DNA METHYLATION OF THE INTERFERON-GAMMA PROMOTER IN ASSOCIATION WITH OVEREXPRESSION OF THE INTERFERON-GAMMA GENE IN PERIODONTAL DISEASE

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A thesis submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Master of Science in the Department of Periodontology, School of Dentistry.

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ABSTRACT

ANTONINO CRIVELLO: DNA METHYLATION OF THE INTERFERON-GAMMA PROMOTER IN ASSOCIATION WITH OVEREXPRESSION OF THE INTERFERON-GAMMA GENE IN PERIODONTAL DISEASE

(Under the direction of Dr. Shaoping Zhang, Dr. David W. Paquette, Dr. Silvana Barros, and Dr. Steven Offenbacher)

The pathogenesis of periodontitis at the biofilm-gingival interface is modulated by a cascade of innate immune mechanisms that create a cytokine network. The dental biofilm and resultant inflammatory response have the capacity to alter the host DNA chromosomal structure through epigenetic mechanisms. The major epigenetic modification in humans is DNA methylation. These modifications have the potential to permanently alter the local gene expression by inducing tissue localized epigenetic changes that can persist within cell lineages and represent a permanently altered gene expression pattern that can affect the metabolism of the tissues and the inflammatory response. The pro-inflammatory cytokine interferon-gamma (IFN-γ) is an important regulator of the innate immune response to oral pathogens. The objective of this study was to compare the level of DNA methylation at the IFN-γ promoter between healthy and inflamed gingival tissues and correlate those findings with IFN-γ expression levels.
ACKNOWLEDGEMENTS

The completion of this work was only possible with the help of several individuals to whom I am indebted to. I would like to thank Silvana Barros for her mentorship and support throughout this endeavor and to Steven Offenbacher for allowing me the opportunity to work within the setting of the Center for Oral and Systemic Disease. Further thanks are extended to David W. Paquette for his leadership and guidance throughout my graduate studies at the University of North Carolina at Chapel Hill. I would also like to thank Ray C. Williams for the all of the wisdom and knowledge that he shared with me during my residency. I would also like to express thanks to Shaoping Zhang who spent countless hours working with me and directing me through this study and to Kevin L. Moss who was always available to assist during the assembling of this thesis.

A status report of a phase II clinical trial has been included in this document evaluating the safety and efficacy of a chlorhexidine diacetate intraoral delivery system for the treatment of periodontal disease in subjects with human immunodeficiency virus. I would like to thank and acknowledge Sid Kalachandra, David W. Paquette, Holly R. Howell, Roland R. Arnold and Steven M. Van Scyoc for their support and collaboration in conducting this research.
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CHAPTER 1

INTRODUCTION

Periodontal diseases are amongst the most prevalent inflammatory conditions in humans (Oliver et al. 1998). The destruction of the tooth’s attachment apparatus has been attributed in part to a microbial etiology (Socransky et al. 1998) and to the host’s inflammatory response to these periodontopathogens (Offenbacher 1996). The bacterial insult at the biofilm-gingival interface results in the direct destruction of host tissues through bacterial virulence factors such as collagenases and leukotoxins, and indirectly through the activation of host inflammatory cells such as neutrophils and macrophages (Offenbacher 1996) which in response to the pathogenic biofilm release effector molecules such as prostaglandin E2 (Offenbacher et al. 1994) and proinflammatory cytokines such as interleukin-1beta (IL-1β) (Giannopoulou et al. 2003), tumor necrosis factor-alpha (TNF-α) (Ikezawa et al. 2005) and interferon-gamma (IFN-γ) (Dutzan et al. 2009). These signaling molecules modulate the inflammatory process through the autocrine and paracrine activation of inflammatory cells, through mechanisms of chemotaxis (Kebschull et al. 2008) and through the regulation of gene expression (Trombone et al. 2008). These inflammatory cascades culminate in the clinical manifestation of periodontal disease.

The pro-inflammatory cytokine IFN-γ has been associated with the chronic inflammatory lesion with conflicting reports regarding the predominant role of IFN-γ in
stable or active periodontal lesion (Takeichi et al. 2000; Lappin et al. 2001). Further functions of IFN-γ include a critical role in T-cell differentiation, specifically in T helper (Th)1-mediated immunity against viral and bacterial infections (Boehm et al. 1997). Th1 cells secrete IFN-γ, promoting the containment of the infectious stimulus via, in part through macrophage and neutrophil function.

The expression levels of IL-1α, IL-1β, IL-6, IL-8, TNF-α, Cluster Differentiation (CD)14, Toll-Like Receptor (TLR)2, and TLR4 in human gingival fibroblasts were found to be significantly higher in inflamed gingival tissues when compared to healthy controls when analyzed with DNA microarray analysis (Wang et al. 2003). Microarray analysis allows for the evaluation of mRNA expression of numerous genes simultaneously. This method of analysis can be used to compare two biological states in order to identify the differential expression of genes and related pathways between them. Papapanou et al. (2004) examined the gene expression profile of gingival tissues from 14 patients with chronic and aggressive periodontitis by microarray analysis. These authors reported differences in gene profiling between diseased and healthy sites within the same patient while no significant differences were noted between the gene expression signature of patients with aggressive and chronic periodontitis in this pilot study (Papapanou et al. 2004). Recently, IFN-γ gene expression was found to be upregulated in patients with chronic periodontitis (Gomes et al. 2006).

The role of epigenetics in the inflammatory response has been studied. Epigenetic modifications are regulatory mechanisms that do not involve the direct modification of the intrinsic nucleotide sequence information encoded within the DNA. Instead, they can involve alterations of nucleosomal histone proteins and of cytosine
residues in the DNA sequence. The methylation of DNA at cytosine-guanine (CpG) dinucleotides is a postreplication event catalyzed by DNA (cytosine-5)-methyltransferase (Rao et al. 2000) through the recognition of established methylation patterns during embryogenesis and reproduction of these patterns during the replication of adult cells (Razin et al. 1994). Genes that are active in a particular tissue or cell type tend to demonstrate decreased methylation of CpG dinucleotides, while those that are inactive are often characterized by a dense CpG methylation pattern (Rao et al. 2000). Hypomethylation of the proximal promoter region of the IFN-γ gene has been correlated with increased transcription of the gene product in human Th1 cell clones; hypermethylation of this region has been demonstrated to inhibit nuclear factor binding to the promoter region with an associated decrease in transcription (Young et al. 1994). Previous studies using methylation-sensitive restriction mapping techniques have identified significant CpG methylation sites within the IFN-γ promoter region. Several groups have studied CpG sites within the IFN-γ promoter that have been demonstrated to be associated with the epigenetic regulation of Th cell differentiation (White et al. 2002; White et al. 2006; Kwon et al. 2008). Frequently evaluated sites include those at positions -295, -186, -54, +122, +128 and +171 with respect to the start of transcription within the IFN-γ gene.

Epigenetic modifications are associated with chronic inflammatory conditions including rheumatoid arthritis and diabetes mellitus (Backdahl et al. 2009). Recently, it has been demonstrated in an animal model that the periodontal pathogens found in the dental biofilm and the subsequent inflammatory response also have the capacity to alter
the host DNA chromosomal structure through epigenetic mechanisms (Bobetsis et al. 2007).

The objective of this study is to characterize the DNA methylation pattern of the IFN-γ promoter region in healthy and periodontally-involved human gingival tissues and to assess differences in the mRNA expression levels between these “healthy” / non-inflamed and “diseased” / inflamed tissues.
CHAPTER 2

MATERIALS AND METHODS

Study Population

Following protocol approval by the University of North Carolina Institutional Review Board (IRB) at the University of North Carolina at Chapel Hill, gingival biopsy samples from 10 non-periodontitis (“healthy” / “non-inflamed” or minimally-inflamed) and 10 chronic periodontitis (“diseased” / “inflamed”) sites were collected from 20 different subjects. The “inflamed” (periodontitis) tissues were collected from sites demonstrating probing depths of 5 millimeters (mm) or greater, bleeding on probing and severe radiographic alveolar bone loss. “Non-inflamed” (non-periodontitis) gingival tissue was collected from sites with probing depths of 4 mm or less, no bleeding on probing and no radiographic evidence of bone loss. “Non-inflamed” tissue samples were collected during crown lengthening procedures while gingiva from the periodontally-involved sites was obtained during periodontal flap surgery. The inclusion criteria included subjects being adult males or females between the ages of 18 and 65 years (inclusive), having at least 20 teeth in the functional dentition, excluding third molars and that the subjects were in good general health. Exclusion criteria included individuals with a chronic disease presenting with oral manifestations, subjects exhibiting gross oral pathology, treatment with antibiotics for any medical or dental condition within 1 month prior to the screening examination, chronic treatment (i.e., two weeks or more) with any
medication known to affect periodontal status (e.g., phenytoin, calcium channel blockers, cyclosporin, coumadin, non-steroidal anti-inflammatory drugs) within one month of the study, ongoing medications initiated less than three months prior to the study (i.e., medications for chronic medical conditions must have been initiated at least three months prior to the study).

