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

E. A. Nicu[1], U. Van der Velden[1], V. Everts[2], A. J. Van Winkelhoff[3], D. Roos[4] and B. G. Loos[1]

Departments of [1]Periodontology, [2]Oral Cell Biology, and [3]Oral Microbiology, Academic Center for Dentistry Amsterdam (ACTA), Universiteit van Amsterdam and Vrije Universiteit, Amsterdam, The Netherlands; [4]Department of Blood Cell Research, Sanquin Research and Landsteiner Laboratory, Academic Medical Center (AMC), Universiteit van Amsterdam, Amsterdam, The Netherlands

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Abstract

Background: 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 131 H/H, is associated with more periodontal breakdown than the R/R. This may be due to hyper-reactivity of the H/H-PMNs upon interaction with bacteria.

Aim: To study whether the FcγRIIa genotype modifies the PMN reactivity in periodontitis patients.

Material and Methods: 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 *Actinobacillus actinomycetemcomitans* (*A.a.*). Phagocytosis, degranulation (CD63 and CD66b expression), respiratory burst and elastase release were assessed.

Results: Patients of the H/H genotype showed more bone loss than those with the H/R or R/R genotype ($p = 0.038$). H/H-PMNs phagocytosed more opsonized *A.a.* than did R/R-PMNs ($p = 0.019$). The H/H-PMNs also expressed more CD63 and CD66b than did the R/R-PMNs ($p = 0.004$ and 0.002, respectively) and released more elastase ($p = 0.001$).

Conclusions: The genotyping results confirm previous reports that more periodontal destruction occurs in the H/H genotype than in the H/R or R/R genotype. The functional studies indicate a hyper-reactivity of the H/H-PMN in response to bacteria, which may be one of several pathways leading to more periodontal breakdown.

Key words: FcγRIIa; genotype; periodontitis; PMN; reactivity

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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 *Actinobacillus actinomycetemcomitans (A.a.)* and *Porphyromonas gingivalis* have been implicated in the pathogenesis of the disease (Slots 1999, van Winkelhoff et al. 2002). However, recent literature indicates a genetically determined hyperactivity of the host response, which constitutes one aspect of the susceptibility to periodontitis (Van Dyke & Sheilesh 2005). Disease resistance seems to be characterized by a proper defence against periodontal pathogens without concomitant damage to the host itself.

Conflict of interest and source of funding statement

The authors declare that they have no conflict of interests.

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During the course of periodontal infection, the human polymorphonuclear neutrophilic leukocyte (PMN) represents the first line of antibacterial defence. The PMNs have also been implicated in periodontal tissue degradation by releasing proteases and reactive oxygen species (Janoff 1985, Van Dyke et al. 1985, Weiss 1989, Fredriksson et al. 2003, Matthews et al. 2006). The PMN constitutively expresses two types of Fcγ receptors: FcγRIIa and FcγRIIIb. The FcγR recognize and bind the constant part of immunoglobulin G. FcγRIIa mediates phagocytosis (Anderson et al. 1990b), killing of opsonized cellular targets via antibody-dependent cellular cytotoxicity (Graziano & Fanger 1987) and respiratory burst (Anderson et al. 1990a). Important to note that FcγRIIa contains a transmembrane domain that facilitates signal transduction to the cell. This is not the case for FcγRIIIb, which

does not contain a cytoplasmic domain but is anchored to the cell membrane via a glycosyl-phosphatidylinosytol (GPI) anchor. The role of FcγRIIIb in the 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 FcγRIIa (Chuang et al. 2000), whereas others have suggested that FcγRIIIb does not contribute to effector functions (Scott-Zaki et al. 2000).

FcγRIIa occurs in two allotypic forms, designated FcγRIIa-H131 and FcγRIIa-R131 due to the genetically determined presence of either a histidine or an arginine residue at amino acid position 131 (Warmerdam et al. 1990). The genotype prevalence strongly depends on ethnicity and is a source of conflicting results. In the general population, the genotype distribution seems similar in African Americans and Caucasians, and distinct from that of Japanese or Chinese individuals (van der Pol & van de Winkel 1998). Among Caucasians, the prevalence of the FcγRIIa-131 H/H genotype has been reported to be higher in chronic or aggressive periodontitis than in healthy subjects (Loos et al. 2003, Yamamoto et al. 2004). 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 Caucasians. Moreover, Caucasian periodontitis patients with the H/H genotype show more periodontal breakdown than those with the H/R or R/R genotype (Loos et al. 2003, Wolf et al. 2006).

