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Application of the checkerboard immunoblotting technique to the quantification of host biomarkers in gingival crevicular fluid

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Abstract

Background—The aim of this study was to describe the development and validation of the checkerboard immunoblotting (CBIB) technique for the high-throughput quantification of multiple inflammatory mediators in gingival crevicular fluid (GCF) samples.

Material and methods—Monoclonal antibodies were used to bind GCF IL-1 β , IL-8 and MMP-8 to the surface of membranes. Biotinylated antibodies were used to detect bound antigens in a checkerboard format. Signals were developed using chemiluminescence, captured on film and quantified using software for array analysis. The assay was tested for potential cross-reactions among the 3 pairs of antibodies. Eleven CBIBs were processed to determine the analytical sensitivity of the assay. Forty GCF samples were analyzed using CBIB and ELISA in parallel and significance of correlations among the results was tested using the Pearson correlation coefficient. 931 GCF samples were collected from 20 periodontally healthy and 20 periodontitis subjects and analyzed using CBIB in order to test the assay's sensitivity and dynamic ranges using clinical samples.

Results—The CBIB was capable of distinguishing among the 3 analytes. The sensitivity and dynamic ranges of the assay were suitable for the detection of the 3 targets in the majority of GCF samples. There were highly statistically significant ($p < 0.0001$) positive correlations between CBIB and ELISA data for all 3 biomarkers. The periodontitis subjects had statistically significantly higher mean levels of IL-1 β and IL-8 compared to healthy subjects.

Conclusions—The CBIB technique is a sensitive and specific assay for the high-throughput quantification of MMP-8, IL-8 and IL-1 β in GCF.

Keywords

Periodontal diseases; Gingival crevicular fluid; Cytokines; Chemokines; Matrix metalloproteinases

Periodontal diseases are initiated by bacterial species living in biofilms at or below the gingival margin and progress largely as a result of the inflammation initiated by specific subgingival species. The magnitude and quality of the host response to the oral bacteria seems to be a major determinant of disease severity¹ and several lines of evidence have demonstrated that immune mechanisms are responsible for the tissue destruction in periodontal diseases². The gingival crevicular fluid (GCF) contains several cellular and molecular components of the

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Key findings: the Checkerboard Immunoblotting technique is a sensitive and specific assay for the high-throughput quantification of MMP-8, IL-8 and IL-1 β in GCF.

immunological response present in serum, as well as mediators and by-products of tissue destruction generated within the tissues. Therefore, the GCF provides a non-invasive, easily collected fluid to measure *in vivo* the inflammatory mediators released during disease processes that affect the periodontal tissues³.

The limitations of commonly employed ELISA assays for measuring host markers of inflammation in GCF, reside in the small number of samples, subjects and host markers that can be conveniently analyzed concomitantly³. Further, a single periodontal site produces very little volume of GCF for analysis, typically 0.5 µl or less, requiring either pooling or dilution of the samples in order to provide enough volume for the analysis of several analytes. The checkerboard immunoblotting (CBIB) technique can potentially examine up to 45 GCF samples screened against up to 45 different inflammatory mediators, resulting in 2,025 antigen-antibody reactions on a single membrane. Hence, the levels of several targets in individual GCF samples could be measured at the same time. By examining GCF biomarkers at the site level one can explore site-specific correlations among these biomarkers and site-specific relationships among host response mediators and clinical and microbiological parameters.