**DNA Isolation and Sodium Bisulfite Conversion**

Genomic DNA was isolated from collected gingival tissue samples using a DNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Genomic DNA was bisulfite treated using established procedures (Grunau et al. 2001). Briefly, 1 µg of genomic DNA in 45 µl of nuclease-free water was denatured at 42°C for 20 minutes with 5 µl of freshly prepared 3 M sodium hydroxide (NaOH). Saturated sodium bisulfite solution (5.20-5.69M) was freshly prepared by adding 5.4 g of sodium bisulfite to 8 ml of water and the pH adjusted to a final pH of 5 with the addition of 1.3 ml of a 3 M NaOH solution. Hydroquinone (0.22 g; Sigma H-9003) was dissolved in 10 ml of water and 500 µL of this solution was added to the dissolved sodium bisulfite solution resulting in a final concentration of 10 mM. 450 µl of the bisulfite-hydroquinone solution was added directly to the denatured DNA and the reaction was performed in the dark water bath at 55°C overnight. The DNA was then desulfonated using Wizard DNA Clean-up Systems (Promega) according to the manufacture’s protocol and resolved in 50 µl of 1 mM Tris-Cl pH 8. This solution was combined with 5.5 µl of a 3 M NaOH solution and incubated at 37°C for 20 minutes. The DNA was then neutralized with 40 µl of a 7.5 M ammonium acetate solution, precipitated with 300 µl of 100% ethanol and 2 µl of seeDNA. After overnight incubation at -20°C, the DNA was precipitated and washed
with 500 µl of 70% ethanol and dried. The DNA was finally resolved in 25 µl of 1mM Tris-Cl pH 8 and stored at -20°C.

**Amplification**

The detailed information of primers/sequencers used in PCRs can be found in Table 1. Five amplicons containing 6 CpG sites within the promoter region of IFN-γ were included in the methylation analysis. Individual PCR condition can be found in Table 2.

**Table 1: Primers/sequencers for amplification of bisulfate-treated DNA samples.**

<table>
<thead>
<tr>
<th>CpG site position</th>
<th>Primers</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Position -295</strong></td>
<td>Forward</td>
<td>5’-[Biotin] TTTGTAAGGGTTTGAAGGTTTTTATAAGAT-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-CAAACCCATTATACCCACCTACACCA-3’</td>
</tr>
<tr>
<td></td>
<td>Sequencer</td>
<td>5’-TTTTATACCTCCCCACTT-3’</td>
</tr>
<tr>
<td><strong>Position -186</strong></td>
<td>Forward</td>
<td>5’-TTAGAATGGTATAGGTGGTATATGG-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-[Biotin] TATTATTTAGGTTTTTCTTTAAACTCTC-3’</td>
</tr>
<tr>
<td></td>
<td>Sequencer</td>
<td>5’-GGGTATAGGTTTGTT-3’</td>
</tr>
<tr>
<td><strong>Position -54</strong></td>
<td>Forward</td>
<td>5’-GGGTTTTGTTTATAGTAAAGGATTTAAGG-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-[Biotin] AATCAAACAATAATACCTACCTCCTCTAA-3’</td>
</tr>
<tr>
<td></td>
<td>Sequencer</td>
<td>5’-TATTCTTTTTTTAAATTTGTG-3’</td>
</tr>
<tr>
<td><strong>Position +171</strong></td>
<td>Forward</td>
<td>5’-[Biotin] TTTTGATTTGATTGTTTGAATAAGAA-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-CATTTTCAACCAACAAACAAATACTATTAA-3’</td>
</tr>
<tr>
<td></td>
<td>Sequencer</td>
<td>5’-ACAACCAAAAAAACCC-3’</td>
</tr>
<tr>
<td>CpG site position</td>
<td>Primers</td>
<td>Sequences</td>
</tr>
<tr>
<td>-------------------</td>
<td>---------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Position +122-128</td>
<td><strong>Forward</strong></td>
<td>5’-[Biotin]TTTTGGATTTTGATTAGTTTGATATAAGAA-3’</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse</strong></td>
<td>5’-AAAAACCCAAAACCATAAAAAACTAAAA-3’</td>
</tr>
<tr>
<td></td>
<td><strong>Sequencer</strong></td>
<td>5’-CTAAAAAACCCAAAATATAACTTAT-3’</td>
</tr>
</tbody>
</table>

Table 2: PCR conditions.

<table>
<thead>
<tr>
<th>Amplicons</th>
<th>Denaturation (°C),T(min)</th>
<th>Denaturation (°C),T(min)</th>
<th>Annealing (°C),T(min)</th>
<th>Elongation (°C),T(min)</th>
<th>Elongation Step (°C),T(min)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CpG site</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-295</td>
<td>94,15</td>
<td>94,30</td>
<td>58,30</td>
<td>72,30</td>
<td>72,10</td>
</tr>
<tr>
<td>-186</td>
<td>94,15</td>
<td>94,30</td>
<td>54,30</td>
<td>72,30</td>
<td>72,10</td>
</tr>
<tr>
<td>-54</td>
<td>94,15</td>
<td>94,30</td>
<td>60,30</td>
<td>72,30</td>
<td>72,10</td>
</tr>
<tr>
<td>+122-128</td>
<td>94,15</td>
<td>94,30</td>
<td>55,30</td>
<td>72,30</td>
<td>72,10</td>
</tr>
<tr>
<td>+171</td>
<td>94,15</td>
<td>94,30</td>
<td>55,30</td>
<td>72,30</td>
<td>72,10</td>
</tr>
</tbody>
</table>

**Pyrosequencing**

PCR products (5-20 µL) were added to a mix consisting of 2 µL Streptavidin Sepharose HP™ (Amersham Biosciences), 40 µL binding buffer (Biotage) and high purity water to achieve a total volume of 82 µl. This mixture was shaken at 1200 rpm for 5 minutes at room temperature. Vacuum Prep Tool™ (Biotage) was used to prepare single-stranded PCR products according to the manufacturer’s instructions. The Sepharose beads with the single stranded templates attached were then released into a PSQ 96 Plate Low™ (Biotage) containing 12 µL annealing buffer (Biotage) with 0.25 µM corresponding sequencer (Table 1). Pyrosequencing reactions were performed in a
PSQ 96MA™ System (Biotage) according to the manufacturer’s instructions, with the PyroGold SQA™ Reagent Kit (Biotage). CpG site quantification was performed with methylation Software PyroQ-CpG™. Criteria for Pyrogram selection were as follows: sufficient peak height of >15 units (arbitrary units for light emission calculated by the software), symmetric peaks without any irregularities or side-peaks, wide reading length with a high reliability until the end of the sequence, and absence of any significant signals at the positions where a bisulfite treatment control was included or where control nucleotides were dispensed to check for unspecific background signals.

**Statistical Analysis**

The Wilcoxon Ranked Sum test was used for the comparison of median methylation levels of all CpG sites observed within each sample group and the resulting p-values were plotted against the start point of the window. The threshold for statistical significance was set at a p-value less than 0.05. All analyses were performed using SAS v.9.2. (SAS Institute, Cary, NC).

