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Systemic acute-phase reactants, C-reactive protein and haptoglobin, in adult periodontitis

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SUMMARY

Capture ELISAs with biotinylated monospecific antibodies were developed to detect both C-reactive protein (CRP) and haptoglobin (Hp) in serum of adult periodontitis (AP) patients and normal subjects. Each acute-phase reactant was significantly increased in serum from AP patients with CRP at 9·12 ± 1·61 mg/*l versus* 2·17 ± 0·41 mg/*l* ($P < 0·001$) and Hp at 3·68 ± 0·37 g/*l versus* 1·12 ± 0·78 g/*l* ($P < 0·001$). Assessment of clinical characteristics of the patients' periodontal disease indicated that CRP and Hp levels were significantly increased in patients with the most frequent disease active episodes ($P < 0·02$ and $P < 0·001$, respectively). Longitudinal examination of the Hp levels showed a significant decrease following scaling and root planing (3·68 *versus* 2·38 g/*l*; $P < 0·01$). After a 2-year administration of 50 mg/b.i.d. Flurbiprofen (a non-steroidal anti-inflammatory drug), significantly decreased Hp levels were noted ($P < 0·005$). CRP levels declined by 35–40% after 1–2 years of treatment with the drug ($P < 0·05$). The findings indicated that localized infections resulting in increased inflammation and tissue loss in the periodontium elicit systemic host changes manifest by increases in two acute-phase reactants. The conclusions are that either these molecules are formed locally and distributed to the serum, or these presumably localized infections impact upon the systemic components of the host protective responses.

Keywords acute-phase reactants periodontitis C-reactive protein haptoglobin NSAIDs

INTRODUCTION

Due to the chronic bacterial colonization of the supra- and subgingival aspects of the teeth, the juxtaposed gingival tissue often demonstrates some level of localized inflammation [1]. As periodontitis ensues, there are alterations in local host inflammatory mediators [2,3], the initiation of a localized specific host response [4–6], and finally, a serum antibody response to the bacteria is observed [7,8]. Consequently, these findings would support some ability of the localized inflammation and/or infection to be manifest systemically within the affected host.

Bacterial infections frequently provide a strong stimulus for a systemic acute-phase response manifest by the increased production of some 25 plasma proteins [9–11]. Although most acute-phase reactants are synthesized by hepatocytes, some are synthesized by other cell types, including monocytes, endothelial cells, fibroblasts and adipocytes [11]. The strong acute-phase proteins include C-reactive protein (CRP), α_2-macroglobulin, and serum amyloid A, which respond rapidly to inflammatory stimuli, and serum levels may increase several 100-fold [12–15]. Moderate acute-phase proteins include haptoglobin, fibrinogen and α_1-antitrypsin, which can increase 2–10-fold [16], while complement component C3 and ceruloplasmin are considered weak acute-phase proteins, which may increase up to two-fold [12].

Cytokines appear to play a major role in the clinical symptoms and tissue destruction associated with progressing periodontitis [2]. There is also strong evidence for cytokines eliciting the systemic acute-phase response in various chronic inflammatory diseases [17,18]. Many of these cytokines are derived from activated macrophages and can act both locally and distally to amplify cytokine production from other cell types (e.g. fibroblasts, endothelial cells), which then emerge from the local tissues and can initiate systemic acute-phase responses [19]. Since multiple cytokines have been detected in both gingival tissues and gingival crevicular fluid (GCF) [2,20,21], changes in local acute-phase reactants might be expected. Increased levels of acute-phase proteins have been noted with gingival inflammation, including during experimental gingivitis and periodontitis, reflecting the locally stressed environment [22–24]. We measured various acute-phase proteins in the serum of early-onset periodontitis (EOP) smokers compared with healthy non-smokers and smokers with only gingivitis, and found extensive variation in both the diseased and healthy subjects, although CRP appeared to be increased in the EOP group [25].