The genetic variation in the FcγRIIa has functional consequences: the FcγRIIa-H131 genotype binds immunoglobulin (Ig) IgG2-opsonized particles more efficiently than the FcγRIIa-R131 genotype (Warmerdam et al. 1991). This phenomenon may have important consequences for the pathogenesis of periodontitis, as has also been speculated by others (Wilson & Bronson 1997). 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 Ig subclass reactive with *A.a.* (Wilson & Hamilton 1992). 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 (Loos et al. 2003, Yamamoto et al. 2004). 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 defence 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 analysed PMN activation in both H/H and R/R genotypes following incubation with opsonized *A.a.* We studied four PMN functional parameters: phagocytosis, degranulation, respiratory burst and elastase activity.

Material and Methods

Screening of periodontitis patients

Ninety-eight periodontitis patients from a cohort within the Department of Periodontology, Academic Center for Dentistry, Amsterdam (Bizzarro et al. 2007), 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 microlitres of the purified DNA solution (concentration 70–100 ng DNA/μl solution) was added to a polymerase chain reaction (PCR) reaction with allele-specific primers for FcγRIIa-H131 and FcγRIIa-R131 (Flesch et al. 1998). 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

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 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.

Table 1. Summary of characteristics of study population; values are means ± standard deviations or numbers of subjects (with percentage in brackets)

	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 in the following categories	
<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%)

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

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 before blood collection. For all the 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 periodontal bone loss of >1/3 of the root length on ⩾2 teeth on dental radiographs.

Bacteria and immune serum

To obtain immune serum against *A.a.*, we selected 10 periodontitis patients who were culture positive for *A.a.*; they were patients from the departmental clinic who were under active

periodontal treatment. The undiluted serum of these patients was tested against *A.a.* 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 h in brain heart infusion broth supplemented with 5 mg/l hemin and 1 mg/l menadione at 37°C in humidified 5% CO_2. Bacteria were harvested, washed twice in phosphate-buffered saline (PBS), checked for purity and the concentration was adjusted to approximately 5×10^8 colony forming units (CFU)/ml. All bacterial preparations were sonicated on ice for 2 min., at 5-s 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 microlitres of undiluted serum and 15 μl of the sonic extract were allowed to precipitate for 48 h at room temperature. *A.a.* serotype c was the only one of the five serotypes tested that induced immunodiffusion bands with the serum from most of the 10 patients: five out of the 10 periodontitis patients' sera tested were positive against serotype c. Subsequently, we pooled all available sera from these five patients [pooled whole serum (Serum)] and stored it at -20°C in 50 μl aliquots to be used as an opsonization source throughout all the experiments. For some experiments, the serum was incubated at 56°C for 30 min. to remove complement activity, and the resulting heat-inactivated serum (HIS) was used as a source of Igs.

Phagocytosis assay

Blood was collected from patients by a 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 $800 \times g$, at 20°C for 20 min. continuously. The 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, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM HEPES, 10 mM glucose, pH 7.4). The purity and viability were >95%, as determined by flow cytometry and trypan blue exclusion, respectively.

A.a. serotype c (5×10^8 CFU/ml) was used in the phagocytosis assay and was labelled with fluorescein isothiocyanate (FITC; 0.015 mg/ml, Sigma) for 30 min. 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 unlabelled or FITC-labelled *A.a.* 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.a.* 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 min., the samples were placed on ice to stop phagocytosis. Samples were centrifuged at $500 \times g$ for 5 min. 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 analysed within 1 h by flow cytometry. Trypan blue 0.064% (w/v) was used to quench fluorescence from adherent, non-ingested FITC *A.a.* 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, USA) 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 described previously (Hildemann et al. 1992). Briefly, the PI takes into account the percentage phagocytic PMNs and the number of fluorescent bacteria per phagocytic PMN (PI = MFI of phagocytic PMNs × % phagocytic PMNs). Phagocytosis of opsonized *A.a.* is plotted as the PI of cells when challenged with opsonized *A.a.* minus background (i.e. negative control, cells incubated with non-opsonized *A.a.*).