**Quantitative Real Time PCR**

Total RNA was isolated from all gingival tissues (n=20) with the use of the RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA from 2 µg of total RNA was synthesized using the Omniscript Kit (Qiagen) and random decamer primers. Real-time PCR was performed with 1 µl cDNA, 12.5 µl TaqMan Universal PCR mix, and 1.25 µl 20X Assay on demand gene expression assay mix (Mm00445273_m1 from Applied Biosystems, Foster City, CA), in a 7000 Sequence Detection System (ABI Prism, Applied Biosystems). Reactions were performed in duplicates and at two independent times. The ribosomal 18s (housekeeping gene) was used as an endogenous control.
Results were evaluated using the delta–delta Ct method, where delta Ct was calculated as (IFN-γ Ct) - (18s Ct), and the relative quantity of IFN-γ mRNA expression was calculated by the delta–delta Ct as $2^{-[(\text{infected sample Ct})-(\text{control sample Ct})]}$. 
A lower level of methylation was found at the six evaluated CpG sites within the IFN-γ promoter region of the “inflamed” gingival tissues compared to “healthy” samples. Figure 1 demonstrates two representative pyrograms of the methylation status of the IFN-γ promoter region at position -295 from healthy and periodontally-involved gingival tissue samples indicating that 56% of the amplification products from “healthy” tissue taken from 1 subject were methylated at position -295 (Figure 1A) while 34% of the PCR products from “diseased” tissue taken from 1 subject were methylated at position -295 (Figure 1B).
Pyrograms of the IFN-γ Promoter Region at Position -295 From Healthy and Periodontally-Involved Gingival Tissues

Figure 1: The pyrograms illustrate a control and test sample. Methylation levels of the CpG site at position -295 in the “non-inflamed” and “inflamed” gingival tissues analyzed with the Pyro Q-CpG system.

A) 56% of the amplification products from “non-inflamed” gingival tissue were methylated B) 34% of the PCR products from “inflamed” gingival tissue were methylated.
Table 1 illustrates the mean percentage (%) of DNA methylation and standard deviations (s.d.) at the evaluated 6 CpG positions within the IFN-γ promoter for the non-periodontitis and periodontitis groups.

Table 3: The mean % DNA methylation, standard deviation (s.d.) and statistical significance at the six CpG positions within the IFN-γ promoter region for the non-periodontitis and periodontitis tissue samples.

<table>
<thead>
<tr>
<th>Periodontal Status / CpG Position</th>
<th>Non-Periodontitis</th>
<th>Periodontitis</th>
<th>Statistical Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% DNA Methylation (Mean +/- s.d.)</td>
<td>% DNA Methylation (Mean +/- s.d.)</td>
<td>p-value</td>
</tr>
<tr>
<td>-295</td>
<td>52.5 +/- 5.7</td>
<td>42.7 +/- 9.7</td>
<td>0.002</td>
</tr>
<tr>
<td>-186</td>
<td>35.4 +/- 7.8</td>
<td>33.6 +/- 5.9</td>
<td>0.84</td>
</tr>
<tr>
<td>-54</td>
<td>59.7 +/- 4.6</td>
<td>58.0 +/- 6.1</td>
<td>0.55</td>
</tr>
<tr>
<td>+122</td>
<td>71.0 +/- 8.6</td>
<td>63.1 +/- 11.5</td>
<td>0.01</td>
</tr>
<tr>
<td>+128</td>
<td>74.9 +/- 4.4</td>
<td>71.1 +/- 7.7</td>
<td>0.03</td>
</tr>
<tr>
<td>+171</td>
<td>51.9 +/- 3.1</td>
<td>48.0 +/- 2.6</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Figure 2 demonstrates the relative levels of methylation at the 6 CpG sites for periodontitis and non-periodontitis tissues. The average percentage methylation for positions -295, -186, -54, +122, +128 and +171 for the non-periodontitis gingival tissues were 52.5%, 35.4%, 59.7%, 71.0%, 74.9% and 51.9%, respectively while for the periodontitis samples, the percentage methylation for the same positions were 42.7%, 33.6%, 58.0%, 63.1%, 71.1% and 48.0%, respectively. The difference in the level of methylation was statistically significant for nucleotide positions -295 (p=0.002), +122 (p=0.01), +128 (p=0.03) and +171 (p=0.03) but not significant for positions -186 (p=0.84) and -54 (p=0.55).
Figure 2: The percentage of methylation in each individual CpG site from periodontitis and non-periodontitis gingival tissues. Methylation levels were consistently lower at all of the evaluated CpG sites within the IFN-γ promoter region in the periodontitis tissues when compared to the non-periodontitis samples.

The overall level of methylation for the IFN-γ promoter was statistically significantly (p=0.01) lower for the periodontally-involved gingival samples (Figure 3). For the non-periodontitis samples, the median percent methylation value was 57% with a maximum value of 87% and a minimum value of 25% methylation. The median percent methylation for the periodontitis gingival samples was 51.5% with a maximum of 84% and a minimum of 22%.
The level of mRNA expression was greater for “inflamed” tissues than “non-inflamed” tissues with the difference not statistically significant (p=0.28). The healthy “non-inflamed” gingival tissues demonstrated a 1.6 +/- 0.5 fold increase in mRNA expression while the “inflamed” periodontal tissues had an increase in IFN-γ mRNA expression of 2.4 +/- 0.6 fold relative to periodontally-healthy gingival samples (Figure 4). The gingival tissues from the periodontitis group had a 1.6 fold increase in IFN-γ gene expression compared to the healthy gingival control.
Figure 4: The levels of mRNA expression between the periodontitis and non-periodontitis samples demonstrated a relatively greater level of expression from the “inflamed” tissues.
CHAPTER 4
DISCUSSION

Differential methylation patterns within the IFN-γ promoter region between diseased periodontal tissues and healthy gingiva were associated with differing levels of IFN-γ gene expression. In this study, periodontally-involved human gingival tissue samples were consistently hypomethylated relative to healthy gingival samples with respect to CpG sites within the IFN-γ promoter. Overall, the promoter region of periodontally-inflamed tissues was relatively hypomethylated. Furthermore, the level of mRNA expression of the IFN-γ gene tended to be greater for “inflamed” tissues than “non-inflamed” healthy tissues.

Periodontitis is an infectious chronic inflammatory state with demonstrable levels of pro-inflammatory cytokines at a systemic level in serum and locally in gingival tissues and in gingival crevicular fluid (GCF). The levels of IL-1β, TNF-α and IFN-γ have all been found to be significantly elevated in serum samples, gingival tissue biopsies and GCF from periodontitis patients (Stashenco et al. 1991; Lee et al. 1995; Gorska et al. 2003). Recently, Dutzan et al. demonstrated higher cytokine IFN-γ levels in the GCF and greater IFN-γ expression in gingival tissues from progressive periodontal lesions in patients with chronic periodontitis (Dutzan et al. 2009).

The production of IFN-γ promotes the phagocytic containment of the infectious stimulus via containment through macrophage and neutrophil function. The neutrophil is the primary effector cell in the inflammatory response to the bacterial biofilm
(Offenbacher 1996). The peripheral blood neutrophils isolated from patients with both chronic (Fredriksson et al. 2003) and aggressive (Gronert et al. 2004) forms of periodontal disease have been characterized as demonstrating a hyperactive phenotype termed hyperresponsivity. These hyperresponsive neutrophils have been proposed to elicit a component of the tissue damage seen in periodontal diseases. Wright et al. noted an increased expression of a significant number of IFN-stimulated genes from hyperresponsive peripheral blood neutrophils isolated from chronic periodontitis patients relative to periodontally healthy controls (Wright et al. 2008). Elevated peripheral blood type I IFN levels in periodontitis patients demonstrated the potential to serve as a priming factor that could contribute to neutrophil hyperresponsivity with respect to FcγR-mediated reactive oxygen species (ROS) generation (Matthews et al. 2007). IFN-γ has also been shown to “prime” for neutrophil ROS generation (Ellis et al. 2004).

