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Myeloid-related protein (MRP)8/14 (calprotectin) and its subunits MRP8 and MRP14 in plaque-induced early gingival inflammation

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Abstract

Background: The inflammatory myeloid-related protein, MRP8/14, also called calprotectin, and its subunits MRP8 and MRP14 have been detected and identified recently in gingival crevicular fluid (GCF). It has been suggested that the type and phase of inflammation can be discriminated on the basis of differences in the expression of calprotectin and its subunits, released during activation and/or death of granulocytes and monocytes. The purpose of this study was to quantify calprotectin and its subunits (MRPs) simultaneously in the GCF during the initial phase of experimentally induced gingivitis, and to examine their inter- and intra-individual variations.

Material and Methods: Fifteen healthy non-smoking subjects, aged 18–30, were involved in this study. An initial hygiene phase (days − 11 to 0) was followed by 10 days of undisturbed plaque accumulation. At days − 11, − 3, 0, 10, 11, clinical parameters were recorded and GCF samples collected with Durapore strips from 12 sites in each subject. Quantitative analyses of total proteins, MRP8/14, MRP14 and MRP8 were performed by ELISA procedures.

Results: During the experimental phase with no oral hygiene (days 0–10), the clinical parameters Plaque Index, Gingival Index (GI) and bleeding on probing increased as expected, confirming that plaque accumulation leads to gingival inflammation. Levels of the MRPs were individually variable. They increased with plaque accumulation in one-half of the subjects, and decreased in the other subjects. The levels of MRP8/14 and MRP14 at subject recruitment (day − 11) could predict a significant part of the GI at day 10. Only minute amounts of the subunits MRP8 and MRP14 were detected in comparison with the complex MRP8/14 throughout the experiment. Considerable variations were noted among sites within subjects.

Conclusion: The expression of calprotectin in the early phase of experimental gingivitis is variable between subjects, and two groups of subjects can be differentiated according to their response patterns. Clinical parameters at the very first visit (day –11) seemed to be different in the two response groups. The results of the present investigation indicate that the inflammatory response to plaque accumulation depends on the initial status of the subjects, which may not be leveled out by the introduction of perfect oral hygiene. Whether these patterns reflect a different susceptibility to periodontal diseases remains to be determined.

Key words: calprotectin; experimental gingivitis; gingival crevicular fluid; L1-protein; myeloid-related protein

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Calprotectin (myeloid-related protein, MRP8/14) is a member of the S100 family of calcium-binding proteins, and is released during activation and/or death of granulocytes and monocytes (Lemarchand et al. 1992, Guignard et al. 1996, Rammes et al. 1997, Fagerhol et al. 1980). MRP8/14 is a 36.5 kDa heterodimeric complex of two non-covalently bound calcium-binding subunits, MRP8 and MRP14, with molecular weight of 10.8 and 13.2 kDa, respectively (Dale et al. 1983, Edgeworth et al. 1991, Teigelkamp et al. 1991, Johne et al. 1997 Hunter & Chazin 1998). The biological role of the S100 proteins is still controversial (Kligman & Hilt 1988). Nevertheless, three general modes of functioning are recognized: regulation of the protein kinases (Murao et al. 1989), antimicrobial activity (McNamara et al. 1989, Steinbakk et al. 1990, Sohnle et al. 1991, Murthy et al. 1993), and a regulatory role in inflammatory reactions (Hogg et al. 1989, Berntzen et al. 1991, Hessian et al. 1993, Kerkoff et al. 1999).

The plasma level of calprotectin is thought to reflect the inflammatory activity in patients with rheumatoid arthritis (Berntzen et al. 1988, 1989, Brun et al. 1992, 1994), Crohn's disease (Lugering et al. 1995a), and ulcerative rectocolitis (Lugering et al. 1995b). High plasma levels of MRP8/14 were detected among patients with septicemia, purulent meningitis, bacterial pneumonia, and vascular insults. In contrast, levels were normal or only slightly increased during viral infections (Sander et al. 1984). The relative levels of the subunits and the complex MRP8/14 in synovial fluid and serum allow the discrimination of active and non-active osteoarthritis from rheumatoid arthritis (Burmeister & Gallacchi 1995).

