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Reactivity of neutrophils, monocytes and platelets in periodontitis

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Publication date
2008

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Citation for published version (APA):

Nicu, E. A. (2008). *Reactivity of neutrophils, monocytes and platelets in periodontitis*. [Thesis, fully internal, Universiteit van Amsterdam].

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Chapter 2

Hyper-reactive PMNs in FcγRIIa 131H/H genotype periodontitis patients

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Journal of Clinical Periodontology 2007; 34:938-945

Abstract

Receptors for the Fc part of IgG (FcγRIIa) on polymorphonuclear leukocytes (PMN) mediate phagocytosis and cell activation. Previous results show that one of the genetic variants of the FcγRIIa, the 131H/H, is associated with more periodontal breakdown than the R/R. This may be due to a hyper-reactivity of the H/H-PMNs upon interaction with bacteria. We aimed to study whether the FcγRIIa genotype modifies the PMN reactivity in periodontitis patients. A cohort of 98 periodontitis patients was genotyped. From these, 10 H/H and 10 R/R consented to participate. PMNs were incubated with immune serum-opsonized *A. actinomycetemcomitans*. Phagocytosis, degranulation (CD63 and CD66b expression), respiratory burst and elastase release were assessed. Patients of the H/H genotype showed more bone loss than the H/R or R/R ($P=0.038$). H/H-PMNs phagocytosed more opsonized *A. actinomycetemcomitans* than did R/R-PMNs ($P=0.019$). The H/H-PMNs expressed also more CD63 and CD66b than did the R/R-PMNs ($P=0.004$ and 0.002 , respectively) and released more elastase ($P=0.001$). The genotyping results confirm previous reports that more periodontal destruction occurs in the H/H genotype than in H/R or R/R. The functional studies indicate a hyper-reactivity of the H/H- PMN in response to bacteria that may be one of several pathways leading to more periodontal breakdown.

Introduction

Periodontitis is a chronic infectious disease of the supportive tissues of the teeth characterized by gradual loss of periodontal attachment and alveolar bone. Periodontopathic bacteria such as *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* have been implicated in the pathogenesis of the disease (1,2). However, recent literature indicates a genetically determined hyperactivity of the host response, which constitutes one aspect of the susceptibility to periodontitis (3). Disease resistance seems to be characterized by a proper defense against periodontal pathogens without concomitant damage to the host itself.

In the course of periodontal infection, the human polymorphonuclear neutrophilic leukocyte (PMN) represents the first line of antibacterial defense. The PMNs have also been implicated in periodontal tissue degradation by releasing proteases and reactive oxygen species (4,5,6,7). The PMN constitutively expresses two types of Fc γ receptors: Fc γ RIIa and Fc γ RIIIb. The Fc γ R recognize and bind the constant part of immunoglobulin G. Fc γ RIIa mediates phagocytosis (8), killing of opsonized cellular targets via antibody-dependent cellular cytotoxicity (9), and respiratory burst (10). Important to note is that Fc γ RIIa contains a transmembrane domain, that facilitates signal transduction to the cell. This is not the case for Fc γ RIIIb, which does not contain a cytoplasmic domain but is anchored to the cell membrane via a glycosyl-phosphatidylinositol (GPI)-anchor. The role of Fc γ RIIIb in activation of PMNs has been debated. Some investigators have shown that this receptor is indeed capable of inducing signal transduction, possibly with the help of Fc γ RIIa (11), whereas others have suggested that Fc γ RIIIb does not contribute to effector functions (12).

Fc γ RIIa occurs in two allotypic forms, designated Fc γ RIIa-H131 and Fc γ RIIa-R131 due to the genetically determined presence of either a histidine or an arginine residue at amino acid position 131 (13). The genotype prevalence strongly depends on ethnicity and is a source of conflicting results. In the general population, the genotype distribution is similar in African-Americans and Caucasians, and distinct from that of Japanese or Chinese (14). Among Caucasians, the prevalence of Fc γ RIIa-131H/H genotype has been reported to be higher in chronic or aggressive periodontitis than in healthy subjects (15). On the other hand, Nibali et al. (2006) found no difference in the genotype frequency between aggressive periodontitis patients and healthy controls when considering either all subjects or only the Caucasians (16). Moreover, Caucasian

periodontitis patients with the H/H genotype have more periodontal breakdown than those with the H/R or R/R genotype (17).

The genetic variation in the FcγRIIa has functional consequences: the FcγRIIa-H131 genotype binds IgG₂-opsonized particles more efficiently than FcγRIIa-R131 genotype (18). This phenomenon may have important consequences for the pathogenesis of periodontitis, as has also been speculated by others (19). It is known that IgG₂ dominates the humoral immune response against polysaccharide antigens that are abundant on the cell wall of the gram-negative periodontal pathogens. For example, IgG₂ is the main immunoglobulin subclass reactive with *A. actinomycetemcomitans* (20). It has been hypothesized that the highly efficient binding of opsonized particles to FcγRIIa in the H/H genotype may result in a hyper-reactive state of the PMN (15,17). The strongly activated H/H PMN may release more of its granule contents, thus contributing to collateral damage, i.e. loss of periodontal connective tissue, periodontal ligament and alveolar bone in the defense process against periodontal pathogens. However, this hypothesis has never been tested.