The immune response includes a family of pattern-recognition receptors called Toll-like receptors (TLRs) that recognize pathogen-associated molecular patterns which are highly conserved structures present in large groups of microorganisms (Takeda et al. 2005). Bacterial lipopolysaccharide, peptidoglycan, bacterial DNA and double-stranded RNA are all examples of pathogen-associated molecular patterns (Rangsin et al. 2007). TLRs are predominantly expressed on cells of the innate immune system including neutrophils, monocytes/macrophages and dendritic cells. They are also present on gingival epithelial cells (Kusumoto et al. 2004). Ligand binding to TLRs initiates a cascade of signaling pathways that lead to the transcription of pro-inflammatory cytokines involved in the inflammatory response to the dental biofilm (Rangsin et al. 2007). TLR2 and TLR4 have been well documented and are associated with the
recognition of pathogen-associated molecular patterns in the lipopolysaccharide and fimbriae from *Porphyromonas gingivalis* and the lipopolysaccharide from *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans* and *Fusobacterium nucleatum*, respectively (Hirschfeld et al. 2001; Asai et al. 2001; Darveau et al. 2004; Mochizuki et al. 2004; Yoshimura et al. 2002). Uehara et al. demonstrated that expression of TLR4 on human gingival epithelial cells could be enhanced by treatment with IFN-γ (Uehara et al. 2002). The expression of CD14 (a TLR-related molecule: a co-receptor for lipopolysaccharide) is up-regulated in human gingival fibroblasts upon increased production of IFN-γ in inflamed periodontal tissues (Mochizuki et al. 2004). Upon stimulation with *Salmonella enteric* and *Aggregatibacter actinomycetemcomitans* lipopolysaccharide, IFN-γ-primed CD14 human gingival fibroblasts demonstrated increased production of the neutrophil recruiting chemokine, IL-8 via the enhanced CD14-TLR4 system (Tamai et al. 2002; Mochizuki et al. 2004).

Overexpression or prolonged production of IFN-γ may lead to direct host tissue destruction or indirect destruction through the activation of effector cells such as the macrophage (Boehm et al. 1997). IFN-γ stimulation of macrophages induces direct antimicrobial mechanisms as well as up-regulation of antigen processing and presentation pathways (Schroder et al. 2004). The effects of IFN-γ on bone metabolism have been contradictory. There is evidence to support an inhibitory function for IFN-γ with respect to the proliferation and differentiation of committed precursor cells into osteoclasts (Takayanagi et al. 2000). IFN-γ suppresses osteoclastogenesis by interfering with the receptor activator of nuclear factor κB ligand/receptor activator of nuclear factor κB (RANKL/RANK) signaling pathway indirectly by inducing the degradation of the RANK
adaptor protein, TNF receptor-associated factor 6, resulting in a strong inhibition of RANKL transcription (Takayanagi et al. 2000). Opposing evidence exists to support a decrease in the amount of trabecular bone and mineralization in osteoporotic mice with respect to elevated IFN-γ levels (Mann GN et al. Endocrinology 1994). IFN-γ is the physiologic inducer of the Major Histocompatibility Complex (MHC) II expression and thus of antigen presentation and consequently, IFN-γ leads to T cell activation and T cell secretion of the osteoclastogenic factors RANKL and TNF-α (Gao et al. 2007). IFN-γ has both direct and indirect anti-osteoclastogenic and indirect pro-osteoclastogenic properties in vivo. Gao et al. demonstrated that under conditions of estrogen deficiency, infection, and inflammation the in vivo effects of IFN-γ in a mouse model were of osteoclastogenesis and bone resorption (Gao et al. 2007).

The immune response to periodontopathic bacteria is in part determined by the balance between Th1 and Th2 cells (Gemmell et al. 2007). The division of Th cells into functional subsets is regulated by differential cytokine secretion (Mosmann et al. 1986). Th1 cytokines include IL-2, IL-12 and IFN-γ and culminate in a cell mediated response while the Th2 cytokines include IL-4, IL-5, IL-6, IL-10, and IL-13 and potentiate humoral immunity (Modlin et al. 1993). Th1 cells secrete IFN-γ and thereby activate macrophages, natural killer cells and CD8+ T-cells. Several studies have demonstrated a predominant Th1 cell response in the stable periodontal lesion and favor a Th2 cell response in active lesions (Lappin et al. 2001). Conflicting results have shown a predominance of Th1 cells or a reduction of Th2 cells in tissues demonstrating disease progression (Takeichi et al. 2000). Recent studies report the involvement of both Th1 and Th2 cells in periodontal disease sites (Prabhu et al. 1996). In a mouse model, both Th1
and Th2 cytokine knockout mice exhibited significantly more natural alveolar bone loss than their respective wild-type control mice with IFN-γ knockout mice demonstrating a two-fold increase in alveolar bone loss compared with wild-type control mice (Ivanovski A et al. 2007). The progression of gingivitis to periodontitis is accompanied by a shift from a predominantly T-cell lesion to a B-cell lesion (Page et al. 1976) so that it can be extrapolated that a shift from a predominantly cell-mediated immunity (Th1) to humoral immunity (Th2) occurs during the pathogenesis of periodontitis. It has been suggested that a susceptibility factor in the development of periodontitis may reside with T regulatory cells and whether they produce Th1 or Th2 responses to the bacterial biofilm (Kinane et al. 2007). Kikuchi et al. demonstrated that Aggregatibacter actinomycetemcomitans lipopolysaccharide-stimulated human monocyte-derived dendritic cells stimulated Th1 differentiation via IL-12 production (Kikuchi et al. 2004).

IFN-γ is the prototypical Th1 cytokine and is expressed from a locus which also contains genes encoding members of the IL-10 family located on chromosome 12 in humans (Bowen H et al. 2008). During Th1 cell differentiation, IL-12 signals via the IL-12R/STAT4-signalling pathway to induce IFN-γ expression. The secreted IFN-γ signals through the IFN-γR/STAT1 pathway to further increase IFN-γ levels resulting in a positive autoregulatory loop reinforcing Th1 differentiation. Transcription factors regulate gene expression by directly or indirectly being recruited to regulatory regions within a gene. The IFN-γ promoter contains multiple potential transcription factor binding domains for notably the activator protein 1 (AP1), cAMP response element binding protein (CREB) and YY1 (White GP et al. 2002).
Honda et al. compared the gene expression profile of inflammatory mediators including IFN-\(\gamma\) in gingivitis and periodontitis lesions. IFN-\(\gamma\) expression was demonstrated to be elevated in the periodontitis group (Honda et al. 2006). Gomes et al. evaluated the levels of mRNA expression in controlled diabetics with moderate and severe chronic periodontitis and found elevated levels of IFN-\(\gamma\) mRNA in two out of thirteen patients (15.4%) with moderate chronic periodontitis and five out of ten patients (50%) with severe chronic periodontitis (Gomes et al. 2006).

One recognized mechanism for gene regulation is the epigenetic regulation of gene expression which involves changes in nucleosomal histones and nucleotide residues in the DNA sequence (Rao et al. 2006). The methylation of DNA at cytosine residues within CpG dinucleotides is the major epigenetic modification found in humans. Transcribed or “active genes” within particular tissues or cells tend to be relatively hypomethylated while those that are not being readily transcribed, or “inactive genes” are often relatively hypermethylated (Rao et al. 2006). These modifications determine the level of cell type-specific gene transcription by modulating the accessibility of transcription factors, coactivator proteins, basal transcription apparatus, and RNA polymerase II to the gene promoter region (Rao et al.). Since most DNA methylation changes are not readily reversed and are usually conserved following DNA replication, these modifications have the potential to permanently alter the local gene expression by inducing local tissue-level epigenetic changes that can persist within cell lineages and represent a permanently altered gene expression pattern that can affect metabolism of the tissues and inflammatory responses (Rao et al. 2006).
Chronic inflammation is associated with genetic and epigenetic modifications (Stenvinkel et al. 2007; Backdahl et al. 2009). Genetic differences exist in the extent of the inflammatory response based on the levels of expression of inflammatory mediators such as IL-1 and TNF-α (Schenkein et al. 2002). Genetic polymorphisms in several genes have been demonstrated to affect the immune and inflammatory response to microbial insults and are associated with an increased risk for chronic and aggressive forms of periodontal disease. The IFN-γ gene contains a microsatellite polymorphism in its first intron. Two single nucleotide polymorphisms (SNPs) in this gene [Intron1 (760 T or A) and 30 (G or A)] combine with the microsatellite polymorphism to form two major haplotypes, I and II (Vandenbroeck et al. 2003). Homozygotes for alleles found on haplotype I produced 1.5–3.0 times more IFN-γ than did homozygotes for marker alleles on haplotype II (Hutchings et al. 2002). Lymphocytes from patients with tuberculosis stimulated with a disease-related antigen (Mycobacterium tuberculosis purified protein derived (PPD) antigen) demonstrated elevated levels of IFN-γ production in haplotype I versus haplotype II carriers over a course of more than six months (Lopez-Maderuelo et al. 2003).

