thus facilitating invasion of the epithelial barrier by oral bacteria and/or their products into the systemic circulation. A distinctive bacterial product that can be found in blood is endotoxin, which is a cell wall component of Gram-negative bacteria. It has been shown previously that dental plaque maturation in the EGM is accompanied by a shift towards increasingly Gram-negative bacteria (Theilade et al. 1966) including periodontal pathogens of the red and orange complex species (Salvi et al. 2005). Indeed, it has been suggested that an infected periodontium is a major source of persistent endotoxemia, even as a consequence of gentle mastication (Geerts et al. 2002). Furthermore, periodontal pathogens have also been detected in atheromatous plaques and in abdominal aortic aneurysms, further supporting the occurrence of bacteremias/endotoxemias by oral bacteria (Haraszthy et al. 2000, Kurihara et al. 2004).

In addition to producing bacteremia/endotoxemia, we hypothesized that dental plaque accumulation may elicit a host systemic inflammatory response to the local dental plaque and/or bacteremia/endotoxemia. In this study potential changes in levels of serum cytokines and acute phase proteins as well as neutrophil oxidative activity as a result of gingivitis were assessed.

Peripheral blood neutrophils are the primary cells of the acute inflammatory response. Neutrophils can mediate tissue destruction in inflammatory diseases (Hansen 1995). Dental plaque accumulation and periodontal disease elicit a peripheral neutrophil response (Kowolik et al. 2001, Matthews et al. 2007, Wahaidi et al. 2009), which includes the release of cytotoxic oxidants and proteolytic enzymes, both of which possess detrimental tissue-damaging effects.

In response to infection, inflammatory cytokines promote a series of events, at the local infection site and systemically (Gabay & Kushner 1999). Dental plaque accumulation in both spontaneous and experimental gingivitis induces changes in gingival crevicular fluid cytokine levels (Ebersole et al. 1993, Offenbacher et al. 2010, Salvi et al. 2010, Trombelli et al. 2010) with potential for systemic dissemination (Ebersole et al. 1999). Human experimental endotoxemia is reported to induce changes in levels of serum cytokines (van Eijk et al. 2007, Dorresteijn et al. 2010). Systemic effects of these cytokines include production of acute phase reactants (Baumann & Gauldie 1994), leukocyte recruitment (Yoshimura et al. 1987), upregulation of endothelial adhesion factors (Munro 1993), and induction of procoagulation (Nachman et al. 1986).

An increase in serum levels of C-reactive protein (CRP) and fibrinogen occurs in response to infection including periodontal infection (Kweider et al. 1993, Noack et al. 2001, Beck & Offenbacher 2002, Mattila et al. 2005, Paraskevas et al. 2008). CRP has prothrombotic effects (Cermak et al. 1993) and activates complement (Volanakis 1982). Fibrinogen increases blood viscosity (Lowe et al. 1997), promotes platelet aggregation (Cook & Ubben 1990), and stimulates smooth muscle proliferation (Ishida & Tanaka 1982).

Systemic translocation of orally-derived endotoxin and the potential subsequent host systemic response has not been previously investigated in gingivitis, including the EGM.

This sub-study was part of a larger investigation (Subject N=128) that examined the systemic response to dental plaque accumulation, using the EGM in a population of black and white healthy adult male and female subjects (Wahaidi et al. 2009). The results of this study indicated that neutrophil activity increased in black but not white subjects in response to experimental gingivitis. No gender differences were noted. The aim of the present study was to compare data from a subpopulation (n=50) of the original study (Wahaidi et al. 2009), to determine if (1) dental plaque accumulation and the ensuing gingivitis results in

endotoxemia, and (2) whether the presence of endotoxin would result in race and gender-associated differences in a subsequent host systemic response.

Materials and Methods

Study Design

The original study has been described in detail previously (Wahaidi et al. 2009). The protocol was approved by the Institutional Review Board of Indiana University Purdue University Indianapolis/Clarian Health (approval number 0405-50). All participants provided written informed consent for participation. One week after all subjects had received a professional oral prophylaxis and oral hygiene (OH) instructions, they entered the three study phases (Table 1). In the “control phase” (weeks 0–3) subjects performed optimal OH practices. In the “experimental phase” (weeks 3–6), subjects refrained from all OH measures including the use of dentifrices, mouthrinses, dental floss, and gum. At the end of the experimental phase, which was also the start of the “recovery phase” (weeks 6–9) subjects received a professional oral prophylaxis and resumed normal OH practices (Table 1). Subjects visited the clinic weekly. During each visit, oral examinations were conducted, dental plaque and gingivitis were assessed, and peripheral blood samples were collected. Serum was separated and stored for laboratory analyses. The blood samples at week 6 were collected prior to the administration of oral prophylaxis.