Calprotectin and its subunits have been detected also in the gingival crevicular fluid (GCF) (Kido et al. 1998, Miyasaki et al. 1998, Kojima et al. 2000). Cross-sectional observations indicated higher calprotectin levels (Kido et al. 1998, 1999), and up to 20-fold higher MRP8 levels (Lundy et al. 2001) in patients with periodontitis than in periodontally healthy subjects. So far, the complex and its subunits have not yet been measured quantitatively in the same GCF sample simultaneously. One may speculate that the relative levels of these proteins in GCF indicate the type and phase of gingival and periodontal inflammation as well (Burmeister & Gallacchi 1995, Johne et al. 1997).

MRP expression may show important variation from site to site, and may depend on the local clinical status of the sites sampled. Surprisingly little is known about topographic patterns in the expression of host factors in general. The problem of ''representative sites'' for sample taking, debated in the context of microbiological tests (Mombelli et al. 1990, 1991b, 1994), has not been studied systematically with regard to inflammatory mechanisms.

In the process of determining the potential value of MRPs in periodontal diagnosis, a number of fundamental issues need to be addressed in subjects without periodontal disease first. Among them are (i) the expression of MRPs in the early development of a gingival inflammation, and (ii) the inter- and intra-individual variation in the expression of these proteins. Repeated sampling in a multitude of sites in the same individual is an essential exercise to determine the normal variability and the consistency of the expression of a biological marker in a given clinical situation.

The purpose of this study was to quantify calprotectin and its subunits (MRPs) simultaneously in the GCF during the initial phase of experimentally induced gingivitis, and to examine their inter- and intra-individual variations.

Material and Methods

Subjects

Fifteen healthy young non-smoking subjects (six males and nine females), aged between 18 and 30, were involved in a 10-day experimental gingivitis trial. They were systemically healthy and had no current therapy with NSAIDs. Pregnant women and all persons presenting periodontal pockets deeper than 5 mm associated with loss of clinical attachment were excluded from the study. The project was approved by the Ethical Committee of the School of Dental Medicine, Geneva, and written informed consent was obtained prior to the start of the study.

Study design

Eleven days prior to the experimental gingivitis, all subjects received instructions for proper oral hygiene and supragingival scaling of all teeth, with the goal of reaching a Gingival Index score (GI, Löe 1967) of 0 or 1 at all sites within 1 week (day -3). Between days 0 and 10, the subjects refrained from oral hygiene procedures to allow for an undisturbed accumulation of bacterial deposits on the tooth surfaces. At day 10, after the clinical measurements and GCF sampling, the whole dentition was cleaned professionally. The participants were examined five times during the course of the study by one examiner (M. L. Q.): before the first professional tooth cleaning (day -11), twice before the non-brushing period (days -3 and 0), at the end of the non-brushing period (day 10), and 1 day after re-institution of oral hygiene (day 11). The following clinical parameters were recorded at each time point: Plaque Index (PlI, Silness & Löe 1964), GI, probing pocket depth (PPD) using a force control probe (Paro Audio Probe, Esro AG, Thalwil, Switzerland) and bleeding on probing (BOP). Presence or absence of bleeding was recorded dichotomously for each site as 1 or 0, respectively. A mean value, representing the percentage of bleeding sites, was calculated for each subject.

Site selection and sampling procedures

Clinical parameters were assessed and GCF samples were collected from 12 sites in each subject at each of the five visits. The mesio-buccal aspects of the teeth from the right to the left upper molar were used as experimental units. The PlI was assessed first. Next, the supragingival plaque was removed with a curette and cotton pellets, the teeth were isolated with cotton rolls, and gently dried using an aspiration tip. GCF was collected, using 2×6 mm strips of polyvinylidene difluoride membrane (Durapore Membrane, Millipore, Bedford, MA, USA), placed at the entrance of the gingival sulcus, and left in situ for 15 s. GCF sampling was repeated after 3 min at each site. The two paper strips from each site were transferred into a microtube and stored at -20°C until analyzed.

Laboratory procedures

Eighty microliters of a 10 mM Tris-HCl solution, pH 7.4, was added to each sample. The tubes were gently vortexed for 30 s to elute the GCF-proteins. The paper strips were wedged at the top of the tubes with the caps and centrifuged at 5000 rpm for 7 min. After centrifugation, the strips were removed and the supernatant transferred into new tubes. All procedures were performed at 4°C to avoid protein degradation.