Interleukin 1 beta (IL-1β), matrix-metalloproteinase 8 (MMP-8) and interleukin 8 (IL-8) were selected as initial targets during the development of the CBIB, based on their key role in the pathogenesis of periodontal diseases. Levels of GCF IL-1β have been found to be elevated in periodontitis patients^{4–6}, to be higher in progressing versus non-progressing sites⁷ and to decrease after periodontal therapy^{6–8}. In addition, blocking of this cytokine resulted in reduced radiographic bone loss in a primate model⁹. IL-8 assures the selective chemotaxis of neutrophils into the gingival crevice. This chemokine has been detected in GCF at higher levels in periodontitis patients compared to healthy individuals and in actively progressing sites compared to stable sites^{7,10}. Further, after scaling and root planing, GCF levels of IL-8 also tended to decrease¹¹. MMP-8 is one of the main collagen-degrading enzymes in GCF¹². GCF MMP-8 levels decrease as a consequence of periodontal therapy^{13,14} and the blocking of this enzyme through the use of selective cyclooxygenase inhibitors (meloxicam)¹⁵ and low dose doxycycline¹⁶ also resulted in clinical improvements in the periodontal condition.

Therefore, the purpose of the present study was to develop a technique based on the principles of the CBIB for the simultaneous quantification of IL-1β, MMP-8 and IL-8 in a large number of GCF samples.

Material and methods

Checkerboard Immunoblotting (CBIB)

Coating of polyvinylidene difluoride (PVDF) membranes with capture antibodies—PVDF membranes[‡] were pre-wetted in 100% methanol for 10 sec, washed in distilled water for 5 min, equilibrated in coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6) for 10 min and assembled in a MiniSlot®[§]. The PVDF membrane was laid on top of 10 layers of Whatman paper filters. A mixture of capture monoclonal antibodies: anti-human IL-1β (catalog # MAB601), anti-human MMP-8 (catalog # MAB208) and anti-human IL-8 (catalog # MAB908)^{||} (2 µg/ml of each), was loaded into each slot (150 µl/slot). The apparatus was disassembled, and the Whatman paper replaced by a plastic cushion. The membrane was then blocked with 0.5% Hammersten casein[¶] in TBS-T buffer [1% Tween 20 in Tris-buffered saline (20 mM Tris base, 137 mM NaCl, pH 7.6)] (1 ml/slot), for 1 h at room temperature.

[‡]Hybond-P, Amersham Biosciences, Arlington Heights, IL, USA

[§]Immunetics, Cambridge, MA, USA

^{||}R&D systems, Minneapolis, MN, USA

[¶]VWR International Ltd. Poole, England

Incubation of antigen standards and samples—In order to create a standard curve to compare signal intensities from unknown samples, a mixture of recombinant human IL-1 β , MMP-8 and IL-8^{||} was serially diluted (five 2-fold dilutions) in PBST (0.5% Tween 20 in Phosphate-buffered saline). The dynamic ranges for the 3 inflammatory mediators were: 7.0 to 150 pg; 6.3 to 100 ng and 18.8 to 600 pg for IL-1 β , MMP-8 and IL-8, respectively. Standards and samples (150 μ l/slot) were loaded into the MiniSlot and incubated for 2 h at room temperature. After incubation, samples and standards were aspirated from the slots, the MiniSlot was disassembled, the PVDF membrane released from the apparatus and washed 3 times for 10 min with 250 ml of TBS-T on a rotator. Nonspecific binding sites on the remaining surface of the membrane were then blocked with 120 ml of 1% Hammersten casein in TBS-T buffer by incubation at 4°C overnight.

Blocking of endogenous biotin—GCF samples contain biotin derived from bacteria present in the gingival crevice. This endogenous biotin can result in non-specific binding of streptavidin-horseradish peroxidase conjugate, increasing considerably both background and non-specific signals. Hence, endogenous biotin was blocked with a Streptavidin/Biotin blocking kit[#]. Membranes were incubated in 120 ml of a solution of streptavidin diluted in TBS-T (2 drops of streptavidin solution/10 ml of buffer) for 15 min at room temperature. After 3 $\times$ 10 min washes with 250 ml of TBS-T, the membrane was incubated in 120 ml of a solution of biotin diluted in TBS-T (2 drops of biotin solution/10 ml of buffer) for 30 min at room temperature.