PMN degranulation assay

PMNs (1×10^7 cells/ml) and serum-opsonized *A.a.*, HIS-opsonized *A.a.* or non-opsonized *A.a.* (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 min., 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 min. 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 analysed by flow cytometry. Data are expressed as the relative MFI, calculated as the MFI of cells when challenged with *A.a.* (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 using the 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.a.*, HIS-opsonized *A.a.* and non-opsonized *A.a.* in a PMN to *A.a.* ratio of 1:100. Fifty microlitres of the PMN suspension and 50 μl stimulus were added to 100 μl of a reaction buffer in 96-well plate. The increase in absorbance at 590 nm was monitored for 90 min. The maximal increase in fluorescence was calculated over a 30-min. interval. Data are expressed as relative H_2O_2 release calculated as the difference between the H_2O_2 release from PMN incubated in the presence of *A.a.* and the H_2O_2 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 (Claesson et al. 1994), with minor modifications. Briefly, 75 μl from the supernatants collected following a phagocytosis assay (see *Phagocytosis assay*) were added to 25 μl 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.a.* and the elastase activity from PMN incubated in HEPES buffer as a negative control.

Data analysis

Tabulation of data, box plot generation and data analysis were performed with the SPSS 12.0 package. The 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 analysed in a general linear model (ANCOVA), taking the genotype as a fixed factor, and age and Caucasian race as the covariates. In the functional studies, the distribution of data was skewed; therefore, differences between the FcγRIIa H/H131 and R/R131 patient groups were statistically analysed with the Mann–Whitney *U* test or the χ^2 test, where appropriate. Differences between PMN functional parameters under different conditions within the FcγRIIa H/H131 and R/R131 patient groups were statistically analysed with the Friedman test for related samples. Further, in stepwise linear regression analyses, possible modifying factors for significant results in PMN functional assays seen in non-parametric analyses were explored. In these analyses, the log-transformed experimental data were entered as dependent variables, while the FcγRIIa genotype, age, gender, race and smoking were entered as independent variables. Variables with the lowest *p*-value <0.1 were allowed to enter the model. For all statistical analyses, *p*-values <0.05 were considered to be statistically significant.

Table 2. Periodontal characteristics of the 98 patients screened for FcγRIIa polymorphism, according to genotype (mean values ± standard deviation)

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

*$p=0.038$.

Table 3. Summary of the characteristics of the group of patients used in the present study

	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

Values are means ± standard deviations or numbers of subjects.

Results

Screening of periodontitis patients

A description of the characteristics of the 98 patients initially genotyped is provided in Table 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 ⩾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 analysed 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 the H/R or the R/R genotype, after adjusting for age and Caucasian race ($p=0.038$; Table 2).

Study population for PMN functional assays

A description of the patient group used for functional assays is provided in Table 3. The patients were on average 45 years old and the gender distribution was not significantly different between the R/R and H/H subjects. Eighteen of the 20 patients were of Caucasian origin, eight were current smokers and five were non-smokers, while seven 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; $p=0.08$).

Phagocytosis

From the phagocytosis experiments, a PI taking into account both the percentage of phagocytic cells and the number of fluorescent bacteria per phagocytic cell (MFI of these cells) was calculated. For both H/H and R/R genotypes, the PI was

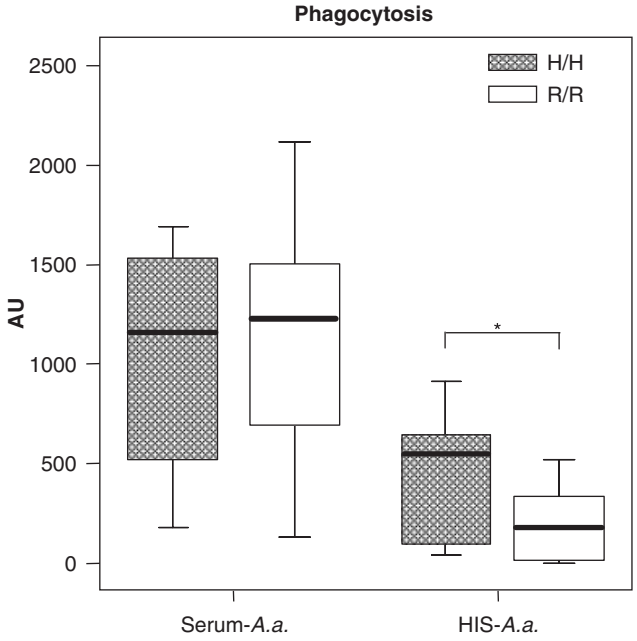

Fig. 1. Box plots showing phagocytic index for H/H and R/R PMNs with whole serum- or heat-inactivated serum (HIS) - opsonized *A.a.* (AU, arbitrary units; * $p = 0.019$).