Total amounts of protein, MRP8/14, MRP8 and MRP14 were measured in the GCF supernatant as follows: the protein content was determined according to the Bradford method (Bradford, 1976, Bio-Rad protein assay, Bio-Rad, Hercules, CA, USA). MRP8/14, MRP8 and MRP14 were measured by ELISA, using microtiter plates (Nunc Maxisorb, Nunc A/S, Fischer Scientific AG, Wohlen, Switzerland) pre-coated with monoclonal mouse anti-MRP8/14, MRP8 and MRP14 as capture antibodies (BMA, Biomedicals AG, Augst, Switzerland). For each of the three assays, the GCF was diluted with a specific assay buffer. The plates were incubated at 37°C for 30 min (MRP8), 60 min (MRP14), or overnight at 4°C (MRP8/14). For the MRP8 and MRP14 assays, biotinylated mouse monoclonal detection antibodies, conjugated to peroxidase in the presence of extravidine, were added. Polyclonal chicken antibodies were used for the MRP8/14 assay.

After adding H_2O_2 and tetramethylbenzidine, the conversion of the substrate to a colored product was read in an MRX microplate reader at 450/630 nm. The amounts of protein, MRP8/14, MRP8 and MRP14 were determined using the MRX revelation Software™ (Dynex Technologies, BioConcept, Allschwil, Switzerland).

Data analysis

The levels of calprotecin and its subunits were expressed as total amounts per 15 s sample and as amounts per total protein. To assess the reproducibility of repeated samples, the values obtained at day −3 were compared with those obtained at day 0 by calculating, for each site, the difference between the two measurements, and then the mean of the differences. Standard deviations of single measurements (S_i) and coefficients of variation (CV) were determined.

An individual mean value was calculated for each participant at each time point from the 12 measurements of every parameter recorded in his dentition. The Mann–Whitney *U* and the Wilcoxon signed-rank tests were used to determine differences between groups of individuals, using the subject as the statistical unit. Stepwise multiple linear regression analysis was used to study the relationship between calprotectin levels and the parameters assessed after a period of plaque accumulation.

Results

Of the 15 subjects entering the study, data from 14 subjects (eight females and six males) completing the experimental gingivitis were statistically evaluated. Local clinical and immunochemical data were available from 162 sites (11 subjects contributed with 12 and three with 10 sites). MRP8/14, MRP14, MRP8 and total protein were analysed quantitatively in 798 GCF samples (missing data for one subject at day 11 (12 samples)).

Reproducibility

Table 1 shows the mean values of the clinical parameters (PlI, GI, PPD), the total amounts of protein, MRP8/14, MRP8 and MRP14 of the samples taken at day −3 (mean day −3) and day 0 (mean day 0), the means of the differences of the values from the samples taken at day −3 and day 0 (mean diff. 1st–2nd), the coefficient of variation of the first measurement (CV 1st) and the standard deviation of a single measurement (S_i). The statistical unit in this table is the site. This implies that for each parameter the data of 162 sites are taken into account.

Variation from site to site

Fig. 1 shows the distribution profile of GCF-MRP8/14 in the maxillary dental arch as represented by calprotectin levels in all samples taken at day 10. Similar profiles were found at days 0 and 11, and when the levels of calprotectin and its subunits were expressed as amounts per total protein. As the graph indicates, there was a tendency for higher calprotectin levels in more posterior locations (linear regression, $p = 0.01$).

Longitudinal changes

The longitudinal development of the mean clinical parameters is shown in

Table 1. Mean values of local clinical, biochemical and immunochemical parameters (ng/site) assessed at days −3 and 0, means of the difference of the assessments at days −3 and 0 (mean diff. 1st–2nd), coefficients of variation of the first measurements (CV 1st), and standard deviation of a single measurement (S_i). $n = 162$

	Mean (day −3)	Mean (day 0)	Mean diff. (1st–2nd)	CV 1st	*p*	S_i
PlI	0.09	0.02	0.07	3.1	0.002	0.22
GI	0.12	0.18	−0.06	2.6	0.071	0.27
PPD	2.32	2.23	0.09	0.3	0.127	0.50
total protein	4645	4868	−223	1.0	0.526	3153
MRP8/14	459	576	−117	2.7	0.488	1515
MRP8	0.32	0.38	−0.06	2.9	0.649	0.58
MRP14	1.06	1.16	−0.10	0.6	0.139	1.03

PlI, Plaque Index; GI, Gingival Index; PPD, probing depth; MRP, myeloid-related protein.