The purpose of the present study was to investigate whether the FcγRIIa H/H and R/R genotypes have functional consequences for the reactivity of the PMN from periodontitis patients. This might contribute to the periodontitis phenotype in patients. We analyzed PMN activation in both H/H and R/R genotypes following incubation with opsonized *A. actinomycetemcomitans*. We studied four PMN functional parameters: phagocytosis, degranulation, respiratory burst, and elastase activity.

Materials and methods

Screening of periodontitis patients

98 periodontitis patients from a cohort within the Department of Periodontology, Academic Center for Dentistry Amsterdam (21) were genotyped for this study. Genomic DNA was extracted from blood by means of the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN, USA). Five μL of the purified DNA solution (concentration 70 - 100 ng DNA / μL solution) was added to a PCR reaction with allele-specific primers for FcγRIIa-H131 and FcγRIIa-R131 (22). Based on these PCR results, all FcγRIIa-H/H131 and FcγRIIa-R/R131 patients were selected as two contrasting groups for the PMN functional assays.

Recruitment of patients

	Periodontitis patients n=98
Age	44.9 ± 8.4
Gender	
Male	44 (44.9 %)
Female	54 (55.1 %)
Ethnicity	
Non-caucasian	20 (20.4 %)
Caucasian	78 (79.6 %)
Smoking	
Non-smoker	23 (23.4 %)
Former smoker	27 (27.6 %)
Current smoker	48 (49.0 %)
Number of teeth	
Total	26.0 ± 3.3
With bone loss	
< 40 % bone loss	15.8 ± 6.6
40-60 % bone loss	6.9 ± 4.0
>60 % bone loss	3.2 ± 3.4
Genotype FcγRIIa*	
R/R	22 (22.4 %)
H/R	48 (49.0 %)
H/H	28 (28.5%)

Table 2.1 Summary of characteristics of the 98 patients screened for FcγRIIa polymorphism. Values are means ± standard deviations or numbers of subjects (with percentage in brackets)

* Genotyping results for Caucasian patients only are 18 R/R (23.7%), 38 H/R (50%) and 20 H/H (26.3%).

From the genotyping results of the 98 patients, it appeared that 48 (49%) were heterozygous H/R, 28 (29%) homozygous H/H, and 22 (22%) homozygous R/R (Table 2.1). All 28 H/H and all 22 R/R subjects were searched within the computer system of the Dental Faculty and approached to participate, but only 10 in each group consented to donate blood for PMN functional assays. All subjects were informed both verbally and in writing about the purpose of the study. The Ethics Committee of the Academic Medical Center of the University of Amsterdam approved the study. The 20 participants had received active periodontal therapy and were in periodontal maintenance. They were free from systemic diseases and had no clinical symptoms of bacterial, viral or parasitic infections at the time of the study. None of the subjects had taken any form of medication that could affect their immune status, such as anti-inflammatory agents, antibiotics or immunosuppressants during the last 2 weeks prior to blood collection. For all participants,

smoking status and smoking history were recorded and subjects were classified as non-smokers (never smokers or those who quit > 10 years ago), former smokers (those who quit smoking in the last 10 years) and current smokers. All patients showed on dental radiographs periodontal bone loss of > 1/3 of the root length on ≥ 2 teeth.

Bacteria and immune serum

To obtain immune serum against *A. actinomycetemcomitans*, we selected 10 periodontitis patients who were culture positive for *A. actinomycetemcomitans*; they were patients from the departmental clinic who were in active periodontal treatment. The undiluted serum of these patients was tested against *A. actinomycetemcomitans* serotype a strain HG 569, serotype b strain HG 90, serotype c strain HG 683, serotype d strain 3381, and serotype e strain HG 1650. Bacteria were grown for 18 hours in brain heart infusion broth supplemented with 5 mg/L hemin and 1 mg/L menadione at 37°C in humidified 5% CO₂. Bacteria were harvested, washed twice in PBS, checked for purity and the concentration was adjusted to approximately 5×10^8 CFU/mL. All bacterial preparations were sonicated on ice for 2 min, at 5-sec intervals, amplitude 18, by means of a Soniprep-150 ultrasonic disintegrator (MSE, London, UK). Immunodiffusion of whole serum was carried out in 1% agarose (Sigma Chemicals Co., St. Louis, MO, USA) in 50 mM Tris-HCl buffer, pH 7.6. Fifteen μ L of undiluted serum and 15 μ L of the sonic extract were allowed to precipitate for 48 h at room temperature. *A. actinomycetemcomitans* serotype c was the only one of the five serotypes tested that induced immunodiffusion bands with the serum from the most of the 10 patients: 5 out of the 10 periodontitis patients sera tested were positive against serotype c. Subsequently we pooled all available sera from these 5 patients (pooled whole serum [Serum]) and stored it at -20°C in 50- μ L aliquots to be used as an opsonization source throughout all experiments. For some experiments, the serum was incubated at 56°C for 30 minutes to remove complement activity and the resulting heat-inactivated serum (HIS) was used as a source of immunoglobulins.

