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ORIGINAL ARTICLE

Elevated platelet and leukocyte response to oral bacteria in periodontitis

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Summary. *Background:* Periodontitis is associated with an increased risk for cardiovascular diseases (CVD), but the underlying mechanisms are poorly understood. Recently, we showed that platelets from periodontitis patients are more activated than those from controls. *Objective:* Given the regularly occurring bacteremic episodes in periodontitis patients, we hypothesized that platelets and/or leukocytes from periodontitis patients are more sensitive to stimulation by oral bacteria, in particular the known periodontal pathogens, than platelets from control subjects. *Methods:* Three-color flow cytometry analysis was performed to quantify activation of platelets (P-selectin, PAC-1, CD63) and leukocytes (CD11b) in whole blood from patients with periodontitis (n = 19) and controls (n = 18), with and without stimulation by oral bacteria. Phagocytosis was assessed by using green-fluorescent protein (GFP)-expressing *Aggregatibacter actinomycetemcomitans* (*Aa*). *Results:* Neutrophils and monocytes were activated by all species of oral bacteria tested, but no differences were observed between patients and controls. In response to several species of oral bacteria, platelets from periodontitis patients showed, compared with controls, increased exposure of P-selectin (P = 0.027) and increased formation of platelet-monocyte complexes (P = 0.040). Platelet-leukocyte complexes bound and/or phagocytosed more GFP-*Aa* than platelet-free leukocytes (for neutrophils and monocytes, in both patients and controls, P < 0.001). *Conclusions:* In periodontitis, increased platelet response to oral bacteria is paralleled by increased formation of platelet-leukocyte complexes with elevated capacity for bacterial clearance. We speculate that activated platelets and leukocytes might contribute to increased atherothrombotic activity.

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Introduction

Systemic inflammation may have an atherogenic effect at different levels [1]. In addition to induced endothelial dysfunction and induced secondary dyslipidemia, systemic inflammation can activate the coagulation cascade [2], a process mainly mediated by tissue factor [3]. Platelets also contribute to activation of coagulation when they are primed during systemic inflammation; this process is closely related to atherothrombosis [4]. Activated platelets release chemokines and cytokines, and expose proinflammatory receptors, facilitating their binding to leukocytes and endothelial cells [5]. Platelet-leukocyte (i.e. platelet-neutrophil and platelet-monocyte) complexes are a sensitive marker for platelet activation and have a proposed role in plaque instability, thrombosis and inflammation [6]. Increased numbers of circulating complexes have been reported in patients with unstable angina, myocardial infarction and stroke [7–9]. The correlation between systemic inflammation and atherothrombosis is sustained by an increased incidence of cardiovascular disease (CVD) in patients with systemic lupus erythematosus, rheumatoid arthritis, inflammatory bowel disease or periodontitis as compared with subjects without an inflammatory disease [2,10].

Periodontitis is an infectious disease of the supportive tissues of the teeth, characterized by gradual loss of tooth supporting alveolar bone, and affects up to 10% of the population in its most severe form [11]. The primary etiologic factor of periodontitis is the subgingival infection with a group of Gram-negative pathogens. The major bacterial species associated with periodontitis are *Aggregatibacter actinomycetemcomitans* (*Aa*), *Porphyromonas gingivalis* (*Pg*) and *Tannerella forsythia* (*Tf*) [12]. Inflammation in the periodontal tissues

results in areas of ulceration of the epithelium in the periodontal pocket, which leads to dissemination of oral bacteria into the circulation during mastication [13]. Transient bacteremias in periodontitis patients underlie chronic production and systemic increases of various proinflammatory mediators, including interleukin (IL)-1β, IL-6, C-reactive protein and tumor-necrosis factor (TNF)-α [14,15].

In a previous study, we have shown that platelets from periodontitis patients have an increased activation status compared with platelets from healthy controls [16]. Strains of the periodontal pathogen *P. gingivalis*, but also species of the oral commensal microflora, such as *Streptococcus sanguis* (*Ss*), induce platelet activation *in vitro* and in animal studies [17,18]. Given the regularly occurring bacteremic episodes in periodontitis patients, we hypothesized that platelets and/or leukocytes from periodontitis patients are more sensitive to stimulation by oral bacteria, in particular the known periodontal pathogens, than cells from control subjects. If true, the association between platelet activation and oral bacteria improves our understanding of the underlying mechanisms contributing to the higher risk of CVD in periodontitis patients. Therefore, the aim of the present study was to investigate whether platelets and/or leukocytes from periodontitis patients are more sensitive to stimulation by oral bacteria than cells from matched controls.