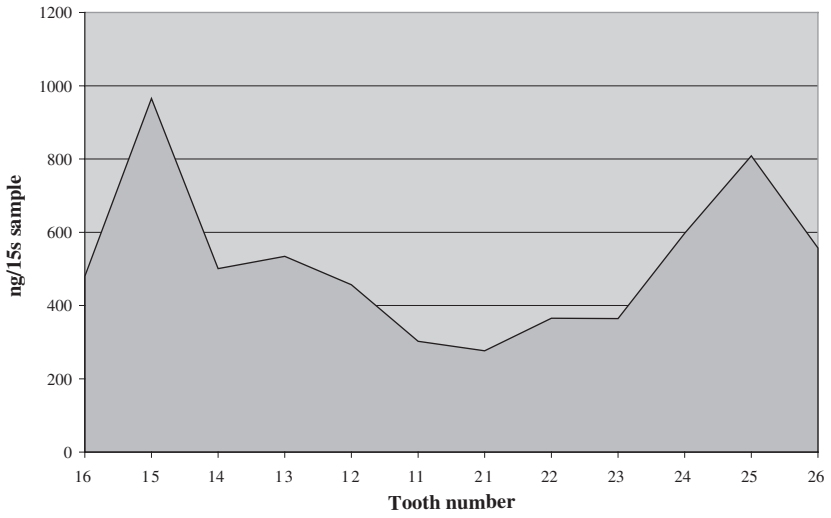

Fig. 1. Distribution profile of gingival crevicular fluid-myeloid-related protein 8/14 in the maxillary dental arch (calprotectin levels in all samples taken at day 10).

Table 2. Longitudinal changes of mean clinical parameters

Day		PII	GI	PPD	BOP
− 11	pre-experimental phase	0.20 ± 0.40	0.18 ± 0.38	2.51 ± 0.70	0.19 ± 0.39
− 3		0.09 ± 0.29	0.12 ± 0.33	2.32 ± 0.77	0.09 ± 0.29
0	experimental phase	0.02 ± 0.14	0.18 ± 0.38	2.23 ± 0.77	0.04 ± 0.19
10	(non-brushing period)	1.78 ± 0.70	1.30 ± 0.71	2.41 ± 0.72	0.33 ± 0.47
11	post-experimental assessment	0.01 ± 0.12	0.61 ± 0.54	2.49 ± 0.73	0.15 ± 0.35

PII, Plaque Index; GI, Gingival Index; PPD, probing depth; BOP, bleeding on probing.

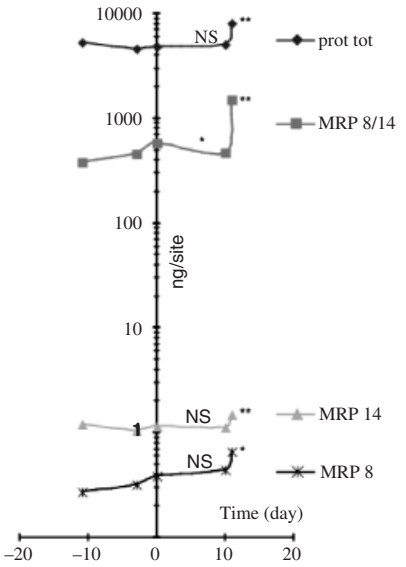

Fig. 2. Expression of mean total protein (prot tot), myeloid-related protein 8/14, MRP14 and MRP8 during the study period (averages of 162 sites in 14 subjects). $^{*}p<0.05$, $^{**}p<0.001$, not significant (NS).

Table 2. The expression of the mean parameters MRP8/14, MRP8, MRP14 and total protein during the experiment are shown in Fig. 2. In mean, calprotectin and its subunits did not increase between days 0 and 10. However, highly significant increases were noted between days 10 and 11 (Wilcoxon matched pairs, $p<0.001$). Only minute amounts of the subunits MRP8 and MRP14 were detected in comparison with the complex MRP8/14 throughout the experiment.