Incubation of biotinylated detection antibodies—After 3 $\times$ 10 min washes with 250 ml of TBS-T, the membrane was loaded in a Miniblotter 45[®] apparatus[§], perpendicularly to the original orientation. Biotinylated goat anti-human antibodies to IL-1 β (catalog # BAF201), MMP-8 (catalog # BAF908) and IL-8 (catalog # BAF208)^{||}, diluted in blocking buffer, were pipetted into different channels of the Miniblotter (110 μ l/channel). Three 2-fold serial dilutions of each antibody were used: 1:1,000 to 1:4,000; 1:32,000 to 1:128,000 and 1:16,000 to 1:64,000 for IL-1 β , MMP-8 and IL-8, respectively. After 1 h of incubation at room temperature, the membrane was washed using a Manifold system[§] with 500 ml of TBS-T, followed by 3 washes with 250 ml of TBS-T on a rotator (10 min each).

Streptavidin-horseradish peroxidase (HRP) conjugate—Streptavidin conjugated with HRP^{||} was diluted in blocking buffer (1:1,000) and the membrane incubated with 120 ml of the reagent for 1 h at room temperature on a rotator. The membrane was washed once more with 250 ml of TBS-T (3 $\times$ 10 min).

Detection of signals—Detection was performed using the LumiGLO ELITE[®] chemiluminescent substrate kit^{**}, containing a luminol solution and a reaction buffer. A sensitive autoradiography film^{††} was used to capture light emission from the membrane. Films were exposed for 1–10 min (depending on the development of the standard curve), and developed in an automated developing machine[‡].

Quantification of antigens—The films were scanned using a flatbed scanner^{§§} and the scanned film analyzed using software for array analysis^{|||}. Arbitrary units called Volume, proportional to the intensity of the signals, were assigned to standards and unknowns. The

[#]Vector Laboratories, Burlingame, CA, USA

^{**}KPL, Gaithersburg, MD, USA

^{††}Hyperfil ECL, Amersham, Arlington Heights, IL, USA

[‡]Kodak M35A X-OMAT Processor, Kodak, Rochester, NY, USA

^{§§}EPSON 1640 Excel, Epson, Long Beach, CA, USA

^{|||}TotalLab, Nonlinear Dynamics, Durham, NC, USA

concentrations of the unknown samples (antigens in GCF samples) were estimated from the standard curve using a logit-log model and expressed as pg/site (IL-1 β and IL-8) or ng/site (MMP-8). Use of the logit-log model permitted estimation of values outside of the linear portion of the curve¹⁷.

Analytical specificity

In order to check for cross-reactivity among the pairs of antibodies a mixture of capture antibodies to the 3 targets was loaded onto a PVDF using the first two lanes of the MiniSlot following the optimized protocol for the CBIB. The subsequent 3 pairs of lanes received single monoclonal antibodies with distinct specificities for MMP-8, IL-1 β and IL-8. After blocking, a mixture of antigens containing: 50 ng of MMP-8, 150 pg of IL-1 β and 300 pg of IL-8 was loaded into all grooves of the MiniSlot. Secondary biotinylated antibodies with distinct specificities for the different targets were loaded into the channels of the Miniblotter, perpendicularly to the orientation of the capture antibodies and standards. Detection of antigen-antibody reactions was performed as described in the protocol above. Since standards do not contain endogenous biotin, the biotin blocking step was omitted.

Analytical sensitivity of the assay

Assay sensitivity was determined based on the results of 11 sequential CBIBs. For each of the 3 analytes the mean background Volume was calculated from slots to which all reagents but no sample had been added. The minimum detectable level of each analyte was then determined by calculating the corresponding concentration of the background mean + 2 standard deviations (SD) from a standard curved constructed using the mean values of the 11 replicates using a fourth order polynomial model.

Testing the assay sensitivity and dynamic ranges using GCF samples

The dynamic ranges of the standard curves of the CBIB were initially defined using values reported in the literature as a reference. In order to determine if the level of sensitivity and the dynamic ranges of our assay were suitable for the levels of IL-1 β , IL-8 and MMP-8 present in GCF, 931 individual GCF samples were processed using CBIB and the percentage of sites within the quantifiable range of the curve calculated.