Study Population

This sub-study analyzed data received from healthy adults (n=50; whites=34; blacks=16; males=22; females=28) (mean age: 24.7, range: 18–30 years) during weeks 3 (end of the control phase and beginning of the experimental phase), 6 (end of the experimental phase and beginning of the recovery phase), and 8 (2 weeks of the recovery phase) of the study. The subjects were in possession of a minimum of 20 natural teeth and showed no signs of periodontal disease (periodontal probing depths <4mm) or gross dental caries. Exclusion criteria included the current use of tobacco products, any medication known to affect the oral soft tissue or local/systemic response, or antimicrobial products within 3 months preceding the study.

Oral Examinations

Oral examinations and assessments of dental plaque and gingivitis were performed by a single trained and calibrated examiner (weighted Kappa for intra-examiner reproducibility >0.7) using the Plaque Index (PI) (Silness and Løe, 1964) and Gingival Index (GI) (Løe and Silness, 1963), respectively. Measurements were made on six sites per tooth (distobuccal, buccal, mesiobuccal, mesiolingual, lingual, distolingual) on all teeth present, with the exception of third molars.

Laboratory Analyses

Endotoxin assay—Serum endotoxin levels were assayed using a Limulus Amebocyte Lysate commercial kit. The ENDOSAFE® Endochrome-K™ test (Charles River Laboratories, SC, USA), a kinetic colorimetric assay, was used for quantification of endotoxin levels. All samples were processed in duplicate.

Serum biomarkers—Neutrophils were isolated by a density gradient centrifugation method (Wahaidi et al. 2009). Total neutrophil activity was measured via luminol-enhanced chemiluminescence using a 1251 Bio-Orbit Luminometer (Bio-Orbit, Turku, Finland) for 90 minutes (Wahaidi et al. 2009) and expressed in millivoltage.min. Samples were processed in triplicate.

Serum cytokine levels were assayed using commercial immunoassay kits. The LINCOplex™ multiplex immunoassays kit (Linco Research Inc., MO, USA) was used for quantification of IL-1 β , IL-6, and IL-8. Samples were processed in duplicate.

Plasma fibrinogen levels were measured using an automated clot detection system in the Clarian Health Laboratories. A commercial kit, the Active™ C-reactive protein ELISA (Diagnostic Systems Laboratories Inc, TX), was used for quantification of CRP. Samples were processed in duplicate.

Statistical Analysis

Means and 95% confidence intervals were calculated. Comparisons between means of the endpoints of the three different phases were performed using repeated measures ANOVA, using the log-transformed data due to the skewed distributions of the measurements. The level of statistical significance was set at 0.05.

Results

Serum Endotoxin

No measurable endotoxin was detected in weeks 4 and 5 of the experimental phase. The percentage of endotoxin-positive subjects at week 6 was overall 56% (whites: 59%, blacks: 50%, males: 50%; and females: 61%). In the endotoxin positive subjects mean serum endotoxin levels (EU/ml) were significantly higher at week 6 versus week 3 ($p<0.0001$) (Table 2) with a mean of 0.74 EU/ml for whites, 0.78 EU/ml for blacks, 0.61 EU/ml for males and 0.81 EU/ml for females. These levels decreased significantly at week 8 to levels similar to those reported at baseline ($p<0.0001$).

Plaque and Gingivitis

PI and GI increased significantly at week 6 versus week 3 ($p<0.0001$) in all subjects as reported previously (Wahaidi et al. 2009). This increase was independent of endotoxin status (Table 2). PI and GI decreased significantly at week 8 versus week 6 ($p<0.0001$), although week 8 PI values were still significantly higher than baseline ($p<0.001$).

Serum Biomarkers

Total chemiluminescence increased significantly during the experimental phase with a p value of <0.001 in the endotoxin-negative group and 0.009 in the endotoxin-positive group (Table 2). This change was dependent on race in the endotoxin-negative group with an increase of 5.88 in blacks ($p<0.001$) but only 2.95 in whites ($p=0.051$) (Fig. 1). In the endotoxin-positive group gender influenced overall neutrophil activity with a significant increase of 5.77 in females ($p<0.001$) versus a decrease in activity by 1.94 in males ($p=0.131$) (Fig. 1). Neutrophil activity for blacks ($p=0.038$) and females ($p=0.006$) returned to baseline levels during the recovery phase.