Variations from subject to subject

With regard to changes of the immunochemical parameters, considerable variation was noted among subjects. Seven subjects showed a stabilization, or even a decrease of calprotectin levels during the experimental phase, followed by a rise of the values, just 1 day after mechanical polishing (Fig. 3A, response pattern 1). Subjects of this group showed higher MRP8/14 levels at days − 11 and 0 than the other participants (Mann–Whitney U, $p=0.003$). Five of those seven subjects were females. The other six subjects showed a significant rise of calprotectin (MRP8/14) levels during the experimental gingivitis phase (Fig. 3B, response pattern 2). In four of these subjects, the calprotectin level decreased after the experimental phase, while in the remaining two subjects, the calprotectin level continued to rise even after the mechanical removal of plaque, i.e. from day 10 to day 11.

Clinical parameters at the very first visit (day –11) seemed to be different in the two response groups (Fig. 4). Pattern 1 subjects had significantly lower PPD values than pattern 2 (Mann–Whitney U, $p=0.026$). Fig. 5 shows the relationship between the mean amounts of subunit MRP14 and the mean amounts of the complex MRP8/14 at days − 11, 0 and 11. Pattern 1 subjects showed a significantly lower ratio between MRP14 and MRP8/14 on day 11 than pattern 2 (Mann–Whitney U, $p=0.046$).

Association between immunochemical parameters and the clinical response to plaque accumulation

Stepwise multiple linear regression analysis was used to study the relationship between pre-baseline calprotectin levels and clinical and immunochemical parameters assessed after a 10-day period of no oral hygiene. These analyses revealed that the levels of MRP8/14 and MRP14 at subject recruitment (day − 11) could predict a significant part of the GI at day 10 (Table 3, $p<0.0001$). Moreover, the level of MRP8/14 at day − 11, i.e. at subject recruitment was significantly correlated to the level of MRP8/14 at day 10 ($p<0.0001$).

Discussion

Calprotectin has been detected in the serum/plasma (Sander et al. 1984, Muller et al. 1994, Golden et al. 1996) and several body fluids, such as the cerebrospinal fluid (Dunlop et al. 1991), synovial fluid (Berntzen et al. 1991), urine (Holt et al. 1983), feces (Roseth et al. 1999) and saliva (Muller et al. 1993, Brun et al. 1994, Cuida et al. 1995). In the present longitudinal study, the levels of calprotectin and its subunits MRP8 and MRP14 were quantified in the GCF simultaneously during a short period of experimentally induced gingivitis. The experimental gingivitis model (Löe et al. 1965, Theilade et al. 1966) has been used successfully to investigate the relationship between microbial colonization and host defense. However, this approach does not allow validating the hypothesis that expression of MRP8/14 in GCF may be a marker of active periodontitis. The present study rather provides elementary data regarding intra- and inter-individual variation, and the response to a simple change in the inflammatory state of the gingiva, induced by 10 days of no oral hygiene, in the absence of periodontal disease. This may serve as a basis for future research on detection of periodontal disease activity.

The levels of calprotecin and its subunits were expressed as total amounts per sample and as amounts per total protein. The patterns of expression were very similar if the MRP levels were taken as total amounts or as amounts per total protein. As has been discussed in the literature, concentrations are more prone to error in samples of small volume whereas total amounts are more appropriate (Lamster 1997). In fact, studies investigating differences of GCF components such as Il-1β and PGE2 in various clinical conditions have indicated that total amounts were more sensitive discriminators than concentrations (Tsai et al. 1998, Tuter et al. 2001).

A high degree of reproducibility was noted for the clinical parameters repeatedly assessed at days − 3 and 0 (Table 1). It should be noted that these values reflect a status of perfect gingival health. In this situation, all parameters show low values and have a small range of variation. During the experimental phase with no oral hygiene (days 0–10), the clinical parameters PII, GI and BOP increased as expected (Table 2), confirming that plaque accumulation leads to gingival inflammation (Löe et al. 1965). In contrast to these findings, the expression of total proteins, MRP8/14, MRP8 and MRP14 did not seem to be