Phagocytosis assay

Blood was collected from patients by venous puncture in the antecubital fossa with minimal stasis, in sodium heparine-containing vacuum tubes (Vacutainer, BD, Alphen a/d Rijn, the Netherlands). Heparinized blood was diluted 1:1 in PBS containing 10% (w/v) sodium citrate, layered on Percoll ($\delta=1.078$ g/mL) and centrifuged at 800 x g, at 20°C for

20 min without brake. Supernatant was discarded and the pellet, containing erythrocytes and PMNs, was washed with ice-cold NH_4Cl buffer (155 mM NH_4Cl , 10 mM KHCO_3 , 0.1 mM EDTA at pH 7.4) to lyse the erythrocytes. After a centrifugation step, PMNs were washed in PBS, counted and the concentration was adjusted to 1×10^7 cells/mL in HEPES buffer (123 mM NaCl, 5 mM KCl, 1mM CaCl_2 , 1mM MgCl_2 , 25 mM HEPES, 10 mM glucose, pH 7.4). Purity and viability was >95%, as determined by flow cytometry and trypan blue exclusion, respectively.

A. actinomycetemcomitans serotype c (5×10^8 CFU/mL) was used in the phagocytosis assay and was labeled with fluorescein isothiocyanate (FITC 0.015 mg/mL, Sigma) for 30 minutes at 37°C. After a wash step to remove unbound FITC, the bacteria were resuspended in PBS and stored in 1 mL aliquots at -20°C until use. Opsonization of unlabeled or FITC-labeled *A. actinomycetemcomitans* was performed with 3% (v/v) serum or HIS for 30 min at 37°C. After opsonization, bacteria were washed and resuspended in HEPES buffer.

PMNs and opsonized FITC-*A. actinomycetemcomitans* were mixed at a ratio of 1:25 in Eppendorf vials containing 175 μL of HEPES buffer and were incubated in a shaking water bath at 37°C. After 30 minutes, the samples were placed on ice to stop phagocytosis. Samples were centrifuged at 500 x g for 5 minutes at 4°C and the supernatants were collected and stored at -20°C for later use. Cells were fixed with 2% paraformaldehyde in PBS and analyzed within one hour by flow cytometry. Trypan blue 0.064% (w/v) was used to quench fluorescence from adherent, non-ingested FITC-*A. actinomycetemcomitans*. The percentage (%) of phagocytic PMNs and the mean fluorescent intensity (MFI) of the phagocytic PMNs were measured in the samples by means of a FACScan flow cytometer (Becton-Dickinson, San Jose, CA) equipped with a laser beam emitting at 488 nm. PMNs were gated according to their forward and side-scatter properties and at least 10 000 cells per sample were counted using the CellQuest software (Becton-Dickinson). The phagocytic index (PI) was calculated as previously described (23). Briefly, the PI takes into account the percentage phagocytic PMNs and the number of fluorescent bacteria per phagocytic PMN ($\text{PI} = \text{MFI of phagocytic PMNs} * \% \text{ phagocytic PMNs}$). Phagocytosis of opsonized *A. actinomycetemcomitans* is plotted as the PI of cells when challenged with opsonized *A. actinomycetemcomitans* minus background (i.e. negative control, cells incubated with non-opsonized *A. actinomycetemcomitans*).

PMN degranulation assay

PMNs (1×10^7 cells/mL) and serum-opsonized *A. actinomycetemcomitans*, HIS-opsonized *A. actinomycetemcomitans* or non-opsonized *A. actinomycetemcomitans* (5×10^8 CFU/mL) were mixed at a ratio of 1: 25 for PMN and bacteria, respectively, and incubated in a shaking water bath at 37°C. After 30 minutes, the reaction was stopped by placing the samples on ice. Samples were washed and the PMNs were resuspended in HEPES buffer. Fusion of primary (azurophilic) and secondary (specific) granules with the plasma membrane was quantified by measuring the appearance of the granule markers CD63 and CD66b, respectively, at the cell surface. The samples were incubated for 30 minutes on ice with mouse anti-human phycoerythrin (PE)-conjugated CD63, mouse anti-human FITC-conjugated CD66b, IgG1-PE isotype control and IgG1-FITC isotype control (Sanquin Reagents, Amsterdam, The Netherlands) at a final concentration of 10 µg/mL. After incubation, cells were washed twice with HEPES buffer, resuspended in 2% (v/v) paraformaldehyde in PBS and analyzed by flow cytometry. Data are expressed as the relative MFI, calculated as the MFI of cells when challenged with *A. actinomycetemcomitans* (serum-, HIS-opsonized or non-opsonized) minus background (i.e. negative control, cells incubated in HEPES buffer).