Methods

Chemicals and antibodies

All chemicals were from Sigma Chemical Co. (St Louis, MO, USA). CD61-PerCP and PAC-1 FITC were obtained from Becton Dickinson Immunocytometry Systems (San Jose, CA, USA), PE-labeled CD62P from Immunotech (Marseille, France), CD14-PE, CD11b-FITC and PE-labelled IgG_1 from Sanquin (Amsterdam, the Netherlands) and IgM-FITC from Beckman-Coulter (Fullerton, CA, USA).

Patients

We selected a consecutive series of periodontitis patients who were referred to the Department of Periodontology of the Academic Centre for Dentistry Amsterdam for diagnosis and treatment of severe periodontitis, who met the inclusion criteria and who consented to participate. Inclusion criteria were generalized gingival inflammation, deepened periodontal pockets and ≥8 teeth with radiographic bone loss ≥30% of the root length. Age-, gender-, race- and smoking status-matched controls were recruited among subjects registered for restorative dental procedures or who visited the dental school for regular dental check-ups. Inclusion criteria for control subjects were (i) missing ≤1 tooth per quadrant (third molar excluded), and (ii) showed on ≤1-year-old dental bitewing radiographs a distance of ≤3 mm between the cemento-enamel junction and the alveolar bone crest. After initial inclusion, one control subject was excluded from analysis because subgingival calculus masked initially the presence of minor loss of periodontal attachment (mild periodontitis). Exclusion criteria for patients and controls were: the presence of systemic disease (especially cardiovascular disorders, diabetes mellitus, and allergies), a recent history or the presence of any acute or chronic infection, systemic antibiotic treatment within the last 3 months or usage of any medication (including sporadic NSAIDs), and pregnancy. Subgingival microbiological samples were taken from all subjects to determine the presence of the periodontal pathogens *Aa*, *Pg* and *Tf*; sampling, laboratory procedures and identification of *Aa*, *Pg* and *Tf* were performed as previously described [12]. All subjects were informed verbally and written to about the purposes of the study and had signed an informed consent. The Medical Ethical Committee of the Academic Medical Center of the University of Amsterdam approved the study.

Bacterial cultures used in the stimulation assays

Aa (Y4) was grown aerobically in enriched brain-heart infusion broth (enriched BHI; hemin 5 mg L^{-1}, menadione 1 mg L^{-1}). *Pg* (W83) was grown anaerobically in enriched BHI. For the anaerobic culturing of *Tf* (ATCC 43037), the enriched BHI was supplemented with 5% v/v fetal calf serum, 1 g L^{-1} L-cysteine and 15 mg L^{-1} *N*-acetylmuramic acid. *Ss* (HG1470) was grown aerobically in BHI. The bacterial suspensions were washed by centrifugation, reduced to an optical density of 1 at 600 nm (corresponding to $5*10^8$ cells μL^{-1}) in HEPES buffer (137 mM NaCl, 2.7 mM KCl, 1.0 mM $MgCl_2$, 5.6 mM glucose, 20 mM HEPES, 1 mg mL^{-1} bovine serum albumin, 3.3 mM NaH_2PO_4; pH 7.4), and stored in aliquots at −20 °C.

Platelet activation

Fasting venous blood samples were collected as previously described [19]. For whole-blood flow cytometry, 0.32% citrate-anticoagulated blood was processed within 5 min after collection. Aliquots of blood (5 μL) were diluted in 30 μL HEPES buffer. Platelets were incubated with and without 30 μL of *Aa*, *Pg*, *Tf* or *Ss* for 30 min at room temperature. Adenosine diphosphate (ADP, 10 μM) was used as a positive control. The reaction vials contained PerCP-labeled anti-CD61, FITC-labeled PAC-1 and PE-labeled anti-CD62p (4 μg mL^{-1} of each monoclonal antibody, final concentration) or PerCP-labeled anti-CD61 (4 μg mL^{-1}) and PE-labeled anti-CD63 (10 μg mL^{-1}). To set fluorescence thresholds, 4 μg mL^{-1} PE-IgG_1 and 4 μg mL^{-1} FITC-IgM isotype control antibodies were used. After mixing and 30-min incubation at room temperature in the dark, HEPES buffer containing 0.2% paraformaldehyde (PFA; 2.5 mL) was added. Flow cytometry was performed as described previously and the geometric mean fluorescence intensity (MFI) was recorded [19]. After fixation, blood samples were analyzed in a FACScan flow cytometer with CellQuest software (Becton Dickinson). Forward and side scatter were set at logarithmic gain. Platelets were identified by characteristic forward and side scatter, and binding of anti-CD61. Exposure of platelet activation markers was determined

on 5000 platelets. The threshold for platelet activation was set at 1% of the appropriate isotype control-antibody. Platelet activation was expressed as the ratio between the MFI of the indicated activation marker after stimulation and the MFI of the same marker in the absence of stimulation (cells in HEPES buffer).