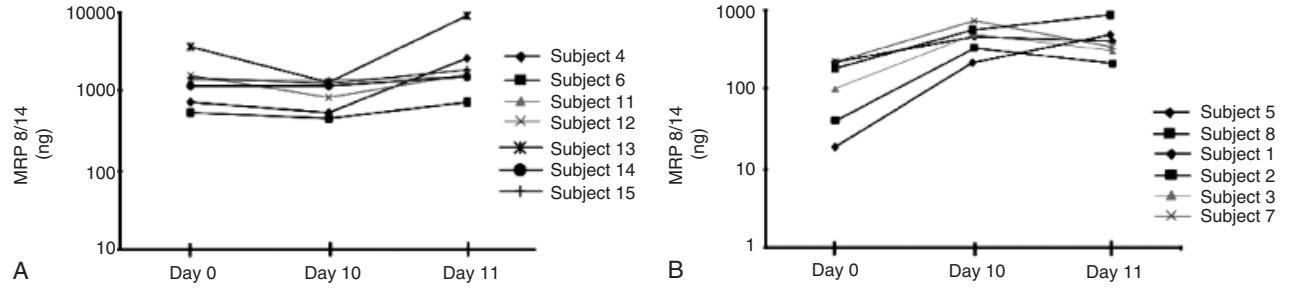

Fig. 3. Calprotectin (myeloid-related protein 8/14) expression during the experimental phase, corresponding to response pattern 1 (A) and pattern 2 (B).

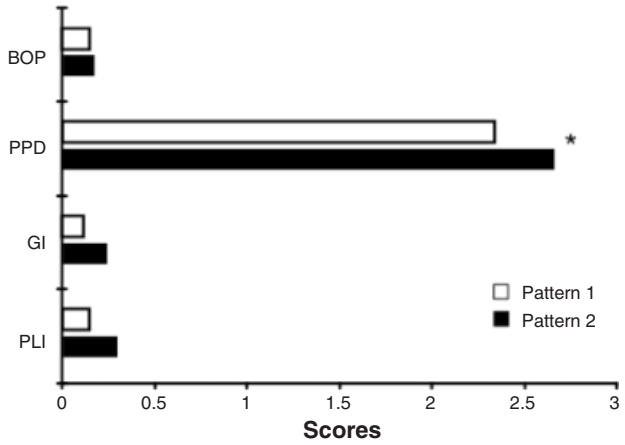

Fig. 4. Mean clinical parameters (bleeding on probing (BOP), probing pocket depth (PPD), Gingival Index (GI), Plaque Index (PLI)) of the two response patterns at the initial visit (day -11). $^{*}p = 0.026$.

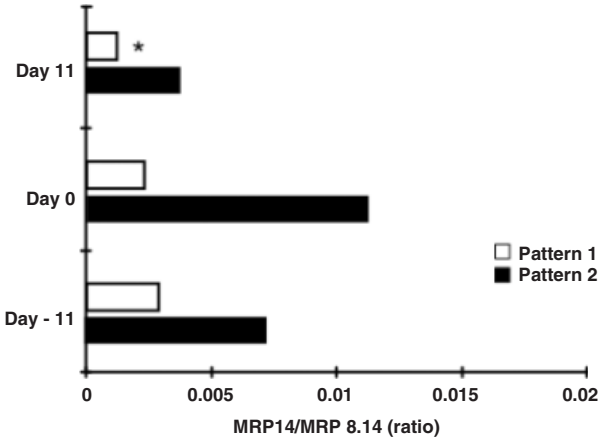

Fig. 5. Relationship between the mean amounts of subunit myeloid-related protein 14 and the mean amounts of the complex MRP8/14 at days -11, 0 and 11. $^{*}p = 0.046$.

Table 3. Local GI after 10 days of undisturbed plaque accumulation

Variable	Estimate	SE	*p*
intercept	1.5	0.08	<0.0001
MRP8/14 (ng/site)	0.00033	0.08	<0.0001
MRP14 (ng/site)	-0.24	0.06	<0.0001

Stepwise multiple linear regression. Final subset of predictor variables, recorded at subject recruitment (day -11), for local GI after 10 days of undisturbed plaque accumulation. $R^2 = 0.12$, total model $p<0.0001$, $n = 150$.
GI, Gingival Index; MRP, myeloid-related protein.