The purpose of this study was to examine the characteristics of serum CRP and haptoglobin (Hp) within periodontitis patients of

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differing disease severity. Furthermore, we examined the impact of mechanical and anti-inflammatory treatments on the levels of these acute-phase proteins. The results suggest that local aspects of periodontitis appear to influence systemic host responses and the resulting reactions may reflect the severity of periodontal destruction.

PATIENTS AND METHODS

Patient population

The adult periodontitis (AP) group included patients from 35 to 55 years of age. The patients had no uncontrolled medical conditions or abnormal blood chemistries (SMAC-20), no need for prophylactic or regular antibiotics, absence of pregnancy, and no need for drugs that would significantly affect the immune system (e.g. glucocorticoids or immunosuppressants). Furthermore, patients were excluded if there was a history of frequent periodontal abscesses. Oral clinical features included moderate to severe radiographically evident alveolar interproximal bone loss with loss of attachment, bleeding on probing, and periodontal pockets ≥ 5 mm. Patients included in this group had to have at least four teeth meeting these disease criteria. In addition, each patient had to have at least two pre-molar or molar teeth in at least two quadrants meeting the disease criteria.

A normal control population was included in a cross-sectional study and was 35–63 years old. These subjects could exhibit mild to moderate gingivitis, with no probing pocket depths > 4 mm and no radiographic evidence of alveolar bone loss.

Experimental protocols

Phase I. In this cross-sectional study, a single serum sample from AP patients ($n = 40$) and normal subjects ($n = 35$) was obtained by venipuncture. The serum sample from the periodontitis group was collected at their initial visit of the longitudinal protocol. The serum was stored at −20°C until analysed for CRP and Hp.

Phase II. This was a longitudinal design in which 38 of the AP patients from Phase I were included. In addition, this group included patients only if they had no medical need for non-steroidal anti-inflammatory drugs (NSAIDs), no sensitivity to NSAIDs or history of peptic ulcers, and no metabolic bone disease other than age-related osteoporosis. The patients were clinically evaluated at the initial visit and approximately every 2 months for 6 months. The clinical examination included a dichotomous plaque index, gingival index, and bleeding on probing [26]. Probing pocket depth and attachment level were determined at six sites per tooth [26]. Finally, disease active sites were determined as attachment loss of ≥ 2 mm between any two visits, or from the initial sampling to any subsequent visit. Serum was collected at each visit and stored at −20°C.

Phase III. This was a longitudinal study which included 34 AP patients from Phase II. Following the 6-month baseline study (above), the patients were randomized into four groups. In this double-blind study, one group received a placebo treatment, and three groups received Flurbiprofen at 5, 15 or 50 mg/b.i.d. for 24 months. The patients received mechanical debridement (scaling and root planing) every 6 months. Serum was obtained every 3 months during this 2-year interval and stored at −20°C.

ELISA procedures for CRP and Hp

We developed a capture ELISA for quantifying CRP and Hp in the serum samples. Briefly, rabbit anti-human CRP or Hp (Calbiochem, La Jolla, CA) was used to coat microtitre plates (Linbro, ICN/Flow, Costa Mesa, CA) at a concentration of 1·0 μg/well. The human sera were diluted 1:500 and 1:1000 for CRP or 1:25 000 and 1:50 000 for Hp and incubated in the wells for 2 h. After washing, biotinylated rabbit anti-human CRP or Hp (NHS-LC-Biotin reagent; Pierce, Rockford, IL) was added and incubated for 2 h. The plates were washed and incubated overnight with streptavidin-alkaline phosphatase (Zymed, South San Francisco, CA). The ELISA was developed with *p*-nitrophenylphosphate (Sigma, St Louis, MO) as we have described previously [27]. The dynamic range of the CRP and the Hp assays was ≈ 1–100 ng/0·1 ml with inter- and intraplate coefficients of variation at 7% and 11%, respectively.

Statistical analysis

Comparison between the CRP and Hp levels in serum of periodontitis patients and normal subjects in Phase I was accomplished using a Mann–Whitney Wilcoxon rank analysis. The levels of these acute-phase proteins in serum from the periodontitis patients exhibiting differences in disease severity (i.e. disease active episodes, Phase II) were compared using a Kruskal–Wallis analysis. Finally, changes in the levels of these two molecules in the serum (Phase III) were assessed using a Friedman two-way ANOVA. All analyses were performed using CSS:Statistica software (SOFTSTAT) on a microcomputer.