Epigenetic modifications have been associated with systemic conditions. DNA methylation has been correlated to varying degrees with cancer (Laird 2005), diabetes mellitus (Poirier et al. 2001; Ling et al. 2008), atherosclerosis (Hiltunen et al. 2003) and rheumatoid arthritis (Takami et al. 2006). DNA methylation in human peripheral blood cells from chronic kidney disease patients correlated with different degrees of inflammation classified by C-reactive protein (CRP) levels (Stenvinkel et al. 2007). The association between chronic inflammation and DNA methylation exists both in the
presence and absence of microbial infection (Shames et al. 2007). Maekita et al. analyzed the effect of *H. pylori* infection on the quantity of methylated DNA molecules within eight regions of seven CpG islands in noncancerous gastric mucosae and examined its association with gastric cancer risk (Maekita et al. 2006). Gastric mucosae were collected from 154 healthy volunteers (56 *H. pylori*-negative and 98 *H. pylori*-positive) and 72 cases with differentiated-type gastric cancers (29 *H. pylori*-negative and 43 *H. pylori*-positive) by endoscopy. Among healthy subjects, methylation levels of all the eight regions were 5.4 to 303-fold higher in *H. pylori*-positives than in *H. pylori*-negatives (p < 0.0001). Methylation levels of the *LOX*, *HAND1*, and *THBD* promoter CpG islands and *p41ARC* exonic CpG island were as high as 7.4% or more in *H. pylori*-positive individuals. Among *H. pylori*-negative individuals, methylation levels of all the eight regions were 2.2- to 32-fold higher in gastric cancer cases than in age-matched healthy volunteers (p ≤ 0.01). Among *H. pylori*-positive individuals, methylation levels were highly variable, and that of only *HAND1* was significantly increased in gastric cancer cases (1.4-fold, p = 0.02). The group concluded that *H. pylori* infection potently induced methylation of CpG islands and that methylation levels of specific CpG islands in noncancerous gastric mucosae may be associated with gastric cancer risk in *H. pylori* – negative individuals. (Maekita et al. 2006). Distant infection with oral microorganisms has been demonstrated to lead to epigenetic alterations. Bobetsis et al demonstrated using a mouse model that pregnant mice exposed to infection with *C. rectus*, presented epigenetic modification in a promoter region of the insulin-like growth factor -2 (*igf2*) gene with specific increases in DNA methylation that played a critical role in IGF2 expression and subsequent fetal growth (Bobetsis et al. 2007).
In the present investigation, lower levels of methylation were found at all six CpG sites within the IFN-γ promoter for the periodontally-involved tissues compared to the healthy controls (Figure 2). The promoter region was relatively hypomethylated in “inflamed tissues” (Figure 3). IFN-γ mRNA expression levels for the periodontally-involved tissues were greater than for the “non-inflamed” samples (Figure 4). Potential transcription factor binding domains within the IFN-γ promoter region include the activator protein 1 (AP1), cAMP response element binding protein (CREB) and YY1 (White GP et al. 2002). Methylation of CpG sites corresponding to these regulatory regions could potentially affect the transcription of the IFN-γ gene. The decreased level of methylation within the promoter region of the IFN-γ gene seen in inflamed periodontal tissues allowed for greater expression of IFN-γ transcripts and consequently a greater expression of the pro-inflammatory cytokine within “diseased” tissues. It is hypothesized that the relatively hypomethylated state of the promoter region allows for more nuclear factor binding and promotion of transcription. Consequently, these findings demonstrate that inflamed periodontal tissues are hypomethylated compared to healthy gingival tissues within the IFN-γ gene promoter and subsequently produce more transcripts and accordingly more IFN-γ at the tissue level, findings consistent with reports of elevated levels of IFN-γ in the gingival tissues from progressive periodontal lesions in patients with chronic periodontitis (Dutzan et al. 2009). Approximately half of the CpG islands in the genome are not associated with annotated promoters, but are intra- or intergenic (Delcuve et al. 2009). These CpG islands may be associated with the transcriptional regulation of non-coding RNAs (Illingworth et al., 2008) as has been documented in various tumors demonstrating an abnormal expression of numerous micro RNAs.
miRNAs) linked to abnormal DNA methylation (Guil et al. 2009). miRNA are regulatory molecules that contribute to differential gene expression. Meng et al. demonstrated that the differential expression of a specific miRNA was associated with tumor cell growth in cholangiocarcinoma (Meng et al. 2008). The group recognized that overexpression of IL-6 was associated with an increased methylation activity via upregulation of expression of DNA methyltransferase enzyme DNMT-1. Further, miRNA expression profiling indicated that miRNA were aberrantly expressed in a methylation-dependent manner by IL-6 and that this differential expression of the miRNA was associated with tumor cell growth involving the mitogen-activated protein kinase (MAPK) cascade (Meng et al. 2008). As such, a mechanism was identified by which inflammation-associated cytokines may epigenetically modulate gene expression through DNA methylation of CpG islands not associated with annotated promoters and contribute to tumor biology.

The findings from this study imply a potential role for the epigenetic modification of genomic DNA in the pathogenesis of chronic inflammatory periodontal disease. Differential levels of methylation of the IFN-γ promoter region demonstrated differences in IFN-γ gene expression in diseased periodontal tissues in comparison to healthy gingiva. The evaluation of DNA methylation patterns of known inflammatory and anti-inflammatory genes may serve as a potential diagnostic tool in adjunct with current methods of diagnosing periodontal disease. With further research, it will be interesting to assess if these markers precede the clinical signs of periodontitis and allow for the detection of early and progressing lesions or the identifications of patients at a greater risk for periodontitis. Indications of this may be inferred by the fact that in this study,
clinically healthy sites demonstrated a baseline level of methylation at the IFN-γ promoter and that epigenetic control may be dose dependent. The modulation of DNA methylation as a means to regulate the inflammatory response to periodontal pathogens and subsequent destruction of the tooth’s attachment apparatus is a potentially interesting therapeutic modality for the management of periodontal disease. To date, the majority of research has been tailored to the development of demethylating epigenetic drugs in an effort to reactivate tumor suppressor genes. Hypermethylated targets have demonstrated therapeutic potential, notably in the treatment of cancer with the advent of inhibitors of DNA methyltransferases that restore the expression of pathologically silenced genes such as tumor suppressor genes. The prototype inhibitors of DNA methylation are the nucleoside analogues 5-aza-CR and 5-aza-CdR which are incorporated in place of cytosine into replicating DNA resulting in the formation of heritable demethylated DNA. Further studies are required to better understand the role of DNA methylation in periodontal inflammation.
CHAPTER 1
INTRODUCTION

Chlorhexidine (CHX) is a broad spectrum antimicrobial agent with demonstrated activity against both Gram-negative and Gram-positive bacteria, yeast, fungi, and viruses (Hugo et al. 1964; Matisheck et al. 1978; Hiom et al. 1992; Harrison et al. 1998). The delivery of CHX at therapeutic doses at a constant rate over an extended period of time is desirable for the treatment of chronic periodontal conditions. A novel intraoral delivery system has incorporated 2.5% (by weight) chlorhexidine diacetate (CDA) into an ethylene vinyl acetate (EVA) mouthguard and has demonstrated sustained concentrations of chlorhexidine for up to 14 days (Tallury et al. 2007).

A recent single-center, 5-week, open-label trial evaluated six medically healthy subjects with generalized moderate plaque-induced gingivitis who wore the EVA delivery system 12 hours per day for 21 days in the maxillary arch (Van Scoyoc et al. 2008). The group found that all patients tolerated the delivery system with 18 non-severe adverse events (AEs) recorded. All AEs were categorized as Grade 1 according to the Common Terminology Criteria for Adverse Events (CTCAE) v3.0 scale. The most frequently reported AEs were oral lesions. There were nine lesions in three subjects. Six
of the lesions were categorized as “unlikely related” or “not related” to the mouthguard due to patients reporting a history of recent non-specific oral trauma. Three lesions were categorized as “likely related” to the study mouthguard, and all three ulcerations were reported in the same subject over a 1 month time period. No treatment was required for any of the oral lesions, and all lesions resolved upon completion of the study. The second most reported AE was headache (7 reports in 3 subjects) and graded as either “not related” or “unlikely related” to the study mouthguard. The remaining two AEs were hematomas attributed to venipuncture, and no treatment was required. The delivery system did not appear to increase calculus or intra-oral discoloration indices. No significant changes in blood chemistries, hematology or urinalysis occurred during the course of the study. Ninety-four percent of serum samples had levels of CDA below the detectable limit of 0.010 µg/ml, and all samples were ≤ 0.012 µg/ml. Over the 21-day course of treatment, salivary CDA levels increased two hours post-dosing at all time points. Analysis of covariance revealed a significant decrease in mean pocket depth in the maxillary arch over 21 days (p<0.05) when controlling for changes in the mandibular arch. The group concluded that CDA was released from the EVA polymer over 21 days of dosing and that this drug delivery system was safe for human use with minimal systemic exposure. The group also noted that trends in the data suggested that the locally-released CDA may reduce the clinical signs of gingival inflammation.