Gingival crevicular fluid samples

Gingival crevicular fluid samples were obtained from the mesiobuccal site of every tooth present (excluding third molars) from 20 chronic periodontitis subjects and 20 periodontally healthy subjects for a total of 931 samples. Following isolation of the site with cotton rolls to prevent contamination with saliva, supragingival plaque was removed, the tooth air dried and a 30-second GCF sample was collected on filter strips^{¶¶}. Periapaper strips were gently inserted into the orifice of the periodontal pocket, 1–2 mm subgingivally. Samples visibly contaminated with blood were discarded. GCF volume was determined using a Periotron 8000[®] ^{##}, calibrated following the protocol described by Chapple et al.¹⁸. The samples were immediately placed in Eppendorf tubes containing 150 μ l of elution buffer [PBST (0.05% Tween 20 in phosphate-buffered saline)], transported to the laboratory and stored at –80°C until assayed. Immediately before analysis, samples were eluted by vortexing for 30 min followed by centrifugation at 10,000 RPM for 10 min.

^{¶¶} Periapaper[®], Interstate Drug Exchange, Amityville, NY, USA

^{##} Oraflow Inc., Plainview, NY, USA

Enzyme linked immunosorbent assay (ELISA)

The same antigens, primary and secondary antibodies, buffers and overall incubation conditions used in the CBIB were used in the ELISA. Briefly, 96 well microtitre plates^{***}, were coated separately with 150 µl/well of mouse monoclonal anti-human MMP-8, IL-8 and IL-1β^{||} diluted in coating buffer (2 µg/ml) and incubated for 1 hour at room temperature on a rotator. After the incubation, plates were washed 3 times (5 min each) with 200 µl/well of TBS-T on a rotator and blocked with 1% bovine serum albumin (BSA) and 5% sucrose in TBS-T (1 h at room temperature on rotator). After washings (3 × 5 min), serial dilutions of the standard antigens (covering the same dynamic ranges described for the CBIB), and samples were loaded in duplicate (150 µl/well) and incubated for 2 hours at room temperature on a rotator. Endogenous biotin was blocked with a Streptavidin/Biotin blocking kit[#]. Plates were incubated (150 µl/well) with a solution of streptavidin-HRP conjugate diluted in TBS-T (2 drops/10 ml of buffer) for 15 min at room temperature. After 3 × 5 min washes the plates were incubated (150 µl/well) with a solution of biotin diluted in TBS-T (2 drops/10 ml of buffer) for 30 min at room temperature, followed by 3 washes. Biotinylated antibodies anti-MMP-8, -IL-8 and -IL-1β^{||} were added to the respective plates (150 µl/well) and incubated for 1 hour at room temperature on a rotator. Substrate Reagent Pack^{||} was prepared following instructions of the manufacturer, 150 µl added to each well and after 10–30 min of incubation the reaction was stopped by the addition of 50 µl of 1 M sulfuric acid. Conversion of substrate was determined in optical density (OD) units at 450 nm using an ELISA reader^{†††}. The concentrations of the unknown samples were estimated from the standard curve using a logit-log model and expressed as pg/site (IL-1β and IL-8) or ng/site (MMP-8).

Validation of the results of the CBIB system using ELISA

In order to compare data from the CBIB with the “gold standard” ELISA, 4 sequential 30-second GCF samples were obtained from 15 sites from 4 periodontally healthy subjects and 25 sites from 7 chronic periodontitis subjects. Each of the 40 sets of 4 sequential GCF samples was pooled and eluted using 600 µl of elution buffer, providing enough sample material to be processed using the CBIB and ELISA assays (150 µl for each assay). Therefore, a total of 40 pooled GCF samples, representing 40 individual sites, were analyzed using 1 CBIB for 3 targets (IL-1β, IL-8 and MMP-8) and 3 ELISA assays (1 for each target), performed in parallel. The levels (pg/site or ng/site) of each mediator were determined in each assay using the respective standard curves and correlations between CBIB and ELISA data examined using the Pearson correlation coefficient.