Platelet-leukocyte interaction and leukocyte activation

Fresh citrated blood (25 μL) was incubated with and without bacteria or ADP as described above (see *Platelet activation*). The samples contained 4 μg mL^{-1} (final concentration) of each CD14-PE, CD11b-FITC and CD61-PerCP. The mix was incubated for 60 min at room temperature. In the control tubes, whole blood was incubated with HEPES buffer containing no bacteria. After incubation, 1 mL of ice-cold lysis solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA; pH 7.4) was added to the samples, thoroughly mixed and placed on ice for 15 min. After lysis, the cells were fixed by addition of 1 mL of HEPES containing 0.3% PFA. Neutrophils and monocytes were identified on their characteristic light scatter patterns and extent of CD14-PE expression (see Fig. 2A). Dim CD14 and 90° light scatter define neutrophils. Bright CD14 and 90° light scatter define monocytes. At least 5000 neutrophils and 1000 monocytes were gated within each measurement. In turn, of the gated neutrophils and monocytes, the extent of binding of anti-CD61 was determined, reflecting platelet binding. Based on control antibody binding, a fluorescence threshold was set and neutrophils and monocytes were considered to be CD61-positive when fluorescence was above this threshold. The CD61-positive neutrophils/monocytes are the platelet-neutrophil complexes (PNCs) and the platelet-monocyte complexes (PMCs), respectively (see Fig. 2B,C). The results are plotted as the % PNCs/% PMCs from the total neutrophil/monocyte population.

Leukocyte activation in response to oral bacteria or ADP was monitored by measuring the exposure of CD11b on neutrophils, monocytes, PNCs and PMCs. CD11b or integrin α_m is part of MAC-1 (macrophage-1 antigen), a receptor implicated in leukocyte adhesion to other cells, phagocytosis and cellular activation [20].

Phagocytosis

Green fluorescent protein (GFP)-*Aa* strain ATCC29522, containing plasmid pNP3M, a kind gift of Dr D. Galli, was grown in TSBYE (3% trypticase soy broth, 0.6% yeast extract) with 100 μg mL^{-1} ampicillin at 37 °C in humidified 5% CO_2; the construct pNP3M is a derivative of the previously described pNP3 [21]. The GFP-containing bacteria allowed us to identify phagocytosing neutrophils and monocytes by flow cytometry. The GFP-*Aa* suspension was washed, reduced to an optical density of 1 at 600 nm in HEPES buffer, and stored in aliquots at −20 °C. Fresh citrate-anticoagulated blood (25 μL) was

Table 1 Summary of the characteristics of the study population

	Control $n = 18$	Periodontitis $n = 19$	*P*-value*
Age	40.8 (36.1–45.4)	42.0 (37.5–46.4)	0.713
Gender (males)	6 (33%)	7 (37%)	0.823
Ethnicity (Caucasian)	14 (78%)	15 (79%)	0.931
Smoking (smokers)	5 (28%)	6 (32%)	0.800
Education (< high school)	3 (17%)	8 (42%)	0.091
BMI (kg m^{-2})	24.4 (22.7–26.0)	25.5 (24.2–26.8)	0.293
Leukocytes (× 10^9 L^{-1})	5.8 (5.1–6.5)	6.5 (5.6–7.5)	0.217
Neutrophils (× 10^9 L^{-1})	3.3 (2.8–3.8)	3.8 (3.1–4.4)	0.300
Monocytes (× 10^9 L^{-1})	0.4 (0.4–0.5)	0.5 (0.4–0.6)	0.166
Lymphocytes (× 10^9 L^{-1})	1.9 (1.6–2.2)	2.1 (1.8–2.3)	0.333
Platelets (× 10^9 L^{-1})	240.3 (216.5–264.2)	276.6 (253.4–299.8)	0.040
Blood pressure (mmHg)			
Systolic	130.2 (121.2–139.2)	127.4 (121.0–133.7)	0.611
Diastolic	84.4 (79.6–89.1)	85.9 (81.3–90.6)	0.649
Total cholesterol (mmol L^{-1})	4.8 (4.4–5.3)	5.1 (4.5–5.6)	0.605
Triglycerides (mmol L^{-1})	0.9 (0.6–1.2)	1.7 (0.6–2.8)	0.171
Fibrinogen (g L^{-1})	3.0 (2.8–3.2)	3.6 (3.4–3.9)	0.001
C-reactive protein (mg L^{-1})	2.2 (0.4–4.1)	3.6 (1.3–5.9)	0.363
Subgingival colonization			
Aa-positive	3 (17%)	9 (47%)	0.046
Pg-positive	1 (5%)	9 (47%)	0.004
Tf-positive	6 (33%)	17 (89%)	0.0001
Number of teeth			
Total	28.7 (28.0–29.5)	27.2 (26.0–28.3)	0.033
With bone loss ≥30%	0	17.1 (14.8–19.4)	–
With bone loss ≥50%	0	6.7 (4.8–8.6)	–