RESULTS

Acute-phase reactant levels in AP

Normal subjects showed a serum CRP level of 2·17 ± 0·41 mg/*l*, while the level in the AP group was significantly elevated ($P < 0{\cdot}001$; Fig. 1). The Hp level in normal subjects was 1·12 ± 0·78 g/*l* and a significantly increased level was detected

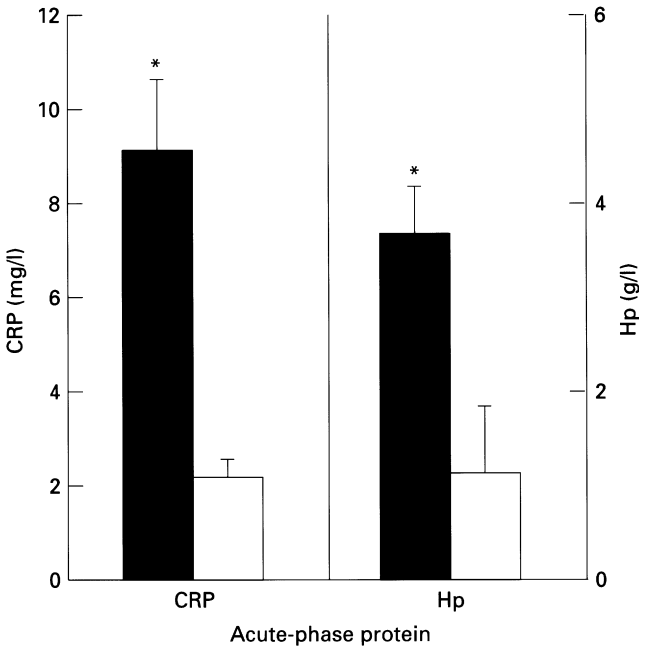

Fig. 1. Levels of C-reactive protein (CRP) and haptoglobin (Hp) in serum from adult periodontitis (AP, ■; $n = 40$) and normal (N, □; $n = 35$) subjects. Bars denote the mean level of each protein and vertical lines 1 s.d. *CRP and Hp levels were significantly elevated in AP *versus* N at $P < 0{\cdot}001$ (Mann–Whitney *U*-test).

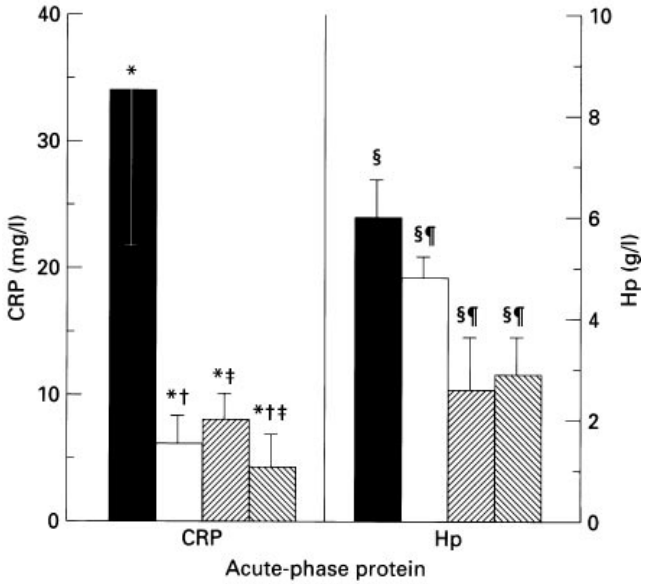

Fig. 2. Levels of C-reactive protein (CRP) and haptoglobin (Hp) in serum from adult periodontitis patients (AP) at an initial sampling time point. Patients were stratified into groups based upon the frequency of disease active sites during a subsequent 6 months monitoring interval: AP-1 (■; mean active sites = 36, $n = 4$); AP-2 (□; mean active sites = 12, $n = 11$); AP-3 (▨ mean active sites = 4, $n = 12$); and AP-4 (▧; mean active sites = 1, $n = 11$). Bars denote mean levels and vertical lines 1 s.d. Statistical comparisons demonstrate matching symbols different at: *$P < 0{\cdot}01$; †$P < 0{\cdot}05$; ‡$P < 0{\cdot}005$; §, ¶$P < 0{\cdot}001$ using a Kruskal–Wallis and Mann–Whitney *U*-test.