Determination of levels of IL-1β, MMP-8 and IL-8 in clinical samples

In order to demonstrate the utility of the CBIB technique, individual 30-second GCF samples were collected from the mesiobuccal sites of all teeth present (excluding third molars) in 20 chronic periodontitis subjects and 20 healthy subjects for a total of 931 samples. These samples were individually evaluated for their levels of IL-1β, MMP-8 and IL-8 using the CBIB (same samples used to test the assay sensitivity and dynamic ranges). Study subjects were recruited from the clinic of the Department of Periodontology, Dental School, Aristotle University of Thessaloniki, Greece between January and July 2004. The Ethics Committee of the Dental School at Thessaloniki University approved the protocol, including the clinical examination and the collection of gingival crevicular fluid samples. All subjects signed informed consent prior to their enrollment into the study. To be included in the study, the periodontally healthy subjects had to be ≥ 20 years of age, have at least 24 natural teeth and no probing pocket depth (PD) > 3 mm. The chronic periodontitis subjects had to be ≥ 20 years of age have at least 15

*** Costar[†], Corning Incorporated, Lowell, MA, USA

††† BIO-TEK Instruments, Winooski, VT, USA

natural teeth and a minimum of 8 teeth with PD of ≥ 5 mm and AL ≥ 3 mm. Subjects were excluded if they had any systemic condition which would influence the course of periodontal disease or treatment, and medical conditions which would require antibiotic coverage for routine dental procedures. Individuals who had taken antibiotics in the previous 3 months or were either pregnant or nursing were also excluded. Clinical measurements were taken at 6 sites per tooth (mesiobuccal, buccal, distobuccal, mesiolingual, lingual and distolingual). All teeth (excluding third molars) were measured (maximum of 168 sites per subject) by a calibrated examiner. Plaque accumulation (0 or 1), gingival redness (0 or 1), bleeding on probing (BOP) (0 or 1), PD (mm) and clinical attachment level (AL) mm were measured. PD and AL were measured using a North Carolina probe. Mean clinical parameters were calculated for each subject and then averaged across subjects. Due to age differences between groups, mean clinical parameters and mean levels of GCF biomarkers were compared using ANCOVA adjusting for age.

Results

Fig. 1 is an example of a checkerboard immunoblotting used to quantify the levels of IL-1 β , IL-8 and MMP-8 in full-mouth samples from a periodontally healthy subject and a chronic periodontitis subject. The vertical lanes represent the 6-point standard curves and GCF samples from different teeth. The horizontal lanes represent the 3 detection biotinylated antibodies loaded in 3 different dilutions. The signals were revealed using chemiluminescence, captured on film and their intensities measured using software for array analysis.

Analytical specificity

Fig. 2 illustrates signals obtained when capture antibodies are loaded onto the membrane surface in a mixture compared to signals obtained when capture antibodies for the 3 different targets were loaded separately into different slots. After monoclonal antibodies bound to the membrane, a mixture of all antigens were loaded to the same lanes. The presence of each analyte (IL-1 β , IL-8 and MMP-8) was detected by secondary biotinylated antibodies specific for each one of the 3 targets, loaded in different horizontal lanes. The results captured on film demonstrated that the pairs of antibodies could distinguish among the 3 targets even when standards were loaded in a mixture.