Respiratory burst assay

Hydrogen peroxide production by PMN was measured by the use of Amplex Red hydrogen peroxide kit (Molecular Probes, Leiden, the Netherlands) according to the manufacturer's protocol. Briefly, the PMN concentration was adjusted to 1×10^6 cells/mL. Cells were stimulated with serum-opsonized *A. actinomycetemcomitans*, HIS-opsonized *A. actinomycetemcomitans* and non-opsonized *A. actinomycetemcomitans* in a PMN to *A. actinomycetemcomitans* ratio of 1:100. Fifty µL of PMN suspension and 50 µL stimulus were added to 100 µL of reaction buffer in 96-wells plate. The increase in absorbance at 590 nm was monitored for 90 minutes. The maximal increase in fluorescence was calculated over a 30-min interval. Data are expressed as relative H₂O₂ release calculated as the difference between the H₂O₂ release from PMN incubated in the presence of *A. actinomycetemcomitans* and the H₂O₂ release from PMN incubated in HEPES buffer as a negative control.

Elastase activity assay

Elastase activity was measured by the hydrolysis of N-methoxysuccinyl-Ala-Ala-Pro-Val-pNA (Sigma) as described (24), with minor modifications. Briefly, 75 μ L from the supernatants collected following phagocytosis assay (see section *Phagocytosis assay*) were added to 25 μ l of 0.1 M Tris-HCl buffer pH 7.2, containing 0.5 M NaCl, and 1 mM N-methoxysuccinyl-Ala-Ala-Pro-Val-pNA. The absorbance was measured at 405 nm for 30 min after the addition of the substrate. Data are expressed as elastase activity calculated as the difference between the elastase activity from PMN incubated in the presence of *A. actinomycetemcomitans* and the elastase activity from PMN incubated in HEPES buffer as a negative control.

Statistical analysis

Tabulation of data, box plot generation and data analysis were performed with the SPSS 12.0 package. Means, standard deviations, medians and frequency distributions were calculated. In the patient screening part of the study, differences between the H/H, H/R, and R/R genotype groups for the radiographic bone levels were analyzed in a general linear model (ANCOVA), taking the genotype as fixed factor, and age and Caucasian race as covariates. In the functional studies, differences between the Fc γ RIIa-H/H131 and R/R131 patient groups were statistically analyzed with the Mann-Whitney U test or the chi-square test, where appropriate. Differences between PMN functional parameters under different conditions within the Fc γ RIIa-H/H131 and R/R131 patient groups were statistically analyzed with the Friedman test for related samples; *P*-values <0.05 were considered statistically significant.

The distribution of our data (phagocytosis, CD63, CD66b, respiratory burst, elastase) was skewed and far from normal. This was the reason for using non-parametric statistics (Mann-Whitney U tests) when comparing the H/H and R/R. In the cases where we found statistically significant differences between H/H and R/R, we log-transformed the experimental data to get a reasonable approximation to a normal distribution. We performed an exploratory linear regression analysis of the PMN activation parameters using the log-transformed data. We have used the values of phagocytosis, CD63, CD66b, or elastase as dependent variable and Fc γ RIIa genotype, age, gender, race and smoking as independent variables.

Results

Screening of periodontitis patients. A description of characteristics of 98 patients initially genotyped is provided in Table 2.1. The mean age of the screened patients was 44.9 years, and slightly more females participated in the study. The great majority was Caucasian and almost half of the participants were current smokers. On average, 26.0 teeth were present and 10.1 (39%) showed bone loss on $\geq 40\%$ of the root length. The genotyping showed that 22% of the patients were homozygous R/R, 49% heterozygous H/R, and 29% homozygous H/H. When we tabulated for Caucasians only, we observed that 24% were R/R, 50% H/R, and 26% H/H.

The scores of radiographic bone loss were analyzed by genotype. While the number of teeth per genotype was not different, it was observed that patients with the H/H genotype had more teeth with bone loss in the category 40-60% of the root length than patients with H/R or R/R genotype, after adjusting for age and Caucasian race ($P=0.038$; Table 2.2).

Genotypes	R/R	H/R	H/H
	n=22	n=48	n=28
Number of teeth present	25.8 \pm 0.6	25.6 \pm 0.4	26.9 \pm 0.5
Number of teeth with bone loss			
< 40 % bone loss	17.7 \pm 1.3	15.2 \pm 0.9	15.3 \pm 1.2
40-60 % bone loss *	5.4 \pm 0.8	6.6 \pm 0.5	8.3 \pm 0.7
> 60 % bone loss	2.6 \pm 0.7	3.6 \pm 0.5	3.1 \pm 0.6

Table 2.2 Periodontal characteristics of the 98 patients screened for FcγRIIIa polymorphism, according to genotype. Values are means \pm standard deviation. * $P=0.038$

Study population for PMN functional assays. A description of the patient group used for functional assays is provided in Table 2.3. The patients were on average 45 years old and gender distribution was not significantly different between the R/R and H/H subjects. Eighteen of the 20 patients were of Caucasian origin, 8 were current smokers, and 5 were non-smokers, while 7 were former smokers. Smoking status was not different between the H/H and R/R groups ($P=0.129$). There was a trend towards more bone loss in the H/H patients than in the R/R patients (number of teeth with bone loss in the category 40-60% of the root length: 8.2 ± 3.4 and 5.5 ± 4.0 for H/H and R/R groups respectively; $P=0.08$).