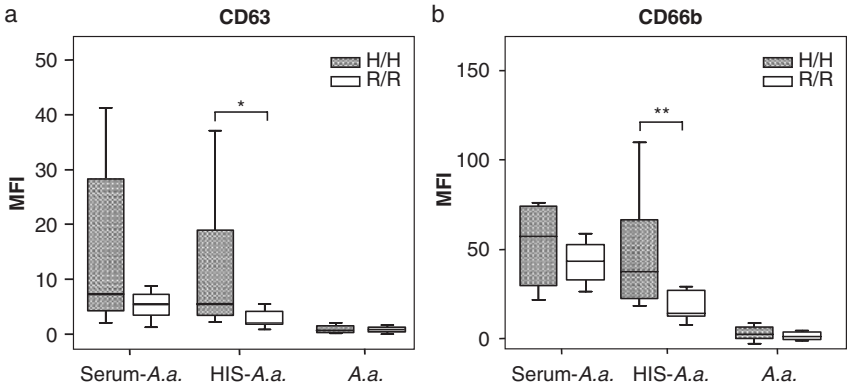

Fig. 2. Box plots showing markers of degranulation of primary granules (a), (CD63) and secondary granules (b), (CD66b) for H/H and R/R PMNs with whole serum- or HIS-opsonized or non-opsonized *A.a.* (MFI, mean fluorescence intensity; * $p = 0.004$; ** $p = 0.002$).

higher using serum than when using HIS for opsonization of *A.a.* (Fig. 1; $p = 0.009$ and 0.005, respectively). The PI of *A.a.* 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.a.*) was significantly higher in the FcγRIIa 131 H/H group than in the FcγRIIa 131 R/R group ($p = 0.019$).

Degranulation

Primary granules

Degranulation of primary PMN granules is a measure of PMN activation and was analysed by the expression of CD63 (Fig. 2a). In the H/H subjects, the expression of CD63 was higher when *A.a.* was opsonized with whole serum (MFI median 7.25) than when HIS was used for *A.a.* opsonization (median 5.49), while non-opsonized *A.a* led to only a minimal CD63 expression (median 0.7; $p = 0.0002$). This pattern was also seen among R/R patients, i.e. MFI of CD63 with serum-opsonized *A.a.* (median 5.46) was higher than with HIS-*A.a.* (median 2.05), while again non-opsonized *A.a.* was essentially not inducing any CD63 up-regulation (median 0.84; $p < 0.0001$).

When comparing the CD63 expression between groups, H/H and R/R patients showed the release of similar amounts of primary granules in response to serum-opsonized *A.a.* ($p = 0.190$). Interestingly, however, when HIS was used for opsonization of bacteria, indicating the FcγR stimulation only, the PMNs from H/H patients expressed significantly more CD63 than the PMNs from R/R patients ($p = 0.004$; Fig. 2a).

Secondary granules

Degranulation of secondary PMNs granules is also a measure of PMN activation and was analysed by the expression of CD66b; the results are summarized in Fig. 2b. In the H/H subjects, the expression of CD66b was higher when *A.a.* was opsonized with serum (median 57.01) than when HIS was used for *A.a.* 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.a.* was higher (median 43.52) than in response to HIS-*A.a.* (median 14.08), while non-opsonized *A.a.* was not inducing CD66b expression (median 1.365; $p < 0.0001$).

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

Respiratory burst

Respiratory burst is a major anti-bacterial function of the PMN and it was analysed by measuring extracellular H_2O_2 release (Fig. 3). In both H/H and R/R patients, serum-opsonized *A.a.* induced a more intense H_2O_2 release (medians 803.0 and 701.5, respectively) than HIS-opsonized *A.a.* (medians 503.5 and 387.5, respectively), which in turn was higher than the response induced by non-opsonized *A.a.* (medians 55.0 and 64.0, respectively; $p < 0.0001$).

The release of H_2O_2 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.a.* $p = 0.739$; HIS-opsonized *A.a.* $p = 0.796$; non-opsonized *A.a.* $p = 0.739$).