Analytical sensitivity and dynamic ranges of the assay

Fig. 3 depicts the standard curves generated based on mean values obtained from 11 CBIBs. Arrows indicate the minimum detectable levels calculated using the mean blanks + 2 SD: 0.3 ng/site; 4.6 pg/site and 14.8 pg/site for MMP-8, IL-8 and IL-1 β , respectively. Out of the 931 GCF samples processed, 77% were above the minimum detectable levels for MMP-8 and IL-8 while 64% of the samples were positive for IL-1 β . All samples were below the upper limit of the standard curves for IL-1 β and IL-8, while 11% of the samples were above the upper limit of quantification for MMP-8. In summary, the percentages of GCF samples within the dynamic ranges of the assay were: 66%, 77% and 64% for MMP-8, IL-8 and IL-1 β , respectively.

Validation of the results of the CBIB system using ELISA

Fig. 4 shows the scatter plots of the data in pg/site or ng/site obtained with the CBIB compared with data determined using ELISA. All 3 biomarkers presented highly statistically significant ($p < 0.0001$) strong positive correlations, with Pearson r values of 0.91, 0.81 and 0.80 for IL-1 β , MMP-8 and IL-8, respectively.

Determination of levels of IL-1 β , MMP-8 and IL-8 in clinical samples

The mean clinical parameters calculated using all available sites of the study subjects are presented in Table 1. Significance of differences between groups was determined using the unpaired t test. Due to statistically significant differences in age, differences between groups were further examined using ANCOVA adjusting for age. The adjusted means were still statistically significantly different ($p < 0.001$) for all clinical parameters, with the exception of % of sites with suppuration.

Fig. 5 presents the mean levels of IL-1 β , IL-8 and MMP-8 for each individual subject in the two clinical groups. The vast majority of periodontitis subjects had mean levels of IL-1 β and IL-8 above the mean values for the healthy group. Using ANCOVA adjusting for age differences, the periodontitis subjects had statistically significantly higher mean levels (pg/site) ($\pm$ SEM) of IL-1 β (28.0 ± 2.5) and IL-8 (87.3 ± 8.4) compared to healthy subjects: 9.3 ± 2.5 and 57.1 ± 8.4 for IL-1 β ($p < 0.001$) and IL-8 ($p < 0.05$), respectively. Adjusted mean values ($\pm$ SEM) for MMP-8 (ng/site) were 32.1 ± 5.6 and 16.7 ± 5.6 for periodontitis and healthy subjects, respectively.

Discussion

There are several advantages in the use of the CBIB to examine immune mediators in GCF over the traditional ELISA technique. Using the CBIB several analytes can be measured at once, resulting in considerable savings of reagents and time. This allows the high-throughput analysis of a large number of GCF samples for the levels of different biomarkers. For instance, in order to process the 931 samples for 3 inflammatory mediators using ELISA, 70 assays would be required, compared to 11 CBIBs used in this study. A brief review of 43 manuscripts published in the past 3 years, in which measurements of GCF biomarkers were performed, revealed that an average of 374 samples were processed per study. The number of samples per study ranged from 31¹⁹ to 6,134²⁰, but only 3 studies^{20–22} processed a larger number of samples than the 931 GCF samples analyzed in the current paper. GCF is collected in very small volumes, typically less than 0.5 μ l, which limits the number of ELISA assays that can be conducted on a single sample. Investigators are often required to either dilute the samples or to pool several sites to increase the sample volume. Dilution of GCF samples might bring analytes to concentrations below the sensitivity of the assay. Pooling of samples compromises the investigation of associations among local levels of cytokines, the amount and composition of the microbial challenge and the clinical manifestation of the disease at the site level. Moreover, pooling of sites with similar clinical characteristics makes it difficult to explore longitudinal changes such as those that take place in response to therapy or during experimental gingivitis models. By assaying multiple analytes at once, the CBIB obviates the need of dilution or pooling of samples. In order to analyze multiple sites per subject, cost is another crucial consideration. A major advantage of the CBIB lies in its low cost compared to similar techniques. For example, the cost per sample of measuring the 3 mediators quantified in this manuscript using commercially available ELISA kits would be approximately 14 fold higher than the costs involved in the CBIB. Even for newer technologies such as the Multianalyte Microsphere Assay (Luminex) and antibody microarrays, the cost per sample for a 3-plex would be 7 and 2.5 times higher than the cost of the CBIB, respectively. In addition, the CBIB does not require expensive equipment such as an ELISA spectrophotometer, the Luminex machine and a microarray scanner for the quantification of positive signals.

