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Localized Aggressive Periodontitis Immune Response to Healthy and Diseased Subgingival Plaque

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Abstract

Aim—The objective of this case-control study was to compare the inflammatory response of peripheral blood from localized aggressive periodontitis (LAP) patients when stimulated with healthy or diseased plaque samples.

Materials and Methods—Whole blood and subgingival plaque samples were collected from 13 LAP subjects, 14 siblings of LAP subjects and 6 periodontally healthy individuals. Whole blood was stimulated for 24 hours with plaque samples generated from healthy or diseased sites. The levels of 14 cyto/chemokines were detected using multiplex technology.

Results—LAP derived cultures displayed higher levels of GCSF, INF γ , IL10, IL12p40, IL1 β , IL-6, IL-8, MCP-1, MIP-1 α and TNF α , than control cultures regardless of stimulus used. Whole blood from healthy siblings displayed a higher levels of IL-6 compared to control subjects but lower levels than those observed in cultures from LAP participants.

Conclusions—This study suggests that although bacteria is an important factor in eliciting the hyper-inflammatory response observed in LAP patients, the predisposition of host's response to bacterial presence may play a more significant role than the components of the stimulatory plaque.

Keywords

aggressive periodontitis; endotoxin; cytokines; chemokines; microbiology

INTRODUCTION

Periodontitis is a multifactorial disease resulting in destruction of teeth-supporting structures. Aggressive periodontitis (AgP) is a severe form of periodontitis which involves

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Conflict of Interest

No conflicts of interest reported by any of the authors.

rapid alveolar bone resorption, where it often occurs early in life and has a tendency towards familial aggregation (Diehl et al., 2005, Marazita et al., 1994, Vieira and Albandar, 2014, Li et al., 2004). Epidemiological evidence estimates that AgP affects 0.1%–7.6% of children and adolescents worldwide (Albandar and Tinoco, 2002). In the US, African-Americans are reported to have the highest prevalence of any other ethnicity (Albandar and Tinoco, 2002).

An important factor implicated in periodontal disease initiation is the host's immune response to bacteria. Individuals with localized aggressive periodontitis (LAP) display a heightened immune response when whole blood is challenged with periodontopathic bacteria or their byproducts (Shaddox et al., 2010), which could be also attributed to specific cells, such as neutrophils (Kantarci et al., 2003), dendritic cells (Kikuchi et al., 2004) or mononuclear cells (Shapira et al., 1994b, Shapira et al., 1996) and similar immune response defects is observed in families of diseased individuals (Nakagawa et al., 1996, Shaddox et al., 2010, Van Dyke et al., 1985). Furthermore, It is suggested that disease onset may be due to biofilm composition shifting to include more gram negative anaerobes (Berezow and Darveau, 2011). Gram negative organisms are capable of releasing various toxins, including lipopolysaccharides (LPS), which activate innate and adaptive immunity, perpetuating the cycle of disease (Radvar et al., 2008). Studies have associated several specific microorganisms with LAP including *Aggregatibacter actinomycetemcomitans* (Aa), *Tannerella forsythia*, *Peptostreptococcus micros*, *Eikenella corrodens*, *F.Alocis* and *Porphyromonas ginigivalis* (Botero et al., 2007, Nonnenmacher et al., 2001, Shaddox et al., 2012, Zambon et al., 1983). However, a direct observation of the type of the immune response triggered by a complex subgingival plaque in this particular disease has not been described.

Therefore, the objective of this study was to evaluate the immunological response to health and disease-associated-plaque in LAP compared to periodontally healthy subjects. Our hypothesis is that LAP patients will present a hyper-response compared to healthy individuals regardless of stimulant used.