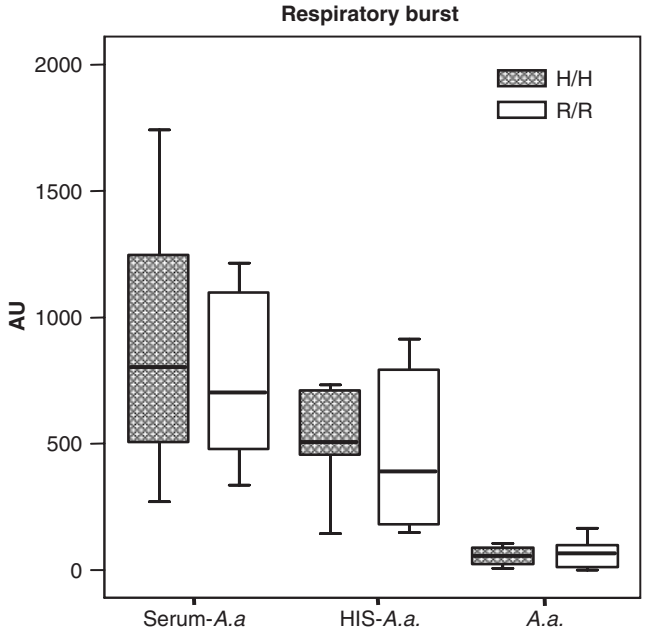

Fig. 3. Box plots showing respiratory burst (H_2O_2 release) by H/H and R/R PMNs with serum- or HIS-opsonized or non-opsonized *A.a*. (AU, arbitrary units).

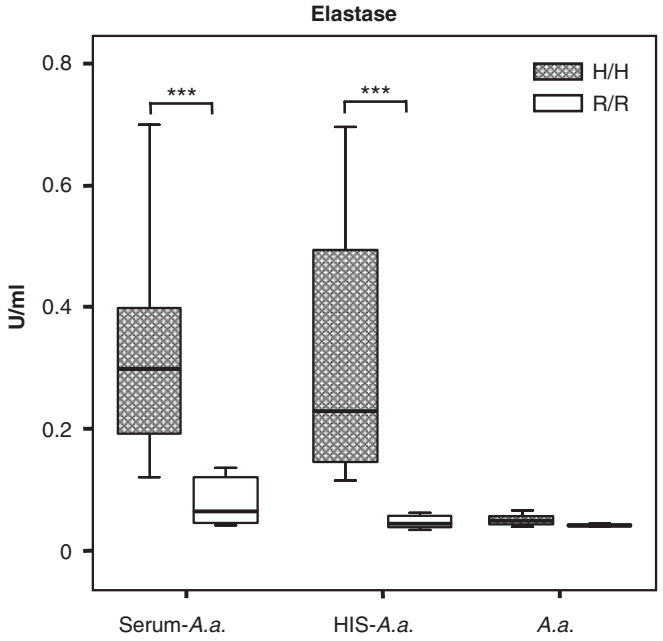

Fig. 4. Box plots showing elastase activity released by H/H and R/R PMNs with serum- or HIS-opsonized or non-opsonized *A.a*. (U/ml, elastase units per ml supernatant; *** $p \leqslant 0.001$).

Elastase activity

Elastase is one of the proteolytic enzymes present in the PMN primary granules, released on 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.a*. is presented in Fig. 4. PMNs from H/H subjects released high amounts of active elastase when stimulated with serum- or HIS-opsonized *A.a*. (median elastase 0.310 and 0.239 U/ml, respectively). The non-opsonized *A.a*. 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 a low release of elastase after incubation with serum-*A.a*. (median 0.075 U/ml), HIS-*A.a*. (median 0.060 U/ml) or non-opsonized *A.a*. (median 0.052 U/ml).

In comparison with the R/R group, H/H PMNs released significantly higher amounts of active elastase in response to serum-*A.a*. and HIS-*A.a*. ($p < 0.001$ and $p = 0.001$, respectively).