generally higher after 10 days of undisturbed plaque accumulation than at the beginning of the experimental phase (they increased with plaque accumulation in half of the subjects only). However, peak values were observed at day 11, i.e. 1 day after professional mechanical tooth cleaning (Fig. 2). Our results may be compared with those of an experiment, counting the crevicular polymorphonuclear leukocytes (cPMNs) during and after a 12-day experimental gingivitis (Jambrec 1993). Interestingly, in this study, mean cPMN counts remained unchanged during the period of undisturbed plaque accumulation, and showed a tendency to increase after the mechanical removal of plaque. This analogy in response points to a link between the expression of GCF-calprotectin and the number of cPMNs during experimental gingivitis, confirming the finding of Miyasaki et al. (1998) who found a direct correlation between calprotectin and lactoferrin levels, and concluded that the major source of GCF calprotectin was neutrophils. Forty-five percent of the total neutrophil cytosolic proteins in fact consist of MRP8 and MRP14 complexes (Edgeworth et al. 1991). The sudden rise of cPMNs and calprotectin levels at day 11 may be as a result of mechanical irritation of the already inflamed gingiva by the cleaning performed on day 10.

Fig. 1 shows the intra-individual variation of the parameter MRP8/14 at day 10 and demonstrates a distinct topographic pattern of calprotectin expression, emphasizing the relevance of defining ''representative sites''. A previous study has shown that bacterial plaque tends to accumulate in similar, locally determined patterns, that can be recognized already after 4 days without oral hygiene (Mombelli et al. 1990). The expression of calprotectin showed a topographic pattern comparable also

with previously shown distribution patterns of periodontal microorganisms in subjects with periodontal disease (Mombelli et al. 1991a, 1994).

Levels of the complex were also variable from subject to subject and two distinct patterns of response could be recognized. Subjects with response pattern 1 (Fig. 3A) showed lower initial mean values of PII, GI and PPD than pattern 2 subjects (day −11, Fig. 4). Furthermore, pattern 1 subjects showed higher MRP8/14 levels at days −11, −3 and 0 than pattern 2 subjects. These findings corroborate the concept of a protective role of MRP8/14, which is reflected in the alias ''calprotectin'' (Steinbakk et al. 1990). A reassessment of the cPMN data collected in the other mentioned experimental gingivitis trial (Jambrec 1993) also showed the presence of two such response patterns among 12 participants.

Already the first experimental gingivitis trials (Löe et al. 1965, Theilade et al. 1966), suspected inter-individual variation in gingival inflammation following the accumulation of bacterial deposits on tooth surfaces. This variation was thought to be related to quantitative differences in plaque mass or differences in plaque composition. However, inter-individual variation in gingival inflammation has also been observed in the absence of any notable quantitative or qualitative differences in plaque accumulation (Abbas et al. 1986). Individual variation in the gingival inflammatory response to plaque accumulation has also been observed in studies on naturally occurring gingivitis (Müller et al. 2000).

Up to 20-fold higher MRP8 levels have been measured in patients with periodontitis than in periodontally healthy subjects (Lundy et al. 2001). Only minute amounts of the two subunits MRP8 and MRP14 were detected in the present study (Fig. 2), and an increase during plaque accumulation was again seen only in pattern 2 subjects. Low levels of the subunits have been observed also in the presence of acute bacterial infection in serum and synovial fluid (Burmeister & Gallacchi 1995), but not in chronic infections. Whether subjects with a pattern 2 MRP response have a higher tendency to develop periodontal diseases than subjects with pattern 1 should be evaluated in further clinical studies. Slightly higher PPD values were in fact noted in pattern 2 subjects (Fig. 4).

Stepwise multiple linear regression analysis pointed out that 12% of GI variation at day 10 could be predicted by the levels of the complex and the subunit MRP14 at the day of subject recruitment (day −11, Table 3). Moreover, the levels of calprotectin at day −11 were linked to those at day 10. These observations show a connection between the level of baseline calprotectin and the early response to plaque accumulation, suggesting that the initial status of subjects entering an experimental gingivitis trial have to be taken into account, as they influence the outcome.

Conclusion

The expression of calprotectin in the early phase of experimental gingivitis is variable between subjects, and two groups of subjects can be differentiated according to their response patterns. Clinical parameters at the very first visit (day −11) seemed to be different in the two response groups. The results of the present investigation indicate that the inflammatory response to plaque accumulation depends on the initial status of the subjects, which may not be leveled out by the introduction of perfect oral hygiene. Whether these patterns reflect a different susceptibility to periodontal diseases remains to be determined.

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