Patients with deficient immune responses are susceptible to destructive and necrotizing forms of periodontal diseases (Ryder 2002). The long-term goal for the CDA-EVA mouthguard is the development of a sustained release intraoral delivery system for the treatment of human immunodeficiency virus (HIV)-associated oral infections. The
phase I trial demonstrated that the CDA-EVA mouthguard was safe for human use. The present phase II trial of this study is a single center, 14-week, double-blinded, randomized controlled clinical trial. The objectives of the study are to assess the safety of the CDA intraoral delivery system in subjects with HIV infection and periodontitis (necrotizing ulcerative or chronic) and further, to evaluate the efficacy of adjunctive CDA locally administered via an EVA copolymer mouth guard for the treatment of periodontitis in HIV subjects.
CHAPTER 2
MATERIALS AND METHODS

The present study protocol was approved by the University of North Carolina Institutional Review Board (IRB) at the University of North Carolina at Chapel Hill. After a baseline examination, HIV subjects were randomized to one of two groups: 1) periodontal debridement plus 2.5% CDA-EVA mouthguard (to be worn 12 hours per day for 12 weeks), or 2) periodontal debridement plus placebo (EVA copolymer only) mouthguard (to be worn 12 hours per day for 12 weeks). The clinical parameters evaluated included percent papillary necrosis, pocket depth, clinical attachment level, percent bleeding on probing, gingival index and plaque index measured at baseline, 6 and 12 weeks; intraoral pain assessments at baseline, 1, 3, 6, 9 and 12 weeks. Plaque samples and saliva were collected for DNA analysis of oral bacteria (subgingival, buccal mucosa and tongue dorsum sites) at baseline, 6 and 12 weeks and quantitation of salivary HIV-1 load at baseline, 6, and 12 weeks, respectively. At the time of this thesis, plaque and salivary samples were not analyzed. The first twelve subjects to enroll participated in a pharmacokinetic component of the trial (six HIV subjects randomized to 2.5% CDA-EVA mouthguard and six HIV subjects randomized to control EVA mouthguard). For these 12 subjects, the following additional procedures were performed at baseline during continuation of an inpatient visit: 5 ml of whole blood and 2 ml of whole unstimulated saliva were collected prior to dosing (within 15 minutes or immediately prior to insertion of the study mouthguard), the randomized study mouthguard was then inserted...
(dosing/time 0) and 5 ml of whole blood was collected 30 minutes, and at 1, 2, 3 and 6, 12, and 18 hours post-dosing while 2 ml of whole, unstimulated saliva, was also collected at 30 minutes, and at 1, 2, 3, 6, 12 and 18 hours post-dosing. At 12 hours (following blood and saliva collections), the study mouth guard was removed. At 24 hours post-dosing, approximately 5 ml of whole blood and 2 ml of whole, unstimulated saliva was collected (from Day 0). The study mouthguard was then re-inserted (dosing/time 0 for Day 1) and 5ml whole blood and 2ml unstimulated saliva was recollected at 2 hours post-dosing (from time 0 on Day 1).

Oral plaque samples were collected but were not analyzed at the time of this thesis. Samples were collected from subgingival sites (four sites each constituting the deepest pocket per quadrant identified at screening), buccal mucosa and tongue dorsum using a sterile curette. Samples were placed into a separate Eppendorf tube containing 150 µl of TE buffer (i.e., subgingival samples will be pooled). Plaque samples are going to be quantitatively and qualitatively analyzed for the presence of oral pathogens using checkerboard DNA-DNA analysis (Socransky et al. 1998). Briefly, 100 µl of 0.5 M NaOH are to be added to each sample vial and the suspensions boiled in a water bath for 5 minutes. The samples are then neutralized with 800 µl of 5-M ammonium acetate. The released DNA is placed into extended slots of a Minislot-30 apparatus (Immunetics, Cambridge, MA) concentrated onto a 15 x 15 cm positively charged nylon membrane (Boehringer Mannheim, Indianapolis, IN) and fixed to the membrane by cross-linking under ultraviolet light. The membranes are pre-hybridized and placed back in a Miniblotter 45-device (Immunetics) producing a 30 x 45 checkerboard pattern. The membranes are then blocked in buffer containing 1% casein in maleic acid buffer for 1
hour. The signals are detected by scanning the membranes at 1000 volts and 200 microns using a Storm Fluorimag3r (Molecular Dynamics, CA). The signals of the samples are converted to absolute counts by comparison with the standard lanes from the membrane. A total of 24 different strains will be analyzed at a sensitivity of $10^3$ cells including total viable counts (TVD) of each sample. The tested strains include both periodontal and non-periodontal species (P. gingivalis, P. intermedia, P. nigrescens, B. forsythia, T. denticola, A. actinomycetemcomitans, C. rectus, E. corroden3, F. nucleatum, P. micros, C. ochracea, V. parvula, S. intermedius, S. oralis, S. sanguis, B. ureolyticus, G. vaginalis, M. curtisii, P. bivia, L. crispatus, L. acidophilus, A. viscosis, S. noxia and H. pylori).

Analyses will include DNA techniques using whole chromosomal and oligonucleotide probes. Total organisms will be estimated using an oligonucleotide “universal” probe to conserved regions of bacterial 16S rRNA.

Whole unstimulated saliva (2 ml) was collected by expectoration and processed on ice within 2 hrs. Samples were stored at -80°C at the time of this report until analysis. Salivary HIV-1 RNA will be quantitated by the nucleic acid sequence-based (NASBA) NuclisensTm assay (Shugars et al. 2001).

At screening and Week 12, peripheral blood and urine samples were collected for laboratory assessments. Tests with abnormal, clinically significant values as judged by the study physician were repeated to rule out laboratory error. Peripheral blood (approximately 5ml sample) was analyzed for protein, bilirubin (total), alkaline phosphatase, lactate dehydrogenase, alanine transaminase, aspartate transaminase, blood urea, creatinine and serum electrolytes. In addition, a complete blood count (CBC) was performed to quantify hemoglobin, hematocrit, white blood cells including differential,
and platelets. Females of childbearing potential underwent urine pregnancy testing at baseline. Urine samples were analyzed (i.e., urinalysis with dip stick) for glucose, protein, pH and specific gravity.

Blood for pharmacokinetic plasma measurements was collected in appropriate containers (i.e., Vacutainer SST tubes). Blood collection tubes were prelabeled with the study number, subject number and the time of the sample. Five milliliters (5 ml ± 2 ml) of whole blood was collected for each pharmacokinetic sample. Once collected, blood samples were rocked gently to mix blood with the anticoagulant, and within 90 minutes were centrifuged at 4,400 RPM for 20 minutes. The harvested plasma was transferred to a fresh, labeled, screw top polypropylene tube and immediately stored at -80°C or below until analysis. For each sample, the subject number, accession number, date, and exact time of the sample was recorded on the appropriate case report form. An explanation was provided for any sample where the volume deviated by more than 2 ml. At the time of this thesis, blood samples for pharmacokinetic evaluation were not as yet analyzed. Plasma is to be analyzed for chlorhexidine diacetate concentrations using a high pressure liquid chromatography (HPLC) method validated at the appropriate quantitation level (Soskolne et al. 1998).