During the course of the study, no statistically significant changes were observed in levels of the analyzed cytokines ($p>0.05$). Moreover, there were no significant differences in the frequency (defined as a measurable concentration versus no detection) of detection of cytokines between endotoxin-positive and endotoxin-negative subjects ($p>0.05$) (data not shown). High individual variability in the pattern of cytokine profiles was observed (data not shown).

Levels of serum CRP and fibrinogen did not change significantly during the study course for any of the groups ($p>0.05$).

Discussion

To our knowledge, this is the first report that directly links gingivitis to endotoxemia. In this study, levels of systemic endotoxin increased significantly following 21 days of dental plaque accumulation in the majority of subjects, with no differences between individuals of different race/gender. As anticipated, resolution of gingivitis following treatment resulted in the reduction of endotoxin to baseline levels.

Only endotoxemia was evaluated in this study, not bacteremia. Endotoxemia is a surrogate marker for Gram-negative bacteremia. Gram-negative bacteria comprise a significant portion of the mature subgingival plaque flora (Theilade et al. 1966). Whereas endotoxemia occurred at a high frequency in a very short period of time in the present study, only low levels of bacteremia in individuals with periodontal disease and particularly, gingivitis have been reported (Kinane et al. 2005, Forner et al. 2006). This may be due to the challenges associated with blood culture techniques.

Endotoxemia, at sufficient levels, is a potent immune and inflammatory stimulator (Guthrie et al. 1984, Lindemann et al. 1988), and may ultimately, in the long run, result in multiple organ system failure (Brandtzaeg et al. 1989). With a mean of 0.72 EU/ml, the amount of serum endotoxin detected in this study was relatively low. This may be attributed to the low severity of gingivitis as a periodontal infection. Indeed, it has been shown that levels of endotoxemia are positively associated with the severity of periodontal disease (Geerts et al. 2002). Nonetheless, although mild, as a chronic insult gingivitis may cause persistent delivery of endotoxin into the systemic circulation. In fact, a systemic endotoxin level greater than 50 pg/ml (equivalent to 0.5 EU) in individuals with chronic infections has been identified as a risk factor for early atherogenesis (Wiedermann et al. 1999).

Endotoxin is known to stimulate neutrophil activity (Guthrie et al. 1984, Fittschen et al. 1988), although the effect of endotoxin on neutrophils varies depending on whether endotoxin is free or bound. Persistent neutrophil hyperactivity plays a role in tissue destruction (Di Filippo et al. 2007; Wittkowski et al. 2007). In this study, a systemic peripheral neutrophil response was noted following 3 weeks of dental plaque accumulation. We previously reported a heightened neutrophil response in blacks versus whites following 3 weeks of experimental gingivitis in healthy adults (Wahaidi et al. 2009). Results of the current study showed that the neutrophil response following dental plaque accumulation in blacks was independent of endotoxin status. In contrast, an association between endotoxin and neutrophil activity was observed as it relates to gender. Endotoxin-positive females showed a significant increase in neutrophil activity in week 6 that dropped back to baseline levels during the recovery phase. In contrast, neutrophil activity for males decreased in the experimental phase, although the change was not significant. In accordance with our findings, gender disparity has been reported in the acute neutrophil inflammatory response. In a rat model an increase in phagocytic activity in circulating neutrophils was noted in female but not male animals in response to an endotoxin challenge (Spitzer 1999). Furthermore, van Eijk et al (2007) reported a more pronounced inflammatory response in females in a human experimental endotoxin model. Sexual dimorphism in the innate inflammatory response including neutrophil biology has been associated with the effect of sex hormones (Miyagi et al. 1992, Molloy et al. 2003).

In the present study, experimental gingivitis and the subsequent endotoxemia were not associated with changes in serum levels of cytokines and acute phase proteins. However, high individual variability in the pattern of cytokine profiles was observed. In agreement, earlier studies demonstrated no association between clinical parameters of periodontitis and serum levels of cytokines including IL-1 β and IL-6, and a high variability of cytokine

profiles (Chen et al. 1997, Gorska et al. 2003). Likewise, the literature supports a gingival crevicular fluid cytokine response in both spontaneous and experimental gingivitis (Deinzer et al. 2007, Andriankaja et al. 2009, Salvi et al. 2010, Trombelli et al. 2010), but related changes in serum levels of cytokine have not been detected (Andriankaja et al. 2009, Trombelli et al. 2010). The inability to detect systemic cytokines may be explained by the mild nature of gingivitis (Trombelli et al. 2010) and the low level of the resultant endotoxemia. In contrast, changes in cytokine levels have been observed in induced endotoxemia (van Eijk et al. 2007, Dorresteijn et al. 2010) where serum endotoxin levels were significantly higher when compared to the present work.