The CBIB technology has been successfully employed by our and other laboratories for the detection of antibodies specific for subgingival species present in serum^{23–25}, and in the study of serum and GCF IgG subclasses in patients with early-onset periodontitis²⁶. However, this is the first manuscript to describe the application of this technique to the quantification of inflammatory mediators in GCF. Since inflammatory mediators are found in very small

concentrations in GCF (pg/ μ l), chemiluminescence was chosen as the detection system due to its higher sensitivity compared to colorimetric and chemifluorescence detection systems. The efficiency of our elution method was assessed for different concentrations of standard antigens and the percentage of recovery determined to be close to 100% (data not shown). Different membranes were tested and PVDF was chosen because of its optimal signal to background ratio and higher protein binding capacity when compared to nitrocellulose, which also translated into a more sensitive assay. The use of dual level target recognition using high quality capture and detection antibodies resulted in a high analytical specificity, as illustrated in Fig. 2. The sensitivity of the assay and the dynamic ranges for the 3 targets were adequate for the investigation of the levels of these mediators found in GCF. Even when only healthy sites from periodontally healthy subjects were analyzed, 70%, 67% and 49% of the GCF samples were positive for MMP-8, IL-8 and IL-1 β , respectively (data not shown). The coefficient of correlation between the two sets of data generated using ELISA and CBIB after the analysis of 40 GCF samples in parallel demonstrated a statistically significantly high correlation between the values generated with the two techniques (Fig. 4). The data from the clinical samples presented in Fig. 5 illustrate how this technique can be used to examine GCF levels of several biomarkers in different periodontal disease states and health. In addition, the results obtained with the CBIB show similar trends to previous reports that examined the levels of the 3 biomarkers tested using different techniques. These studies reported higher levels of IL-1 β , IL-8 and MMP-8 in chronic periodontitis compared to periodontally healthy subjects^{7,20,27}.

Most studies using GCF as a diagnostic tool suffer from limitations such as a small number of subjects and or sites being investigated, lack of longitudinal data and no evaluation of the microbial burden. These limitations are imposed by the low-throughput technologies currently employed to assay biomarkers in oral fluids. The CBIB technique described in this manuscript should permit the quantification of multiple components of the GCF in a large number of samples and help overcome some of these limitations. A similar approach for the high-throughput quantification of subgingival species using checkerboard DNA-DNA hybridization has been applied in our and other laboratories for the past 15 years^{28–31}. The development of the CBIB will allow us to examine not only the cross-regulation of inflammatory mediators in GCF but also relationships among the levels of these mediators and clinical parameters of periodontitis. Further, the CBIB will enable us to investigate correlations among GCF biomarkers and subgingival periodontal pathogens, quantified using the DNA-DNA hybridization technique. The ultimate goal of our research is to combine these technologies to explore host-bacterial interactions during the onset and progression of periodontal diseases. It is important to stress that this technique was developed to be used as a research tool and it was not meant as a side-chair diagnostic test.

We selected IL-1 β , IL-8 and MMP-8 as our primary targets during the development and optimization phase of the technique due to their well established role in the pathogenesis of periodontal diseases. In addition, these biomarkers represent 3 distinct classes of inflammatory mediators: pro-inflammatory cytokines, chemokines and matrix-metalloproteinases. However, in theory, this technique could be applied to the simultaneous quantification of up to 45 GCF biomarkers on a single membrane, reducing even further the cost per analyte per sample. In conclusion, the CBIB technique is a sensitive and specific assay for the high-throughput quantification of MMP-8, IL-8 and IL-1 β in GCF.

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Fig. 1.