Whole, unstimulated saliva was collected via expectoration into a pre-labeled and pre-weighed vial every 60 seconds until an approximate volume of 2 ml was obtained per timed sample. Saliva samples (identified by study number, sample accession number) were immediately stored at -80°C or below until analysis. For each sample, the subject number, accession number, date, and exact time of the sample was recorded on the appropriate case report form. Saliva will be analyzed for chlorhexidine diacetate
concentrations using a high pressure liquid chromatography (HPLC) method validated at the appropriate quantitation level (Soskolne et al. 1998).

Baseline and demographic data will be summarized using descriptive statistics. Changes in clinical probing parameters (baseline to Weeks 6 and 12) will be expressed as mean (or median) values or severity scores per subject. Adverse events and oral soft tissue findings were summarized for the eleven evaluated subjects. Inter-group differences in mean (median) clinical and microbial values will be tested with t-tests for normally distributed values versus Kruskal Wallis tests for non-normally distributed values.
CHAPTER 3

RESULTS

At the time of this thesis, eleven subjects have completed the trial to date having been dosed with either a 2.5% chlorhexidine diacetate mouthguard or placebo mouthguard. Five potential subjects have undergone telephone screenings out of which 4 subjects have agreed to participate in the trial and have signed informed consent forms. From the eleven subjects that have completed the trial, clinical measures, bacterial, blood, urine and salivary samples have been collected. Further, subject diaries have been evaluated and all adverse events have been recorded. The blind has not been broken at the time of this thesis in lieu of an incomplete recruitment process.

Clinical chemistries from hematological and urine samples demonstrated no alterations from baseline (data not shown). Forty AEs have been recorded amongst the 11 dosed subjects. Thirty-eight were classified as Grade 1 while two AE were classified as Grade 2 according to the Common Terminology Criteria for Adverse Events (CTCAE). The reported AEs for each study subject are presented in Table 4 which indicates the date of AE reporting, AE description, possible relation to the study mouthguard, the date of AE resolution and the Grade of the AE according to the CTCAE.
Table 4: Reported adverse events by participating study subjects.

<table>
<thead>
<tr>
<th>Subject Number</th>
<th>Starting Date</th>
<th>Description</th>
<th>Relation to drug</th>
<th>Treatment</th>
<th>Date Resolved</th>
<th>Adverse Event Grade (CTCAE scale)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2001</td>
<td>6-13-08</td>
<td>Hyperkeratosis</td>
<td>Not related-patient reported trauma</td>
<td>None</td>
<td>7-25-08</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>7-7-08</td>
<td>Traumatized incisive papilla</td>
<td>Not related-patient reported trauma</td>
<td>None</td>
<td>7-25-08</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>7-25-08</td>
<td>Hematoma</td>
<td>Unlikely related-appeared to be from trauma</td>
<td>None</td>
<td>8-15-08</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>8-15-08</td>
<td>Ulceration</td>
<td>Unlikely related-appeared to be from trauma</td>
<td>None</td>
<td>8-21-08</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>8-12-08</td>
<td>Patient reported seizure</td>
<td>Not related-subject’s physician believes the event to be viral</td>
<td>None</td>
<td>8-19-08</td>
<td>2</td>
</tr>
<tr>
<td>2002</td>
<td>8-21-08</td>
<td>Nodule</td>
<td>Unlikely related-appeared to be from trauma</td>
<td>None</td>
<td>9-15-08</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>9-29-08</td>
<td>Ulceration</td>
<td>Not related-patient reported trauma</td>
<td>None</td>
<td>11-5-08</td>
<td>1</td>
</tr>
<tr>
<td>Subject Number</td>
<td>Starting Date</td>
<td>Description</td>
<td>Relation to drug</td>
<td>Treatment</td>
<td>Date Resolved</td>
<td>Adverse Event Grade (CTCAE scale)</td>
</tr>
<tr>
<td>----------------</td>
<td>---------------</td>
<td>------------------------------------------</td>
<td>--------------------------------------------</td>
<td>---------------------------------------------------------------------------</td>
<td>---------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>2003</td>
<td>10-30-08</td>
<td>Ulceration-buccal mucosa</td>
<td>Not related-patient reported trauma</td>
<td>None</td>
<td>11-17-08</td>
<td>1</td>
</tr>
<tr>
<td>2003</td>
<td>12-8-08</td>
<td>Sensitive Teeth</td>
<td>Unlikely related-patient has generalized gingival recession</td>
<td>Subject was treated with Gluma desensitizing agent on 12-22-08</td>
<td>12-22-08</td>
<td>1</td>
</tr>
<tr>
<td>2003</td>
<td>9-19-08</td>
<td>Headache</td>
<td>Unlikely related</td>
<td>Subject took 600 mg Ibuprofen</td>
<td>9-21-08</td>
<td>1</td>
</tr>
<tr>
<td>2004</td>
<td>9-29-08</td>
<td>Ulceration</td>
<td>Unlikely related-appeared to be from trauma</td>
<td>None</td>
<td>10-9-08</td>
<td>1</td>
</tr>
<tr>
<td>2004</td>
<td>10-9-08</td>
<td>Gingival inflammation -subject reported spontaneous bleeding</td>
<td>Not related-severe inflammation heavy plaque deposits</td>
<td>Study personnel reviewed Oral Hygiene Instructions</td>
<td>10-30-08</td>
<td>1</td>
</tr>
<tr>
<td>2004</td>
<td>10-9-08</td>
<td>Ulceration</td>
<td>Unlikely related-appeared to be from trauma</td>
<td>None</td>
<td>10-30-08</td>
<td>1</td>
</tr>
<tr>
<td>2004</td>
<td>11-19-08</td>
<td>Large red area on hard palate-no pain</td>
<td>Unlikely related-appeared to be from trauma</td>
<td>Study personnel reviewed Oral Hygiene Instructions</td>
<td>12-8-08</td>
<td>1</td>
</tr>
<tr>
<td>Subject Number</td>
<td>Starting Date</td>
<td>Description</td>
<td>Relation to drug</td>
<td>Treatment</td>
<td>Date Resolved</td>
<td>Adverse Event Grade (CTCAE scale)</td>
</tr>
<tr>
<td>----------------</td>
<td>---------------</td>
<td>--------------------------------------------------</td>
<td>------------------</td>
<td>-----------</td>
<td>---------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>11-14-08</td>
<td>Gingiva tender to palpation</td>
<td>Unlikely related</td>
<td>None</td>
<td>11-25-08</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>11-22-08</td>
<td>Sore mouth</td>
<td>Likely related</td>
<td>Subject did not wear mouthguard for 3 days</td>
<td>11-25-08</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>11-25-08</td>
<td>Ulceration</td>
<td>Unlikely related-appeared to be from trauma</td>
<td>None</td>
<td>12-19-08</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>11-25-08</td>
<td>Ulceration</td>
<td>Unlikely related-appeared to be from trauma</td>
<td>None</td>
<td>12-19-08</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>12-1-08</td>
<td>Sore Mouth</td>
<td>Likely related</td>
<td>None</td>
<td>12-2-08</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>12-19-08</td>
<td>Gingival edema</td>
<td>Probably not related</td>
<td>None</td>
<td>1-9-09</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>1-1-09</td>
<td>Gingiva tender to palpation and possible tissue sloughing</td>
<td>Likely related</td>
<td>None</td>
<td>1-3-09</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>1-8-09</td>
<td>Sinus Infection</td>
<td>Not related</td>
<td>Subject took Alka Seltzer Cold</td>
<td>1-12-09</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>1-9-09</td>
<td>Palpable lymph node</td>
<td>Not related</td>
<td>Subject took Alka Seltzer Cold</td>
<td>1-12-09/1-30-09</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>1-9-09</td>
<td>Erythematous soft palate and uvula</td>
<td>Not related</td>
<td>Subject took Alka Seltzer Cold</td>
<td>1-12-09/1-30-09</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Subject Number</td>
<td>Starting Date</td>
<td>Description</td>
<td>Relation to drug</td>
<td>Treatment</td>
<td>Date Resolved</td>
<td>Adverse Event Grade (CTCAE scale)</td>
</tr>
<tr>
<td>----------------</td>
<td>---------------</td>
<td>----------------</td>
<td>--------------------------------------------------</td>
<td>-----------------</td>
<td>---------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>2006</td>
<td>11-13-08</td>
<td>Ulceration</td>
<td>Not related-patient had not been dosed</td>
<td>None</td>
<td>11-19-08</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>11-19-08</td>
<td>Ulceration</td>
<td>Unlikely related-applied to be from trauma</td>
<td>None</td>
<td>12-22-08</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>12-22-08</td>
<td>Gingival petechiae</td>
<td>Unlikely related</td>
<td>None</td>
<td>1-12-09</td>
<td>1</td>
</tr>
<tr>
<td>2007</td>
<td>11-13-08</td>
<td>Ulceration</td>
<td>Not related-patient reported trauma</td>
<td>None</td>
<td>11-19-08</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>12-4-08</td>
<td>Ulceration</td>
<td>Unlikely related</td>
<td>None</td>
<td>12-5-08</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1-3-09</td>
<td>Cold</td>
<td>Not related</td>
<td>Over the counter cold medicine (Robitussin Mucinex)</td>
<td>1-7-09</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1-12-09</td>
<td>Ulceration</td>
<td>Not related-patient reported trauma</td>
<td>None</td>
<td>2-4-09</td>
<td>1</td>
</tr>
<tr>
<td>2008</td>
<td>11-18-08</td>
<td>Ulceration</td>
<td>Not related-patient reported trauma</td>
<td>None</td>
<td>11-25-08</td>
<td>1</td>
</tr>
<tr>
<td>Subject Number</td>
<td>Starting Date</td>
<td>Description</td>
<td>Relation to drug</td>
<td>Treatment</td>
<td>Date Resolved</td>
<td>Adverse Event Grade (CTCAE scale)</td>
</tr>
<tr>
<td>----------------</td>
<td>---------------</td>
<td>--------------------------------------</td>
<td>------------------------------</td>
<td>--------------------</td>
<td>---------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>2008</td>
<td>1-22-09</td>
<td>Swollen lymph glands (sublingual) and tonsils</td>
<td>Unlikely related</td>
<td>None</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Patient still enrolled in study; will follow-up at next appointment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2009</td>
<td>11-20-08</td>
<td>Ulceration</td>
<td>Not related - patient reported trauma</td>
<td>None</td>
<td>11-25-08</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>11-30-08</td>
<td>Facial abcess (Methicillin Resistant S. aureus)</td>
<td>Not related - physician diagnosed Methicillin Resistant S. aureus</td>
<td>Subject’s physician prescribed 800 mg Bactrim DS - subject discontinued from study due to antibiotic use</td>
<td>12-30-08</td>
<td>2</td>
</tr>
<tr>
<td>2010</td>
<td>11-6-08</td>
<td>Gingival irritation</td>
<td>Not related - patient had not been dosed</td>
<td>None</td>
<td>11-13-08</td>
<td>1</td>
</tr>
<tr>
<td>Subject Number</td>
<td>Starting Date</td>
<td>Description</td>
<td>Relation to drug</td>
<td>Treatment</td>
<td>Date Resolved</td>
<td>Adverse Event Grade (CTCAE scale)</td>
</tr>
<tr>
<td>----------------</td>
<td>---------------</td>
<td>-------------</td>
<td>------------------</td>
<td>-----------</td>
<td>---------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>2010</td>
<td>11-25-08</td>
<td>Bone spicule from previous extraction in gingiva (tooth was extracted and site had fully healed prior to enrollment)</td>
<td>Not related</td>
<td>Removed with cotton pliers</td>
<td>11-25-08</td>
<td>1</td>
</tr>
<tr>
<td>2010</td>
<td>12-15-08</td>
<td>Pain in mandible (extraoral and intraoral exams within normal limits/could not find cause for pain)</td>
<td>Unlikely related</td>
<td>None</td>
<td>1-26-09</td>
<td>1</td>
</tr>
<tr>
<td>2012</td>
<td>12-3-08</td>
<td>Sensitive Teeth</td>
<td>Not related-gingival recession and patient had not been dosed</td>
<td>None</td>
<td>12-15-08</td>
<td>1</td>
</tr>
<tr>
<td>2012</td>
<td>12-29-08</td>
<td>Floor of mouth tender to palpation</td>
<td>Unlikely related</td>
<td>None</td>
<td>1-23-09</td>
<td>1</td>
</tr>
</tbody>
</table>
The two Grade 2 AEs were facial abscess and seizure and were deemed “not related” to the study mouthguard. The subject experiencing the facial abscess (Subject Number 2009 in Table 4) was treated by his physician with an antibiotic regimen and was subsequently withdrawn from the study while the patient experiencing the seizure (Subject Number 2001 in Table 4) persisted in the study until completion with no further AEs. The other 38 AEs were categorized as Grade 1. Of the 40 AEs, three were deemed “likely related” to the mouthguard. These three lesions occurred in one subject (Subject Number 2005) at three different appointments (gingival tenderness to palpation and possible tissue sloughing and sore mouth). All other lesions were regarded as “not related” or “unlikely related” to the study mouthguard. The most frequently reported AEs were oral lesions. There were 29 oral lesions reported in the 11 subjects. These included ulceration (12 reports), tooth sensitivity (2 reports), gingival irritation (1 report), gingival petechiae (1 report), gingival edema (1 report), hyperkeratosis (1 report), floor of mouth tenderness to palpation (1 report), traumatized incisive papilla (1 report), hematoma (1 report) and nodule (1 report). Extra-oral AEs included headache (1 report) and pain in mandible (1 report) and lymphadenopathy (2 reports). Treatment was rendered in three cases: bone spicules were removed from a previous extraction site in one subject (Subject Number 2010). One subject’s physician prescribed 800 mg Bactrim DS for a facial abscess and the subject was subsequently discontinued from study due to use of antibiotics. Another subject was treated with Gluma desensitizing agent for dental sensitivity (Subject Number 2003). No treatment was required for any of the other oral lesions, and all lesions resolved upon completion of the study.
CHAPTER 4