MATERIALS AND METHODS

Participant Population and Clinical Parameters

This is case-control study to comparatively investigate host response to different subgingival plaque in LAP versus healthy subjects. All data and samples collected were obtained under participants' informed consent, which was approved by the Institutional Review Board at the University of Florida (Gainesville, FL). Participants were recruited from the Leon County Health Department (Tallahassee, Florida) from May 2010 to October 2011. Inclusion criteria were: African-Americans, 5–21 years old, diagnosed with LAP [characterized by having at least 2 sites with 5mm pocket depth (PD) with 2mm clinical attachment level (CAL) and radiographic bone loss on first molars and/or incisors (Armitage 1999)], or periodontally healthy subjects [characterized by having no PD 5mm and absence of CAL and bone loss]. Exclusion criteria were: subjects diagnosed with systemic diseases or conditions or currently taking medications that could influence the progression and/or clinical characteristics of periodontal diseases; having taken antibiotics within the last 3 months; tobacco user; or

pregnant/lactating. This study included 13 LAP subjects, 14 healthy siblings of LAP subjects, and 6 unrelated age-matched healthy controls.

Complete medical and dental histories were recorded along with periodontal clinical parameters including: PD, CAL, bleeding on probing (BoP), presence of plaque, and radiographic examination to ensure proper diagnosis. All measurements were performed by a single calibrated examiner, using a UNC 15 probe, at six sites per tooth, and recorded by Florida probe software (Florida Probe Corp., Gainesville, FL, USA).

Subgingival Plaque Collection and Preparation

Bacterial subgingival plaque was collected from a periodontally diseased site in LAP-affected individuals (PD $\geq$ 5mm, CAL $>$ 2mm and BoP) (DD), and from a periodontally healthy site characterized by a PD of 2–3mm with no BoP in healthy participants (healthy siblings (HS), and healthy controls (HC)). After isolating the area of collection and removing supragingival plaque, subgingival plaque was collected using a sterile endodontic paper point (Henry Schein, Melville, NY) by inserting the point to the depth of the pocket and moving it laterally along the axis of the tooth. The paper point was placed in an eppendorf tube with Amies transport media (US Biological, Marblehead, MA.) on ice. Samples were then sonicated for 10 seconds in cool water and the optical density measured (Bio-Rad SmartSpec Plus Spectrophotometer, Hercules, CA, USA). Values were transformed into log counts using a standard curve and diluted to 10^4 /ml (see below).

In vitro stimulation and detection of cyto/chemokines

Four mL of peripheral blood was collected from each participant after which blood was diluted 1:4 in RPMI1640 (Mediatech, Manassas, VA, USA) and stimulated with 1ug/ml of toll-like receptor 4 (TLR4) agonist (*Escherichia coli* LPS) (InvivoGen, San Diego, CA), 10^4 bacteria from a healthy site (healthy sibling and control), 10^4 bacteria from a diseased site, or were left untreated. Blood cultures from each subject were stimulated with both their own plaque sample, as well as with a plaque sample from the opposite condition (ie disease subjects stimulated by their own diseased plaque as well as by a healthy unrelated individual plaque, and vice versa). A pilot study was conducted to determine 10^4 was the ideal concentration of bacteria to observe induction of inflammation at detectable levels even in healthy individuals and to avoid over exposure/stimulation (data not shown). Culture supernatants were collected 24hrs post-stimulation after which a cyto/chemokine detection kit (Millipore, St. Charles, MO, USA) was used to detect and quantify 14 cyto/chemokines: Eotaxin, IFN γ , IL-1 β , IL-2, IL-6, IL-8, IL-10, IL-12(p40), IP10, G-CSF, GM-CSF, MCP-1, MIP-1 α , TNF α . Briefly, culture supernatants and cytokine capture-bead cocktails were incubated overnight at 4°C and then incubated with biotin-labeled anti-cytokine for 1hr at room temperature. Streptavidin-Phycoerythrin was then added and samples were incubated for 30mins. Data were acquired using a Luminex®200™ and analyzed using Milliplex Analyst Software, (VigeneTech Inc, Billerica, MA), a standard curve and five-parameter logistics. Data were analyzed as stimulated levels minus unstimulated levels in pg/ml and values were log transformed to be presented in graphs.