	R/R n=10	H/H n=10
Age	45 ± 11.4	44 ± 8.5
Gender		
Male	5	4
Female	5	6
Ethnicity		
Non-caucasian	1	1
Caucasian	9	9
Smoking		
Non-smoker	4	1
Former smoker	4	3
Current smoker	2	6
Number of teeth		
Total	25.4 ± 3.6	27.4 ± 2.4
With bone loss		
< 40 % bone loss	16.8 ± 7.7	16.0 ± 7.4
40-60 % bone loss	5.5 ± 4.0	8.2 ± 3.4
>60 % bone loss	3.0 ± 3.3	3.1 ± 3.2

Table 2.3 Summary of the characteristics of the group of patients used in the present study. Values are means ± standard deviations or numbers of subjects.

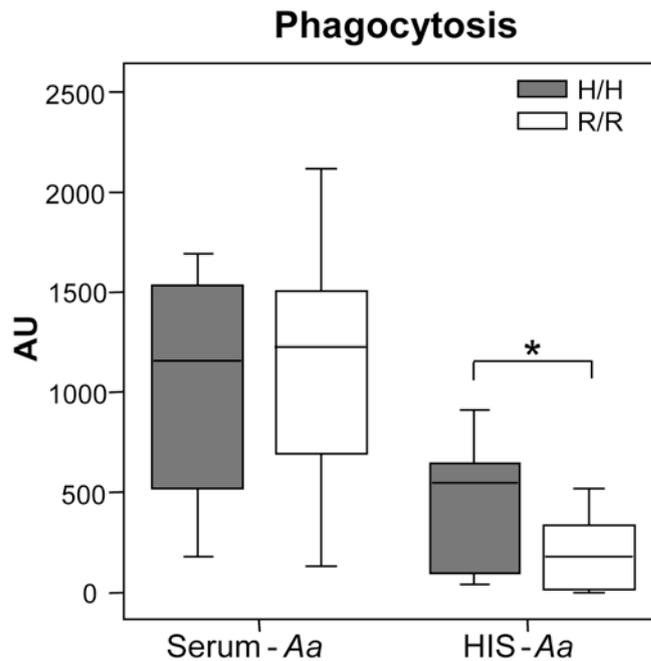


Fig. 2.1 Box plots showing phagocytic index for H/H and R/R PMNs with whole serum- or HIS-opsonized *A. actinomycetemcomitans*. AU, arbitrary units; Aa, *A. actinomycetemcomitans*; HIS, heat-inactivated serum. * $P=0.019$

Phagocytosis. From the phagocytosis experiments, a phagocytic index (PI) taking into account both percentage of phagocytic cells and number of fluorescent bacteria per phagocytic cell (MFI of these cells) was calculated. For both H/H and R/R genotypes, the PI was higher using serum than when using HIS for opsonization of *A. actinomycetemcomitans* (Fig. 2.1; $P=0.009$ and $P=0.005$, respectively). The PI of *A. actinomycetemcomitans* opsonized with serum in H/H and R/R genotypes was comparable, whereas phagocytosis after Fcγ-receptor-mediated activation only (i.e. when using HIS-opsonized *A. actinomycetemcomitans*) was significantly higher in the FcγRIIa131H/H group than in the FcγRIIa131R/R group ($P=0.019$).

Degranulation.

Primary granules. Degranulation of primary PMN granules is a measure of PMN activation and was analyzed by the expression of CD63 (Fig. 2.2A). In the H/H subjects, the expression of CD63 was higher when *A. actinomycetemcomitans* was opsonized with whole serum (MFI median 7.25), than when HIS was used for *A. actinomycetemcomitans* opsonization (median 5.49), while non-opsonized *A.a* provoked only a minimal CD63

expression (median 0.7; $P=0.0002$). Among R/R patients this pattern was also seen, i.e. MFI of CD63 with serum-opsonized *A. actinomycetemcomitans* (median 5.46) was higher than with HIS-*A. actinomycetemcomitans* (median 2.05), while again non-opsonized *A. actinomycetemcomitans* was essentially not inducing any CD63 upregulation (median 0.84; $p<0.0001$).

When comparing the CD63 expression between groups, H/H and R/R patients released similar amounts of primary granules in response to serum-opsonized *A. actinomycetemcomitans* ($P=0.190$). Interestingly, however, when HIS was used for opsonization of bacteria, indicating the Fc γ R stimulation only, the PMNs from H/H patients expressed significantly more CD63 than the PMNs from R/R patients ($P=0.004$; Fig. 2.2A).