in periodontitis sera ($P < 0{\cdot}001$; Fig. 1). The levels of each of these acute-phase proteins in the normal subjects were similar to those previously reported in control populations [12,28].

Acute-phase reactant levels related to disease activity

The periodontitis patients were then stratified into groups based upon the frequency of disease active sites over the initial 6 months of monitoring. Group 1 ($n = 4$) was the most severely affected with a mean of 36 active sites (range 24–49); group 2 ($n = 11$) showed a mean activity of 12 sites (10–20); group 3 ($n = 12$) showed a mean of disease active sites of 4 (range 3–8); and group 4 ($n = 11$) was the least affected, with a mean number of active sites of 1 (range 0–2). Figure 2 demonstrates that patients in group 4 exhibited CRP levels that were within the normal range, while levels in groups 2 and 3 were significantly greater than the normal population level ($P < 0{\cdot}001$). However, the most severely diseased patients (group 1) demonstrated serum CRP levels that were increased by nearly 17-fold over normal ($P < 0{\cdot}02$) and were significantly elevated compared with all other groups.

Hp levels in groups 3 and 4 were within the range of levels detected in the normal population (Fig. 2). In contrast, sera from groups 1 and 2 patients (most severe disease) had Hp levels that were significantly elevated compared with all other patient groups and normal subjects ($P < 0{\cdot}001$).

Acute-phase reactant levels related to treatment

Figure 3 depicts the changes in CRP that occurred in control patients and following treatment with the NSAID, Flurbiprofen. First, data revealed that while the patients were randomized into the four groups, the baseline CRP values were not equal; however,