Microarray analysis

DNA was isolated from all subgingival plaque samples using a DNA Purification kit following manufacturer's instructions (MasterPure, EPICENTRE Biotechnologies, Madison, WI, USA). After purification, DNA concentration was determined using Nanodrop technology (Nanodrop ND-1000 Spectrophotometer, Nanodrop Technologies INC, Wilmington, DE, USA). 200ng of each sample at a concentration of 20ng/μl were submitted to the Forsyth Institute for Human Oral Microbial Identification Microarray (HOMIM) analysis as previously described (Colombo et al. 2009). Briefly, 16S rRNA genes were PCR amplified 16S rRNA primers and labeled with Cy3-dCTP in a second nested PCR. 16S rRNA-based reverse-capture oligonucleotide probes are printed on aldehyde-coated glass slides and probed with labeled PCR products described above hybridized in duplicate. Microarray slides are scanned and crude data extracted using GenePix Pro software (Molecular Devices, Sunnyvale, CA, USA). A mean intensity for each taxon was calculated and signals normalized (Colombo et al. 2009). Signals greater than 1 were categorized into scores from 1 to 5, corresponding to ranked signal levels. Microbial profiles were generated from image files of scanned arrays using a HOMIM online analysis tool (<http://bioinformatics.forsyth.org/homim/>).

Statistics

Using previous data on inflammatory response reported previously (Shaddox et al 2010), we have established that a group of 6 individuals in each group would enable us to detect a 0.6 log difference in markers levels with a standard deviation of 0.3 with a 80% power (Sigma Plot, Systat, San Jose, CA). Statistical analyses were performed using GraphPad Prism software (GraphPad Software, La Jolla, CA). ANOVA analysis (Kruskal-Wallis with Dunn's multiple comparisons) was used for comparisons among experimental groups for clinical data and each cytokine evaluated as well as to compare data among healthy plaque, disease plaque and LPS stimulations among the experimental groups. A *p* value <0.05 was considered significant. A descriptive analysis was conducted for the bacterial profile of each subgingival sample used here.

RESULTS

LAP Hyper-Responsiveness to Subgingival Plaque Samples

The immune responsiveness of 13 LAP affected children, 14 healthy siblings of LAP participants, and 6 unrelated healthy controls was evaluated. Table 1 presents demographic and clinical data on all participants. Differences were found between LAP and healthy individuals for all clinical parameters, except for plaque. No differences were found among groups for age and gender.

Specifically, peripheral blood cell cultures were stimulated with plaque isolated from either periodontally diseased or periodontally healthy sites. We have previously published an LPS-hyper-responsiveness by LAP affected individuals (Shaddox et al. 2010), thus LPS stimulation was utilized as a positive control. Responsiveness of peripheral blood cell cultures was qualitatively and quantitatively evaluated by expression of several cyto/chemokines.

Peripheral blood cell cultures from participants with LAP were found to express higher levels of G-CSF, IFN γ , IL-10, IL12(p40), IL1 β , IL-6, IL-8, MCP-1, MIP-1 α , and TNF α , following stimulation with either diseased or healthy plaque, as well as LPS, when compared to peripheral blood cell cultures of unrelated healthy participants (HC) (Figure 1).

Interestingly, within the LAP cohort there was no significant difference in the levels of cyto/chemokines induced by plaque isolated from diseased sites when compared to those isolated from healthy sites (Figure 2). Similarly, there were no significant differences in the levels of cyto/chemokines induced by plaque when compared to LPS stimulation, with the exception of IL1 β (Fig. 2E). Here LPS induced higher levels of this marker when compared to plaque isolated from either healthy or diseased sites.

Very little responsiveness to plaque isolated from either periodontally diseased or healthy sites was observed in peripheral blood cultures from unrelated healthy control (HC) (Figure 2). On the other hand, LPS was able to induce expression of IL1 β , IL-6, IL-8, MCP-1, MIP1 α and TNF α in these cultures, albeit to a much lower extent than in cultures from LAP participants and their healthy siblings (Figure 1). Interestingly, LPS induced higher levels of IL1 β (Fig. 2E), IL-8 (Fig. 2G), and MIP1 α (Fig. 2I) in blood cultures derived from unrelated healthy control compared to levels from plaque isolated from either healthy or diseased sites. Similarly, LPS induced elevated levels of MIP1 α in blood cultures derived from healthy siblings (HS) (Fig. 2I) when compared to plaque from healthy and disease sites. In addition, there was a trend ($p=0.08$) for the same observation for TNF α release (Fig. 2J) within the unrelated healthy controls and healthy siblings cohorts.