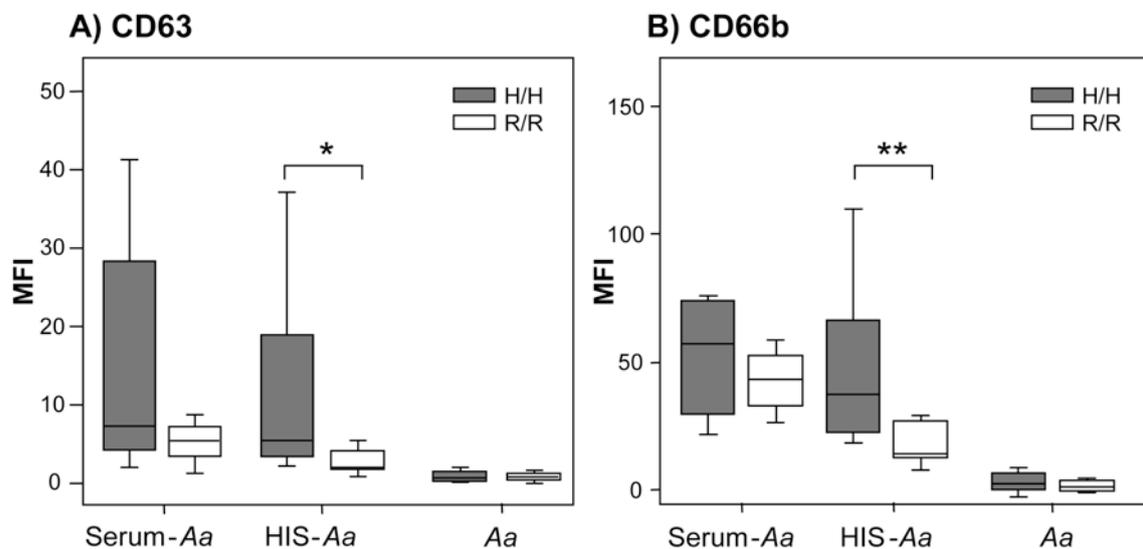


Fig. 2.2 Box plots showing markers of degranulation of primary granules (panel A, CD63) and secondary granules (panel B, CD66b) for H/H and R/R PMNs with whole serum- or HIS-opsonized or non-opsonized *A. actinomycetemcomitans*. MFI, mean fluorescence intensity; Aa, *A. actinomycetemcomitans*; HIS, heat-inactivated serum; * $P=0.004$; ** $P=0.002$

Secondary granules. Degranulation of secondary PMNs granules is also a measure of PMN activation and was analyzed by the expression of CD66b; the results are summarized in Fig. 2.2B. In the H/H subjects, the expression of CD66b was higher when *A. actinomycetemcomitans* was opsonized with serum (median 57.01) than when HIS was used for *A. actinomycetemcomitans* opsonization (median 37.42), while non-opsonized *A.a* induced very limited CD66b expression (median 2.58; $P=0.0001$). The same pattern was

seen in R/R patients, i.e., the MFI of CD66b in response to serum-opsonized *A. actinomycetemcomitans* was higher (median 43.52) than in response to HIS-*A. actinomycetemcomitans* (median 14.08), while non-opsonized *A. actinomycetemcomitans* was not inducing CD66b expression (median 1.365; $P < 0.0001$).

When comparing the two groups, H/H and R/R PMNs released similar amounts of secondary granules in response to serum-opsonized *A. actinomycetemcomitans* ($P = 0.436$). However, when FcγRIIa were stimulated with HIS-opsonized *A. actinomycetemcomitans*, the PMNs from H/H patients expressed significantly more CD66b than did the PMNs from R/R patients ($P = 0.002$).

Linear regression analysis considering genotype, age, gender, race and smoking as independent factors indicated that genotype was the only parameter significantly associated with CD66b expression after stimulation with HIS-*A. actinomycetemcomitans* ($t = -2.417$, $P = 0.03$; Table 2.4).

	Genotype (<i>P</i> -value)	Age (<i>P</i> -value)	Gender (<i>P</i> -value)	Race (<i>P</i> -value)	Smoking (<i>P</i> -value)
Phagocytosis-HIS	0.129	0.798	0.815	0.445	0.550
CD63-HIS	0.068	0.164	0.206	0.515	0.139
CD66b-HIS	0.03	0.119	0.383	0.473	0.186
Elastase-HIS	<0.0001	0.531	0.897	0.308	0.315
Elastase-Serum	<0.0001	0.442	0.897	0.264	0.06

Table 2.4 Linear regression analysis of phagocytosis, CD63, CD66b and elastase activity after stimulation with HIS-*A. actinomycetemcomitans* or Serum-*A. actinomycetemcomitans* using genotype, age, gender, race and smoking as independent variables.

Respiratory burst. Respiratory burst is a major anti-bacterial function of the PMN and it was analyzed by measuring extracellular H₂O₂ release (Fig. 2.3). In both H/H and R/R patients, serum-opsonized *A. actinomycetemcomitans* induced a more intense H₂O₂ release (median 803.0 and median 701.5, respectively) than HIS-opsonized *A. actinomycetemcomitans* (median 503.5 and median 387.5, respectively), which in turn was higher than the response induced by non-opsonized *A. actinomycetemcomitans* (median 55.0 and median 64.0, respectively; $P < 0.0001$). The release of H₂O₂ into the extracellular medium of H/H PMNs was not different from that of R/R PMNs, irrespective of the stimulus used (serum-opsonized *A. actinomycetemcomitans* $P = 0.739$; HIS-opsonized *A. actinomycetemcomitans* $P = 0.796$; non-opsonized *A. actinomycetemcomitans* $P = 0.739$).