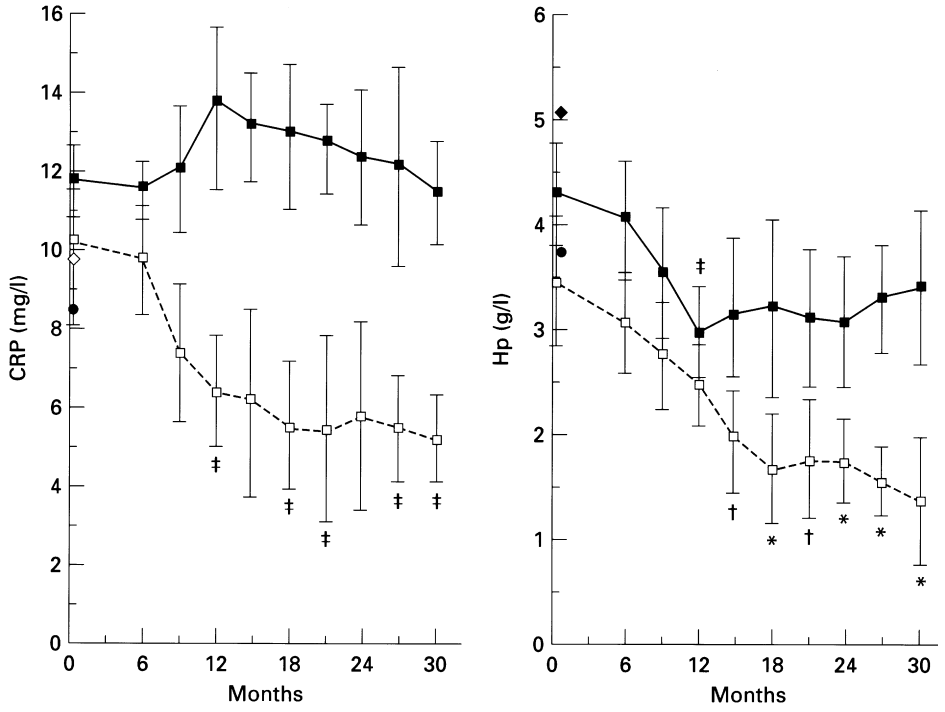

Fig. 3. Levels of C-reactive protein (CRP) and haptoglobin (Hp) in serum from adult periodontitis patients ($n = 34$) during the longitudinal treatment study. Samples at baseline and 6 months were obtained before initiation of treatment. Patient groups then received treatment either with placebo (■; $n = 8$) or Flurbiprofen 50 mg (□; $n = 9$) for the following 2 years. The points denote mean levels, vertical lines enclose 1 s.d., and symbols denote values statistically different from the baseline at: *$P < 0{\cdot}005$; †$P < 0{\cdot}01$; ‡$P < 0{\cdot}05$, using a Friedman two-way ANOVA. Since no consistent changes were noted in the levels following treatment with either 5 (♦) or 15 (●) mg of the non-steroidal anti-inflammatory drug, only the initial levels are presented for these groups.

no significant differences were noted between the groups at this sampling. In the placebo group which received only mechanical hygiene procedures, CRP levels remained elevated throughout the study. Those patients treated with 50 mg Flurbiprofen demonstrated a decrease of ≈ 40–50% over the 2 years, which was decreased from baseline throughout the treatment interval, and reached statistical significance at a few sampling points ($P < 0{\cdot}05$). No consistent differences were noted during treatment with either 5 or 15 mg/b.i.d. of the NSAID (data not shown).

Hp levels showed a significant decrease at 12 months in the placebo group, presumably resulting from the mechanical hygiene (Fig. 3). Treatment with 50 mg/b.i.d. Flurbiprofen caused a continual decrease in Hp, which reached significance by 15 months.

DISCUSSION

Pro-inflammatory cytokines and mediators are significantly elevated with gingival inflammation and during the destructive phase of periodontitis [2,3,22,29–32]. The clinical findings in periodontitis have emphasized the local (periodontium) nature of inflammation and tissue destruction within the oral cavity. One consequence of these localized gingival inflammatory reactions has been the identification of elevated levels of various acute-phase proteins in the gingival crevicular fluid [22–24]. These have included α_2-macroglobulin, α_1-antitrypsin and CRP, which are altered in the crevicular environment, presumably as a result of numerous host–bacterial interactions in the sulcus, and may contribute to the defence of the host in this milieu.

The ability to use acute-phase reactant levels as a measure of infectious processes or inflammatory diseases has substantial support [33]. Acute-phase proteins (including CRP and Hp) have been studied in healthy individuals, and while some biological variability was noted, changes from normal can be detected [34]. Studies of neonates with streptococcal infections and bacteraemias suggested that CRP is a marker for the acute period of infection and α_1-acid-glycoprotein was a marker for recovery [35,36]. CRP levels were proposed as a marker to detect and monitor sepsis in burn patients [37], as well as being useful in monitoring septic patients during treatment [38]. Recently, CRP was shown to increase in *Plasmodium falciparum* infection, was higher in more severe cases, and decreased with treatment [39]. Several studies have demonstrated a correlation between elevated serum acute-phase proteins and the magnitude of joint destruction in rheumatoid arthritis (RA) [40,41]. Evidence is also available suggesting that CRP levels provide a sensitive and objective indicator of disease activity and clearly reflect the response to therapy of RA [15]. In a cross-sectional study of Crohn's disease, acute-phase reactants (including CRP and Hp) changed in parallel with disease activity; however, the increases did not precede disease activity [42].