Overall, the peripheral blood cultures from the healthy siblings (HS) of LAP patients behaved more similar to those from unrelated healthy participants (HC) than those of LAP participants, with the exception of IL6 and IFN γ . Here cultures from healthy siblings (HS) stimulated with plaque isolated from diseased sites had higher levels of IL-6 when compared to those of healthy unrelated controls (HC) (Fig. 1F). In addition, when stimulated with healthy plaque, these siblings displayed higher levels of INF γ compared to those of healthy unrelated controls (HC) (Fig.1B).

Composition of Periodontally Diseased and Periodontally Healthy Subgingival Plaque

The composition of the individual plaque samples was analyzed by HOMIM. The overall diversity of the DD samples was higher than either HS or HH samples, however, all 3 groups contained 69% Gram negative organisms and 39% Gram positive organisms. Both HS and HH samples had higher percentage of Gram negative organisms in the most prevalent species (60%), while DD samples had equal representation. The majority of the most prevalent organisms were shared across all 3 groups, including *Campylobacter gracilis*, *Gemella morbilorum*, and *Streptococcus oralis*, *anginosus* and *intermedius*. Unique to DD samples among the most abundant species were *Parvimonas micra* and *A. actinomycetemcomitans*, which were also highly abundant where present, while *Veillonella sp* was highly prevalent in HS and HH samples. Pathogenic organisms including *A. actinomycetemcomitans*, *Porphyromonas gingivalis*, *Filifactor alocis*, *Tannerella forsythia*, and *Treponema sp* were more abundant in DD samples than in HS or HH samples, and they tended to be often present in the same samples. Interestingly, the DD samples with more

pathogenic species present had overall higher species diversity, yet this was not true for HC samples. For a more comprehensive microbial analysis of this LAP population, please refer to our previous publication (Shaddox et al. 2012).

DISCUSSION

Periodontitis initiates with marginal inflammation of the periodontium (i.e. gingivitis) which is caused by bacterial insult to oral mucosa (Page and Schroeder, 1976). The progression from gingivitis to periodontitis depends on how the host responds to that bacterial insult, yet the mechanism(s) of progression are poorly understood. LAP is a unique form of periodontitis displaying rapid progression and a tendency to affect first molars and incisors. Due to its low prevalence, there are few studies focused on its mechanisms and etiology. In this study, our focus was on the host responsiveness to stimulation by complex subgingival plaque isolated from periodontally healthy and diseased sites. We have observed that LAP individuals have elicited a higher response to bacterial stimuli, regardless if the source of stimuli was coming from a diseased or a healthy subgingival environment.

The host inflammatory response to bacterial products released from the oral biofilm results in exacerbated inflammation and ultimately rapid bone destruction. The hyper-responsiveness described in LAP patients may be the reason they experience such rapid tissue destruction (Shaddox et al., 2010, Shapira et al., 1994b, Kantarci et al., 2003, Shapira et al., 1994a). Whether this hyper-response is directed against specific bacterial pathogens or particular bacterial components is not yet known. Evidence suggests that specific pathogenic bacteria and their unique structures may play a key role in LAP (e.g. *A. actinomycetemcomitans*, *P. gingivalis* or *E.coli* LPS), by eliciting a strong inflammatory response from the host (Nakamura et al., 2004, Shaddox et al., 2010, Shapira et al., 1994b), yet the complex polymicrobial nature of the oral biofilm makes it difficult to pinpoint the source of inflammation.