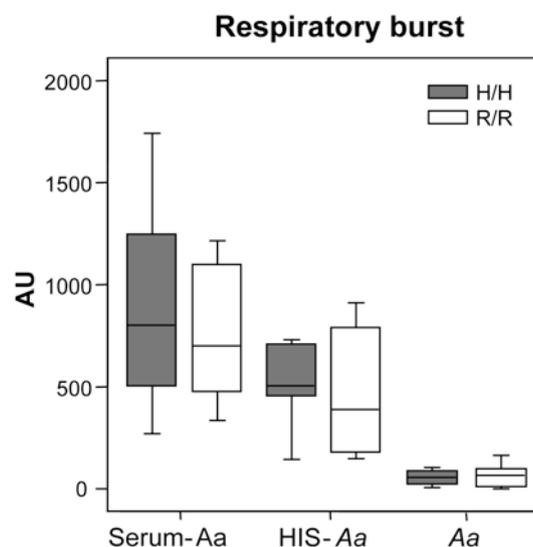


Fig. 2.3 Box plots showing respiratory burst by H/H and R/R PMNs with whole serum- or HIS-opsonized *A. actinomycetemcomitans* or non-opsonized *A. actinomycetemcomitans*. AU, arbitrary units; Aa, *A. actinomycetemcomitans*; HIS, heat-inactivated serum.

Elastase activity. Elastase is one of the proteolytic enzymes present in the PMN primary granules, released upon activation of the PMN and capable of degrading extracellular matrix proteins of the connective tissue. The release of active elastase into culture supernatants following interaction with *A. actinomycetemcomitans* is presented in Fig. 2.4. PMNs from H/H subjects released high amounts of active elastase when stimulated with serum- or HIS-opsonized *A. actinomycetemcomitans* (median elastase 0.310 units/mL and 0.239 U/mL, respectively). The non-opsonized *A. actinomycetemcomitans* induced very low levels of elastase release in PMN supernatants from H/H patients (median 0.058 U/mL). In striking contrast to the H/H PMNs, the R/R PMNs showed low release of

elastase after incubation with serum-*A. actinomycetemcomitans* (median 0.075 U/mL), HIS-*A. actinomycetemcomitans* (median 0.060 U/mL), or non-opsonized *A. actinomycetemcomitans* (median 0.052 U/mL).

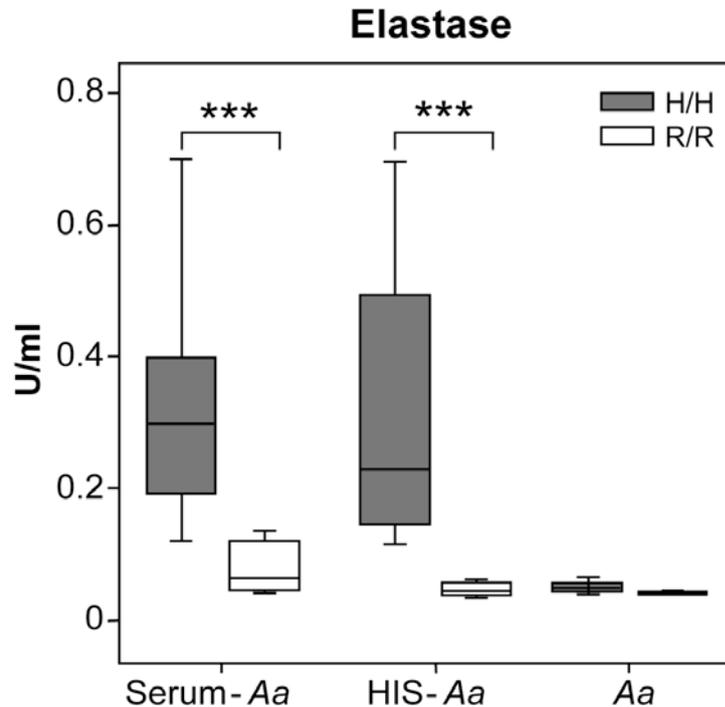


Fig. 2.4 Box plots showing elastase activity released by H/H and R/R PMNs with whole serum- or HIS-opsonized *A. actinomycetemcomitans* or non-opsonized *A. actinomycetemcomitans*. U/mL, elastase units per mL supernatant; Aa, *A. actinomycetemcomitans*; HIS, heat-inactivated serum. *** $P < 0.001$

In comparison to the R/R group, H/H PMNs released significantly higher amounts of active elastase in response to serum-*A. actinomycetemcomitans* and HIS-*A. actinomycetemcomitans* ($P < 0.001$ and $P = 0.001$, respectively). Linear regression analysis considering genotype, age, gender, race and smoking as independent factors indicated that genotype was the only parameter significantly associated with elastase release after stimulation with either HIS-*A. actinomycetemcomitans* or serum-*A. actinomycetemcomitans* ($P < 0.0001$; Table 2.4).

Discussion

We have investigated the relationship between cellular functions of PMNs and the FcγRIIa genotype in periodontally diseased individuals. A significantly higher degree of phagocytosis, degranulation and elastase release from PMNs of 131H/H patients than of

131R/R patients was demonstrated when HIS was used for opsonization of *A. actinomycetemcomitans*.