DISCUSSION

Forty adverse events (AEs) were recorded in eleven subjects. Thirty-eight AEs were classified as Grade 1 and two as Grade 2 according to the Common Terminology Criteria for Adverse Events (CTCAE). The two Grade 2 AEs were facial abscess and seizure and were deemed “not related” to the study mouthguard. Of the 38 Grade 1 AEs, the most frequently reported AEs were oral lesions. There were 29 oral lesions reported in 11 subjects with “ulceration” as the most frequent. Only three of the recorded AEs were deemed “likely related” to the mouthguard while 37 of the remaining AEs were classified as “not related” or “unlikely related”. The three lesions “likely related” to the mouthguard occurred in one subject at 3 different appointments and consisted of gingival tenderness to palpation and possible tissue sloughing and sore mouth. To date, the CDA-EVA mouthguard has demonstrated to be relatively safe in HIV subjects with periodontal disease with regards to patient reported AEs.

Thirty subjects were in the original study protocol. Nineteen more subjects are required to participate in order to achieve the desired sample size that was predetermined for this phase II clinical trial of a 2.5% CDA-EVA controlled-release mouthguard. The recruitment process has identified five more subjects eligible for the study through telephone interviews/screenings out of which four potential subjects have signed informed consent forms and are available to commence the study.
Based on the reported adverse events and findings from hematological and urine sample analyses, the 2.5% CDA-EVA controlled-release mouthguard in the management of periodontal disease in subjects affected with HIV demonstrated relative safety in this phase II clinical trial. Only three of forty reported adverse events were deemed “related to the mouthguard”. No significant changes in blood chemistries, hematology or urinalysis occurred during the course of the study (data not shown). Analyses are ongoing with data from clinical, pharmacokinetic, microbial and salivary outcomes to be reported in the future. Recruitment of subjects meeting the outlined inclusion and exclusion criteria proved to be difficult. Subjects were being recruited from the hospital patient pool at the University of North Carolina and from the Department of Periodontology at the School of Dentistry. The timeline of the present study was subsequently recessed and at the time of this thesis, data from eleven of the desired thirty participants has been presented. The blind was not broken for this thesis and the study is currently ongoing.
REFERENCES


64. Rogus J, Beck JD, Offenbacher S. IL1B gene promoter haplotype pairs predict clinical levels of interleukin-1beta and C-reactive protein. Hum Genet 2008: 123; 387-398.


88. Vandenbroeck K, Goris A. Cytokine gene polymorphisms in multifactorial diseases: gateways to novel targets for immunotherapy? TRENDS in Pharmacological Sciences. 2003: 24(6); 284-289