To better understand the role of subgingival plaque in LAP disease, we stimulated blood samples from healthy and diseased individuals with subgingival plaque samples taken from either healthy or disease sites. Stimulation with whole plaque from an oral site with specific clinical presentations rather than planktonically grown bacteria is a more clinical simulation of the natural disease process (Oscarsson et al., 2008). Previous research has shown that the interactions between bacteria co-existing in a mono-species biofilm induce different behaviors than observed in the planktonic state (Berezow and Darveau, 2011), as well as differences in behavior of different species between mono-species and poly-species biofilms (Zhu et al., 2013). Therefore, studying the interaction of different sources of stimulants contained in subgingival plaque with the host response may help us better understand the clinical inflammatory disease process (Berezow and Darveau, 2011).

Analysis of subgingival plaque samples revealed that *A. actinomycetemcomitans*, commonly identified in LAP (Botero et al., 2007, Cortelli et al., 2005, Slots et al., 1980), was both prevalent and abundant in the plaque from diseased sites, which is consistent with our previous comprehensive microbiological evaluation of this population (Shaddox et al. 2012), which also showed a strong association of *F. alocis* with LAP, yet other pathogenic

organisms are often present in samples with either or both *Aa* or *Fa*. Interestingly, the most notable difference between healthy and diseased subgingival plaque is not in the abundance or prevalence of pathogenic organisms, but in the species diversity. The diseased plaques exhibit greater species diversity than the healthy plaques, with a trend toward higher species diversity when more pathogenic species are represented. Within the diseased samples, species diversity is high, yet distinctly different. These findings indicate that high species diversity may be a more important factor in disease development and progression, which is in contrast to chronic periodontal disease, in which there appears to be a decrease in species diversity (Jorth et al., 2014, Socransky and Haffajee, 2005).

Intriguingly, no significant difference was observed in LAP individuals' response to the diseased site-associated plaque when compared to those of healthy site-associated plaque. While *E. coli* LPS (a TLR4 adjuvant) stimulation resulted in higher levels of IL1 β in LAP and healthy individuals when compared to health and disease-associated plaques, LAP individuals consistently showed higher levels of inflammatory markers when compared to healthy individuals, regardless of the stimulant used, which corroborates our previous report (Shaddox et al., 2010). Our previous investigation reports similar results when using *P.gingivalis* LPS (usually a TLR2 adjuvant) as a stimulant to whole blood cultures. Other studies have also showed *P.gingivalis* LPS, as well as serum of rapidly progressing periodontitis patients, influence hyper-responsiveness of neutrophils (enhanced FMLP-stimulated superoxide production) (Shapira et al., 1994a). Thus, the similarity in response to both types of subgingival plaque may be more due to a generalized or even intrinsic TLR hyper-responsiveness trait in these individuals, which may produce higher levels of inflammatory markers upon the presence of a stimulus, regardless of the type of stimulus.

Another possibility is that the role of the host's vulnerability (i.e. by exhibiting hyper-responsiveness to stimuli) supersedes the role of the pathogenic organisms in propagating the disease. Therefore, if hyper-responsiveness is intrinsic to these individuals, the role of genetic susceptibility in the LAP disease process may be more significant than previously realized. This is further supported by our finding that healthy siblings of LAP patients also demonstrated a strong inflammatory response. Additionally, other reports have shown other immune defects, such as neutrophil chemotaxis, present in nearly one-half of siblings of localized aggressive individuals, which further corroborates the possible genetic role of a specific host response in this disease (Van Dyke et al., 1985).

Thus, some healthy siblings of LAP patients may carry either a hyper-responsive trait/other host immune defect, which may indeed contribute to their predisposition to disease initiation in the future. However, longitudinal evaluation of this population and their responsiveness to bacterial stimuli is needed to confirm this hypothesis.

A possible limitation of this study is the fact that not all individuals were stimulated by the same source of subgingival plaque which could possibly result in reduced variations in the inflammatory responses. However, since whole blood had to be stimulated same day with a fresh subgingival plaque sample, the use of the same biofilm for all patients seen in different visits was unfortunately not feasible in the present study. However, looking at the composition of healthy versus diseased biofilms used here, healthy biofilms usually carried

the commonly health-associated species and the diseased biofilms contained the usual pathogenic bacteria, such as *A.actinomycescomitans*.