The results presented in this study support the hypothesis that the Fc γ RIIa H/H genotype may induce a hyper-reactive phenotype of the PMNs, which in response to periodontal pathogens will release more bioactive molecules that could aggravate the periodontal destruction. Our results are in line with the idea that the periodontal breakdown in periodontitis is partly caused by hyper-reactive PMNs (25,5). However, these latter studies suggested the constitutive nature of the PMN hyper-responsiveness without shedding light on possible molecular mechanisms involved.

The higher activity of the PMNs of the H/H genotype that we have observed is most likely the functional consequence of the strong binding of the IgG₂ on the bacteria to the Fc γ RIIa, because the Fc γ RIIa H/H is the only PMN receptor that efficiently recognizes IgG₂ (18). The HIS employed in this study contains immunoglobulins specific for the *A. actinomycetemcomitans* serotype c strain HG 683 as confirmed in the immunodiffusion assays. We did not further purify IgG₂ from HIS, but it may be the main immunoglobulin subclass reactive with *A. actinomycetemcomitans*, as it dominates the antibody response against polysaccharide antigens that are abundant on the cell wall of the gram-negative periodontal pathogens (20).

In the case of the more reactive genotype of the Fc γ RIIa, the H/H, our data demonstrate a higher release of elastase, contributing to a more severe breakdown of the extracellular matrix in the periodontal tissues.

Another mechanism proposed for the tissue breakdown induced by hyper-reactive PMN is the release of oxygen reactive species (26). We observed no significant difference in H₂O₂ production between the H/H and R/R PMNs, although degranulation was clearly increased in the H/H genotype. This unexpected result could be due to a possible difference in the release of other oxygen species than H₂O₂. For example, using Fc γ R-stimulated PMNs and chemiluminescence as a measure of all oxygen reactive species produced, Fredriksson et al. (1998) showed a higher oxidative burst in periodontitis patients than in controls (27). However, in the same study, there was no difference between patients and controls when intracellular H₂O₂ was assessed (27). Therefore, a future assessment of respiratory burst in H/H and R/R PMNs should take into consideration the production of all reactive oxygen species rather than relying only on H₂O₂ release.

A limitation of our study is the low number of participants. From the 98 periodontitis patients genotyped for Fc γ RIIa 131H/R polymorphism, all H/H (n=28) and all R/R (n=22) patients were approached to participate in this study. However, only 10 H/H and 10 R/R subjects agreed to donate blood necessary for the PMN functional assays. We decided to select the homozygous H/H and R/R as two contrasting groups to be compared with respect to PMN functions. The reason for not including any heterozygous H/R was first the difficulty in recruiting enough subjects and second, if there is a difference between genotypes, it should be more clearly visible when using the homozygous donors. Nevertheless, how PMNs from H/R subjects behave remains to be elucidated. It was important to note that after performing Fc γ RIIa genotyping of the 98 periodontitis patients in this cohort, the H/H genotype was associated with more teeth belonging to the category with bone loss in the 40-60% of the root length. These data confirmed previous reports and support the view that the H/H genotype may be regarded as a putative severity factor for periodontitis in Caucasians (15,17). This may be one important aspect of the genetic make-up of the immune response.

The distribution of smokers and non-smokers was uneven in our R/R and H/H groups. This drawback was due to the limited number of persons with the required genotypes who agreed to participate and hence, unavoidable. However, literature reports on the effect of smoking on the function of PMN are contradictory and do not support a definitive answer on the matter. Ryder et al. (1998) and Sørensen et al. (2004) describe a reduced response to stimulation in PMN from smokers when compared to non-smokers (28,29). Gustafsson et al. (2000) found no difference between the PMN from smokers and non-smokers after Fc γ -receptors stimulation with opsonized bacteria (30). The issue of the possible influence of smoking status or other background characteristics on the measured PMN parameters was a matter of concern for us, as well. We performed an exploratory linear regression analysis using phagocytosis, CD63, CD66b, or elastase as dependent variable, and Fc γ RIIa genotype, age, gender, race and smoking as independent variables. For none of the parameters of interest, smoking was not significantly contributing to the measured values nor did age, gender or race. The only parameter significantly associated with the values of phagocytosis, CD63, CD66b, elastase was the Fc γ RIIa genotype.

The phagocytosis and degranulation were higher for both H/H and R/R groups when whole serum-opsonized bacteria were used in comparison to HIS. This indicates that complement factors in whole serum are important mediators of these processes, thus PMN

reactivity is most likely also affected by complement receptor stimulation. Our data confirm previous reports that cooperative Fc and C3 receptor interaction is required for optimal PMN defense against *A. actinomycetemcomitans* (31).

In conclusion, in the current study we found that Fc γ RIIa polymorphism influences the functions of PMNs in periodontitis patients. Individuals with an H/H genotype show a hyper-reactive phenotype, with increased Fc γ -mediated phagocytosis, degranulation and granular enzymes release, which may be one of the several factors contributing to the severity of the periodontitis in these patients.

Acknowledgements

The present study was supported by The Netherlands Institute for Dental Sciences. We thank Nannette Brouwer and Anton Tool for expert assistance in setting up the phagocytosis and degranulation assays.

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