While most studies of periodontitis have emphasized the local nature of this host–bacterial interaction in the periodontium and gingival sulcus [2,3,32,43], it also appears that systemic manifestations of this disease are also detected. Serum antibody levels are detected to many oral bacteria and appear to be increased with more extensive dental caries [44] or periodontitis [7,8] potentially resulting from transient access of oral bacteria to the circulation [45]. Finally, reports of individual patients and small cohorts have suggested that patients with more severe disease may reflect systemic changes associated with stress [46] and symptoms potentially attributable to more severe bacterial infections [47]. The results of this study showed increased acute-phase proteins in a group of patients identified as AP. These levels presumably reflect both the infection aspect of periodontitis [48], as well as the manifestations of acute and chronic inflammation that exist in the periodontium [2,3,32,43]. Moreover, it was also clear that patients exhibiting the most severe disease exhibited the greatest levels of each of the acute-phase reactants. While the severely diseased patients must be considered an extensively diseased group compared with the general adult periodontitis population, this type of patient may represent that subset of adult individuals who are at highest risk for extensive and rapid disease and can provide a model for evaluating the contribution of the acute-phase response to disease susceptibility or resistance [49]. This finding suggests that an increased burden of infection or elevated inflammatory responses exist in this subset of AP patients, resulting in the elevated acute-phase proteins. However, evaluation of the patients did not support that the quantity of clinical inflammation was greatest in this subset, and previous studies would support that the microbial burden is not routinely increased in patients with more severe periodontitis [50]. Alternative explanations for these findings would be that the quality of the microbial ecology confers a higher probability of systemic manifestations of this localized infection [51], or that there exists a subset of high risk patients with increased susceptibility attributed to altered local (gingival) and/or systemic inflammatory response characteristics [52]. These alternatives could have important implications for host-based variations which contribute to increased susceptibility or resistance to periodontitis progression. Thus, measurement of serum acute-phase proteins may help to identify a subset of patients who are at higher risk for destructive disease, or disclose those patients who are undergoing a process of periodontal breakdown [53].

While prevention of periodontal destruction is of primary importance, it is clear that strategies for early intervention, more specific treatment modalities, and more effective assessment of treatment success are high profile areas of interest in periodontology. Several studies have been performed to evaluate the effects of NSAIDs on periodontitis [54,55], since these pharmaceuticals have been used as palliative agents in treatment of RA. Recently, tenidap sodium, which is a cytokine-reducing anti-rheumatic, was found to decrease CRP in RA patients [40]. Both steroids and disease-modifying anti-rheumatic drugs (DMARDs) also were found to decrease CRP by 30–70% [55]. In both cases these decreases were associated with a positive effect on the inflammatory symptoms of the disease. Numerous studies showed that NSAIDs had minimal effects on CRP, although two studies indicated that Flurbiprofen decreased CRP in a subset (≈ 80%) of patients [56,57]. Clark & Fraser [34] suggested that a change of 150% (CRP) or 70% (Hp) in the levels of these acute-phase reactants is significantly different from the individual subject variabilities. We noted that mechanical hygiene or low doses of the NSAID (Flurbiprofen) had a negligible effect on CRP or Hp levels in the serum. However, as was noted with RA patients, higher doses of the NSAID appeared to cause a decrease in CRP and Hp, with the Hp changes being statistically significant.

The present study suggested that differences in CRP and Hp levels may distinguish a group of AP subjects with more severe disease. Previous reports examining patients with aggressive disease have generally described these patients as a portion of a younger population representing an early onset type of periodontal

disease [49,52,53]. In this study, the subset of patients exhibiting the most significant elevations in these acute-phase serum proteins represented individuals exhibiting a very aggressive, rapid disease in adults. Thus, measurement of acute-phase proteins could provide a valuable tool to identify changes in the periodontal health of patients, particularly in the high risk subset of periodontitis patients. Due to the existing variability in serum acute-phase reactants within this AP population, conclusions on the effect of treatment on these levels remain somewhat equivocal. Nevertheless, the study poses some interesting concepts concerning the identification and monitoring of biological factors associated with progressing periodontitis, when these factors are not homogeneous within the population clinically categorized as AP. The results emphasize the heterogeneity in the AP population, and may be an important consideration in evaluating the effects of various treatment modalities administered to patients. Both mechanical oral debridement and treatment with an NSAID appear to affect these serum glycoprotein markers of infection and inflammation. Additional longitudinal studies of larger, better stratified populations will be required to validate the usefulness of examining acute-phase reactants in monitoring periodontal disease.

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