In conclusion, regardless of the nature of the subgingival plaque (disease or health-associated), LAP subjects display a hyper-inflammatory response compared to periodontally healthy participants. Furthermore, LPS was a more potent stimulant of the host response as compared to whole subgingival plaque cultures. And finally, periodontally healthy siblings of LAP individuals may be at a higher risk for disease initiation as they display a strong inflammatory response to bacterial stimulus as well. Further studies need to be conducted under different conditions to better understand whether plaque diversity or the presence of specific pathogens drives the immune response of these individuals.

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Clinical Relevance

Scientific rationale for the study

LAP patients exhibit hyper-responsiveness of whole blood to stimulation with specific Toll-like receptor agonists. This study examined whether LAP whole blood would exhibit a different inflammatory response upon stimulation by subgingival plaque from healthy and diseased sites as compared to healthy controls and siblings.

Principal findings

We found that LAP whole blood exhibited a hyper-inflammatory response to both health and disease-associated subgingival plaque, as well as to LPS stimulant, when compared to healthy controls. Differences among different stimulants were only found for a few cytokines and mainly in the healthy subjects where LPS produced the highest inflammatory response.

Practical implications

Treatments to reduce specific pathogens may not be sufficient to halt LAP disease progression, rather reduction of biofilm burden as a whole and/or alteration of inflammatory response may be more important for these patients.

Figure 1.

Plaque and LPS induced inflammatory responses among groups within each stimulant. Peripheral blood was diluted 1:4 in RPMI1640 and stimulated with either 1 μ g/ml of toll-like receptor 4 (TLR4) agonist (*Escherichia coli* LPS), 10⁴ bacteria from a healthy site, 10⁴ bacteria from a diseased site. HC = unrelated healthy control; HS = healthy siblings; LAP = localized aggressive subjects. * p < 0.05, ** p < 0.01, # p > 0.05 < 0.1 among groups within the same stimulant, by Kruskal-Wallis test. Error bars indicate standard error of the mean. Bars across columns show differences between groups by Dunn's multiple comparisons.

Figure 2.

Plaque and LPS induced inflammatory responses among stimulants within each group. Peripheral blood was diluted 1:4 in RPMI1640 and stimulated with either 1 μ g/ml of toll-like receptor 4 (TLR4) agonist (*Escherichia coli* LPS) (LPS), 10⁴ bacteria from a healthy site (HP), 10⁴ bacteria from a diseased site (DP). * $p < 0.05$, ** $p < 0.01$, # $p > 0.05 > 0.1$ by Kruskal-Wallis test. Error bars indicate standard error of the mean. Bars across columns show differences between groups by Dunn's multiple comparisons.

Table 1

Age and Clinical Parameters.

	Age	M/F	PD all	%PD>4mm	PD>4mm	Mean CAL	BoP (%)	Plaque (%)
LAP	13.85 ± 2.97	6/7	2.45 ± 0.49 [*]	14.85 ± 13.41 [#]	5.19 ± 0.66 [*]	3.77 ± 1.25 [*]	13.23 ± 5.60 [#]	38.77 ± 26.13
Siblings	11.64 ± 3.46	8/6	1.89 ± 0.36	4.29 ± 4.70	1.43 ± 1.99	0.19 ± 0.48	12.57 ± 7.31 [#]	34.64 ± 29.35
Controls	13.5 ± 4.93	4/2	1.86 ± 0.28	1.17 ± 1.94	1.33 ± 2.07	0.25 ± 0.42	4.50 ± 1.05	22.33 ± 8.41
p value	0.3145	0.6908	0.0052	0.0025	<0.0001	<0.0001	0.0079	0.5503

Means followed by standard deviations. LAP= Localized aggressive periodontitis; M=males; F=females; PD all = mean pocket depth in all sites (mm); %PD>4mm = % of sites with PD >4mm; PD>4mm = mean pocket depth in sites with PD > 4mm (mm); Mean CAL = clinical attachment loss (mm) in affected sites; BoP = % of sites with bleeding on probing; Plaque = % sites with visible plaque.

^{*} p<0.05 siblings vs. controls,

[#] p<0.05 vs. controls Kruskal-Wallis with Dunn's multiple comparison.