Furthermore, it has been proposed that circulating levels of cytokines depend upon multiple factors including not just the type of stimulant but also the duration of exposure to the stimulant (Di Padova et al. 1991). Hence, failure to detect significant changes in serum inflammatory markers may also be attributed to the short duration of the observed endotoxemia. It is important to note that increases in serum endotoxin levels were reported at week 6 of this study, but not at week 5. In fact, it is very likely that an extension of the experimental phase by an additional week would have resulted in an increase in number of endotoxin-positive subjects and possibly endotoxin concentration. Instead, the short time-frame used in our study was probably insufficient to induce prolonged endotoxemia and an ensuing systemic response. Such a finding may be of importance in designing future studies to further understand the temporal aspect of the potential systemic response to endotoxemia following the development of gingivitis.

It should be noted that, although well-controlled, the conditions of the EGM differ from those of spontaneous gingivitis (Deinzer et al. 2007). The EGM involves meticulous OH measures, followed by complete neglect of OH for 21 days. In comparison, during spontaneous gingivitis dental plaque, in some areas, accumulates for a longer duration and some OH is practiced, which results in transient bacteremia (Silver et al. 1977). It is conceivable that endotoxin levels would have been much higher in the present study had the blood samples been collected immediately after the subjects received dental prophylaxis in week 6. These discrepancies between the EGM and spontaneous gingivitis may affect the nature and level of the resultant bacteremias/endotoxemias and the systemic host response.

Although the results from this study did not show a definitive host systemic response to dental plaque accumulation, there were findings of potential clinical relevance. The development of gingivitis was the origin of systemic endotoxemia and hyperactivity of circulating neutrophils, the latter being associated with gender in endotoxin-positive subjects. The increase in neutrophil activity observed in this study could be linked to the local oral infection (i.e. gingivitis), to the circulating bacteria/endotoxin, or to both. As a chronic insult, spontaneous gingivitis may cause persistent delivery of endotoxin into the systemic circulation. In the long-term, this “little and often” endotoxemia and inflammatory response may cumulatively cause detrimental systemic effects. The disparity in the race/gender systemic neutrophil response, in the endotoxin-positive and endotoxin-negative groups, is worthy of further investigation.

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Fig. 1.

Mean ($\pm 95\%$ confidence interval) peripheral blood neutrophil total activity (chemiluminescence) by race and gender at weeks 3 (baseline), 6 (experimental phase), and 8 (recovery phase). Comparisons between means of the control, experimental and recovery phase within each group were performed using repeated measures ANOVA. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$

Table 1

Overview of the clinical procedures performed during the study

	7 Days prior to study			Control Phase			Experimental Phase			Recovery Phase		
Week of Study	0	1	2	3	4	5	6	7	8	9		
Blood Draw	X	X	X	X	X	X	X ^c		X	X		
Plaque and Gingivitis Assessment	X	X	X	X	X	X	X		X	X		
Dental Prophylaxis												
Oral Hygiene Practices	X	X	X	X					X	X		

Only data from weeks 3, 6, and 8 were analyzed in this sub-study.

^a same day of study.

^b same day of study.

^c The blood sample at week 6 was collected prior to the administration of oral prophylaxis.

Table 2

Mean, 95% confidence intervals, and significance for study outcomes over the course of the study. Comparisons between means of the control and experimental phase, and the experimental and recovery phase within each group were performed using repeated measures ANOVA.

Weeks	Endotoxin-negative				Endotoxin-positive			
	3	6	8		3	6	8	
Endotoxin (EU/ml)	<0.08	<0.08	<0.08		<0.08	0.74*** ¹⁾ (0.47, 1.00)	<0.08***	
PI	0.15 (0.13, 0.18)	2.12*** (2.0, 2.24)	0.37*** (0.32, 0.41)		0.14 (0.12, 0.16)	2.08*** (2.0, 2.17)	0.40*** (0.34, 0.45)	
GI	0.46 (0.42, 0.49)	1.22*** (1.16, 1.28)	0.50*** (0.47, 0.54)		0.41 (0.38, 0.44)	1.16*** (1.11, 1.22)	0.47*** (0.44, 0.51)	
Total Neutrophil Activity (millivoltage.min)	5.47 (4.20, 6.74)	9.44*** (7.21, 11.7)	8.51 (6.28, 10.8)		5.77 (3.81, 7.74)	8.39*** ²⁾ (5.88, 10.9)	5.54 (4.78, 6.31)	

1) *** p<0.001,

2) ** p<0.01