Possible modifying factors in relation to PMN functional assays

Linear regression analysis indicated that for the phagocytosis of HIS-opsonized A.a., none of the factors FcγRIIa genotype, age, gender, race and smoking explained the variation in the results (data not shown). For CD63 expression after stimulation with A.a.-HIS, the FcγRIIa genotype explained weakly the results ($p = 0.068$), while all other factors were not associated (p-values > 0.1). In the linear regression analysis for the dependent variable CD66b expression after stimulation with HIS-opsonized A.a., the FcγRIIa genotype was the only parameter significantly associated ($p = 0.03$), while age, gender, race and smoking were not modifying the results (p- values > 0.1). Similarly, genotype was the only parameter significantly associated with elastase release after stimulation with HIS-*A.a*. ($p < 0.0001$), while age, gender, race and smoking were not modifying the elastase results (p values > 0.1). Interestingly, smoking showed a weak relation with elastase release after stimulation with whole serum-opsonized *A.a* ($p = 0.06$); however, the major explanatory variable in this last regression analysis was again the FcγRIIa genotype ($p < 0.0001$), while age, gender and race were not modifying the elastase results (p values > 0.1).

Discussion

We have investigated the relationship between the 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 131 H/H patients than that of 131 R/R patients was demonstrated when HIS was used for opsonization of *A.a*.

The results presented in this study support the hypothesis that the FcγRIIa 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 (Figueredo et al. 1999, Fredriksson et al. 2003). However, these latter studies suggested the constitutive nature of the PMN hyper-responsiveness without shedding light on the 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 FcγRIIa, because the FcγRIIa H/H is the only PMN receptor that efficiently recognizes IgG2 (Warmerdam et al. 1991). The HIS used in this study contains immunoglobulins specific for the *A.a.* 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 Ig subclass reactive with *A.a.*, as it dominates the antibody response against polysaccharide antigens that are abundant on the cell wall of the gram-negative periodontal pathogens (Wilson & Hamilton 1992).

In the case of the more reactive genotype of the FcγRIIa, 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 (Chapple et al. 2002). We observed no significant difference in H_2O_2 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 oxygen species other than H_2O_2. For example, using FcγR-stimulated PMNs and chemiluminiscence as a measure of all oxygen-reactive species produced, Fredriksson et al. (1998) showed a higher oxidative burst in periodontitis patients than in controls. However, in the same study, there was no difference between patients and controls when intracellular H_2O_2 was assessed (Fredriksson et al. 1998). 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 H_2O_2 release.

A limitation of our study is the low number of participants. From the 98 periodontitis patients genotyped for FcγRIIa 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 FcγRIIa 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 (Loos et al. 2003, Yamamoto et al. 2004). 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, the 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 Sorensen et al. (2004) describe a reduced response to stimulation in PMN from smokers when compared with non-smokers. Gustafsson et al. (2000) found no difference between the PMN from smokers and non-smokers after Fcγ-receptor stimulation with opsonized bacteria. The issue of the possible influence of smoking status or other background characteristics on the measured PMN parameters was explored by linear regression analyses after log transformation of the non-normally distributed raw data. Only for *A.a.* opsonized with whole serum was a weak confounding effect for smoking status seen ($p = 0.06$), while the FcgRIIa genotype was a very strong factor ($p < 0.001$). For none of the other parameters of interest, did smoking contribute to the measured values. Age, gender or race were never modifying factors.

The phagocytosis and degranulation were higher for both the H/H and the R/R groups when whole serum-opsonized bacteria were used in comparison with 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 defence against *A.a.* (Wilson & Genco 1989).

In conclusion, in the current study we found that the FcγRIIa polymorphism influences the functions of PMNs in periodontitis patients. Individuals with an H/H genotype show a hyper-reactive phenotype, with increased Fcγ-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.

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Address:
Elena A. Nicu
Department of Periodontology
Academic Centre for Dentistry Amsterdam (ACTA)
Louwesweg 1
1066 EA Amsterdam
The Netherlands
E-mail: E.Comandasu@vumc.nl

Clinical Relevance

Scientific rationale for the study: Epidemiological studies show that periodontitis patients with the FcγRIIa-131H/H genotype on PMNs show more periodontal breakdown than patients with the 131R/R genotype. The objective of this study was to investigate how the genotype influences the functions of PMNs.

Principal findings: PMNs from the 131H/H patients phagocytosed more opsonized *A.a*., underwent more degranulation and released more elastase in ex vivo experiments.

Practical implications: In response to *A.a*., PMNs from 131H/H patients show a genetically determined hyperreactivity compared with 131R/R patients. This intrinsic characteristic may contribute to more collateral damage during host response reactions in periodontitis.