BMI, body mass index; *Aa*, *A. actinomycetemcomitans*; *Pg*, *P. gingivalis*; *Tf*, *T. forsythia*. Values are means (95% confidence intervals) or numbers (%) of subjects. **P*-values calculated by *t*-test or χ^2-test, where appropriate.

added to a mixture containing no bacteria, GFP-*Aa* (35 μL) or GFP-*Aa* (35 μL) plus 10 μL ADP (final concentration 10 μM). The samples contained 4 μg mL^{-1} CD61-PerCP and CD14-PE. Mixtures were incubated for 60 min at room temperature. After incubation, 1 mL of lysis solution was added to the samples, thoroughly mixed and placed on ice for 15 min. The cells were fixed by addition of 1 mL of HEPES containing 0.3% PFA. The same flow cytometry settings and the same gating logic as described above were applied for defining neutrophils, monocytes, PNCs and PMCs.

Statistical analyses

Differences in background characteristics between patients and controls were compared by *t*-tests or χ^2-test (or Fisher-exact test, where needed). Our results of the stimulation assays showed a non-normal distribution and were rank-transformed for subsequent statistical analysis. To analyze the effects of periodontitis and of the different stimulants used, results were compared using repeated-measures analysis of variance (ANOVA). In case of the existence of an overall effect, differences between cells from patients and controls using the different stimulants were analyzed using an ANOVA followed by Bonferroni correction. Data are presented as means ± standard errors of the mean (SEM).

Data analyses were performed with the SPSS package, version 14.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism, version 4.00 for Windows (GraphPad Software, San Diego, CA, USA); *P*-values < 0.05 were considered statistically significant.

Results

Study population characteristics are summarized in Table 1. Periodontitis patients were more often colonized with *Aa*, *Pg* and *Tf* and had increased plasma levels of fibrinogen and increased platelet counts.

Platelet activation by oral bacteria

From Fig. 1 it is evident that the various species of bacteria differently affected platelet activation, as assessed by exposure of P-selectin, CD63 and binding of PAC-1 (i.e. binding of an antibody specifically directed against the activated, fibrinogen-binding conformation of glycoprotein IIb-IIIa) on platelets from patients and controls (overall *P*-values < 0.001 for the within-group analysis for all three markers, Fig. 1A, B, C). When blood from periodontitis patients was incubated with oral bacteria, the exposure of P-selectin was increased compared with controls (overall $P = 0.027$, Fig. 1A). In particular, the response to *Ss* was increased (*post-hoc* $P = 0.003$). The same tendency was observed in response to *Aa* and *Pg* in periodontitis patients (*post-hoc* $P = 0.075$ and $P = 0.066$, respectively). PAC-1 binding was comparable between controls and patients (the between-groups overall $P = 0.410$, Fig. 1B). The overall effects of bacterial stimulation on exposure of