Example of a checkerboard immunoblotting used to detect 3 inflammatory mediators in 28 gingival crevicular fluid samples (GCF) from a healthy subject and 22 GCF samples from a periodontitis subject. The top and bottom portions of the membrane correspond to the 2 sets of 30 grooves of the MiniSlot apparatus. The entire membrane was coated with a mixture of monoclonal capture antibodies for IL-1 β , MMP-8 and IL-8. The first 6 vertical lanes received the serial dilutions of the mixture of standards and the 7th the blank. The remaining vertical lanes were loaded with the GCF samples collected from the mesiobuccal site of every tooth present in each subject. The numbers indicate the tooth from which the sample was obtained. The horizontal lanes were loaded with secondary biotinylated antibodies with different specificities, diluted in blocking buffer. Each detection antibody was loaded in 3 serial dilutions both on the top and bottom portions of the membrane. A signal at the intersection between the vertical and horizontal lanes indicates the presence of a biomarker. The intensity of the signal is related to the levels of each inflammatory mediator in the sample.

Fig. 2.

Checkerboard immunoblotting demonstrating the analytical specificity of the assay. The first two vertical lanes were coated with a mixture of capture antibodies for IL-1 β , MMP-8 and IL-8, while the subsequent lanes were coated with single monoclonal antibodies. After a blocking step (see “material and methods” for details), a mixture of the 3 antigens in two dilutions was loaded into the lanes coated with capture antibodies. The total amounts of each target were: 150 pg, 50 ng and 150 pg for IL-1 β , MMP-8 and IL-8, respectively for lanes 1, 3 and 5, while a two-fold dilution of this mixture was added to lanes 2, 4 and 6. The horizontal lanes were loaded with secondary biotinylated antibodies with different specificities, diluted

in blocking buffer. Detection of signals was carried out using chemiluminescence and autoradiography, as described in the “material and methods” section.

Fig. 3.

Example of typical standard curves generated using the CBIB. The circles represent mean values for 11 replicates and whiskers the standard error of the mean (SEM). Detection limits were defined as mean background + 2 SD and determined using the standard curves and a fourth order polynomial model. Arrows on the x-axis indicate the values for the lower limit of detection for each analyte.

Fig. 4.

Scatter plots comparing the levels of IL-1 β , MMP-8 and IL-8 measured using CBIB and ELISA in parallel. In each plot the x-axis represent the results from the CBIB, while the y-axis represents data obtained using the ELISA assays. Each circle represents the value of IL-1 β , MMP-8 or IL-8 for each of 40 individual sites (15 sites from 4 periodontally healthy subjects and 25 sites from 7 chronic periodontitis subjects). Regression lines, r-values and p-values are presented for each graph. Correlations between CBIB and ELISA data were examined using the Pearson correlation coefficient (r).

Fig. 5.

Point plots of the mean levels of IL-1 β , MMP-8 and IL-8 for each subject in the two clinical groups. Significance of difference between groups was tested using ANCOVA adjusting for age. Circles represent the mean data for each subject and horizontal lines and whiskers represent adjusted mean ($\pm$ SEM) for each group.

Table 1Mean ($\pm$ SEM) clinical characteristics of subject groups

	Healthy (n = 20)	Periodontitis (n = 20)
Age (years) ***	38 $\pm$ 13	55 $\pm$ 12
% males	40	35
Mean pocket depth (mm) ***	1.82 $\pm$ 0.24	3.66 $\pm$ 0.86
Mean recession (mm) ***	0.11 $\pm$ 0.11	1.38 $\pm$ 0.71
GCF volume (ul) ***	0.16 $\pm$ 0.07	0.49 $\pm$ 0.21
% sites with:		
Plaque ***	22 $\pm$ 18	75 $\pm$ 23
Bleeding on probing ***	10 $\pm$ 8	66 $\pm$ 16
Gingival redness ***	3 $\pm$ 5	49 $\pm$ 32
Suppuration ***	0 $\pm$ 0	3 $\pm$ 4

p < 0.001 using unpaired t test