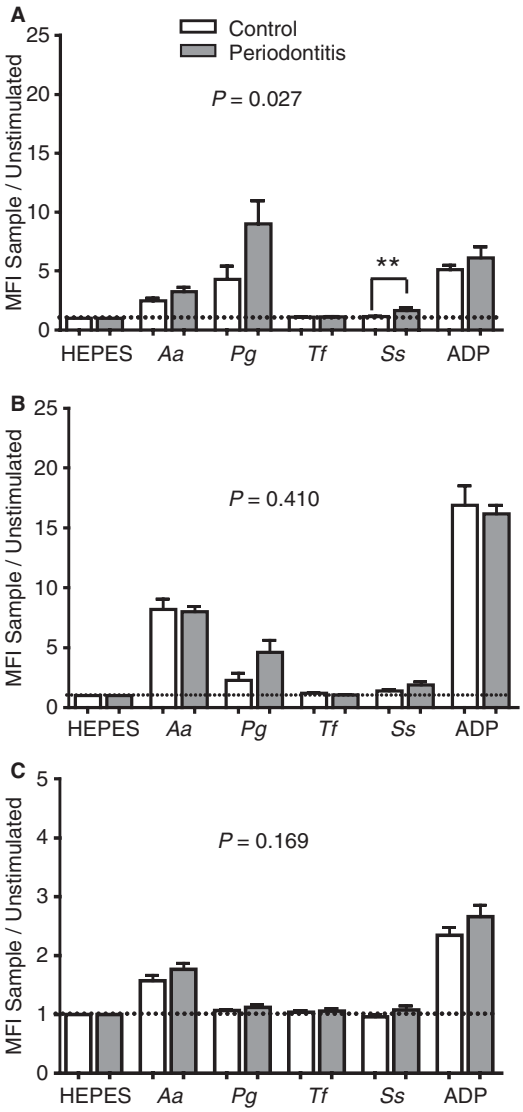

Fig. 1. Platelet activation in response to stimulation with *Aggregatibacter actinomycetemcomitans* (*Aa*), *Porphyromonas gingivalis* (*Pg*), *Tannerella forsythia* (*Tf*), *Streptococcus sanguis* (*Ss*) or adenosine diphosphate (ADP). Graphs represent the increase in mean fluorescence intensity (MFI) of (A) P-selectin, (B) PAC-1 and (C) CD63 expressed as ratio [MFI after stimulation]/[MFI of unstimulated cells]. Data are means ± SEM. After transformation, data were analyzed by repeated-measures ANOVA and the overall *P*-values of the comparison between patients and controls are provided in the graphs; overall *P*-values of the different stimulations both within the patient group and within the control group were < 0.001. $^{**}P < 0.01$ after Bonferroni correction in *post-hoc* testing.

CD63 were also comparable between controls and patients (the between-groups overall $P = 0.169$, Fig. 1C). In contrast to stimulation with *Aa* or *Pg*, stimulation with *Tf* hardly changed the activation status of platelets.

Platelet-neutrophil- (PNCs) and platelet-monocyte- (PMCs) complexes

All bacterial species induced significant formation of PNCs and PMCs, both in controls and patients (overall *P*-values

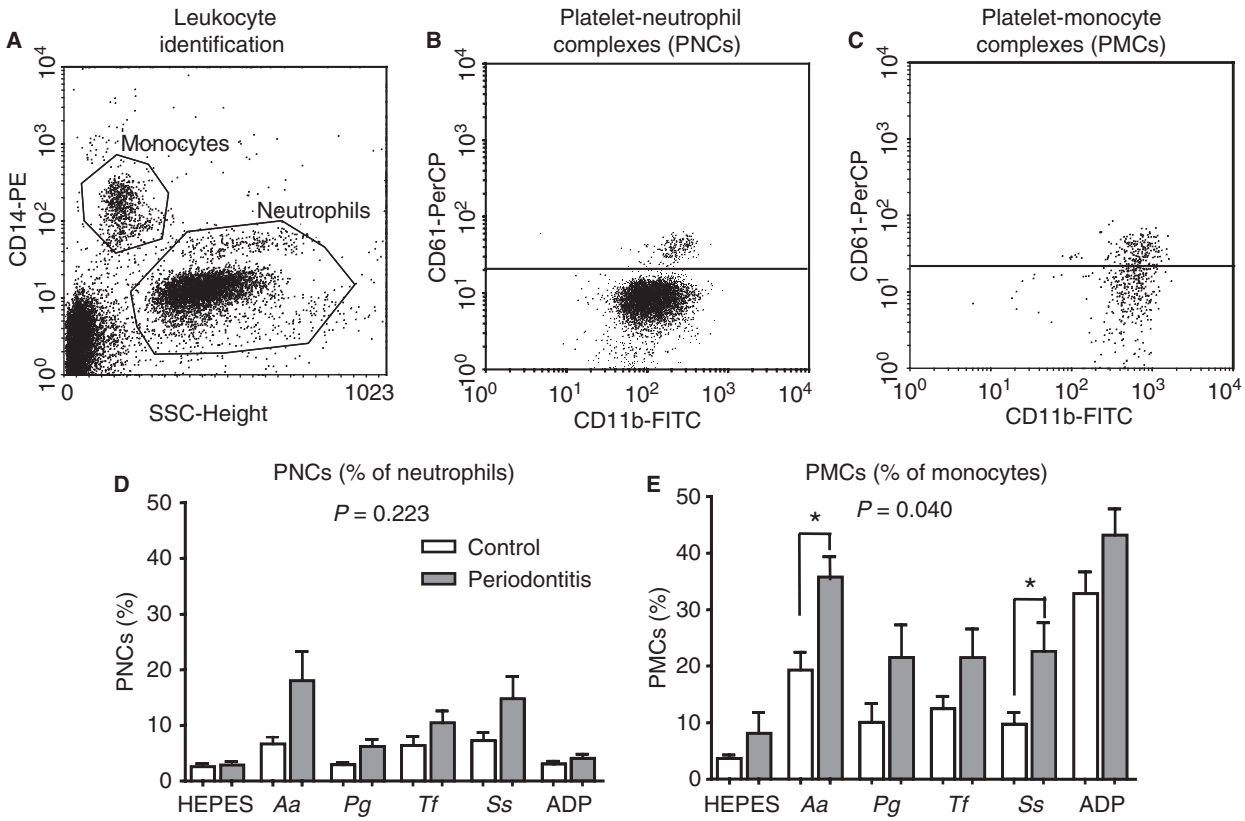

Fig. 2. Formation of platelet-neutrophil (PNCs) and platelet-monocyte complexes (PMCs) in response to oral bacteria or ADP. (A) Identification of neutrophils and monocytes by whole blood flow cytometry as outlined in Methods; (B, C) Identification of PNCs and PMCs (above threshold on Y-axis); (D, E) Percentage of (D) PNCs or (E) PMCs as fraction of the total population of neutrophils or monocytes, respectively. Abbreviations for stimulation conditions are as in Fig. 1. Data are means ± SEM. After transformation, data were analyzed by repeated-measures ANOVA and the overall *P*-values of the comparison between patients and controls are provided in the graphs; overall *P*-values of the different stimulations both within the patient group and within the control group were <0.001. *$P < 0.05$ after Bonferroni correction in *post-hoc* testing.

<0.001). PNC formation was comparable in patients and controls (Fig. 2D, $P = 0.223$). In the presence of oral bacteria, overall more PMCs were formed in patients' blood than in blood from control subjects (Fig. 2E, $P = 0.040$). The most marked difference was observed in response to *Aa* and *Ss* ($P = 0.024$ and $P = 0.034$, respectively), whereas the response to *Pg* followed a similar trend ($P = 0.061$). As expected, activation of platelets in whole blood with ADP resulted in strong, about 4–5-fold, increase in the numbers of PMCs (Fig. 2E), but formation of PNCs was unaffected (Fig. 2D).

Leukocyte activation

Aa, *Pg*, *Tf* and *Ss* triggered significant increases in exposure of CD11b on both neutrophils (Fig. 3A) and monocytes (Fig. 3C) of both patients and controls (overall *P*-values <0.001). In general, exposure of CD11b showed a similar pattern in patients and controls (overall $P = 0.450$ for neutrophils and $P = 0.909$ for monocytes). The exposure of CD11b on PNCs (Fig. 3B) was consistently lower compared with platelet-free neutrophils in response to oral bacteria or ADP. In contrast, exposure of CD11b on PMCs was roughly comparable to its exposure on platelet-free monocytes (Fig. 3D).

Phagocytosis

PNCs bound and/or phagocytosed more GFP-*Aa* as based on the GFP-fluorescence than uncomplexed neutrophils (Fig. 4C). Also, PMCs showed increased GPF-fluorescence compared with uncomplexed monocytes (Fig. 4D), albeit on average less prominent than was observed for PNCs and uncomplexed neutrophils. GFP-fluorescence of PNCs and PMCs was similar in patients and controls, indicating that comparable numbers of GFP-*Aa* were bound and/or phagocytosed irrespective of disease status ($P = 0.549$ and $P = 0.838$, respectively).

Discussion

Low grade, transient bacteremia is a common feature in periodontitis patients, occurring daily during activities such as tooth brushing and chewing [22]. Well-established assays [23] were employed to test the possibility that oral bacteria activate platelets and leukocytes in whole blood of periodontitis patients. Neutrophils and monocytes were activated by all species of oral bacteria tested, but no differences were observed between patients and controls. Interestingly, we found that platelets from periodontitis patients have an increased sensitivity to activation by oral bacteria. Furthermore, platelets in

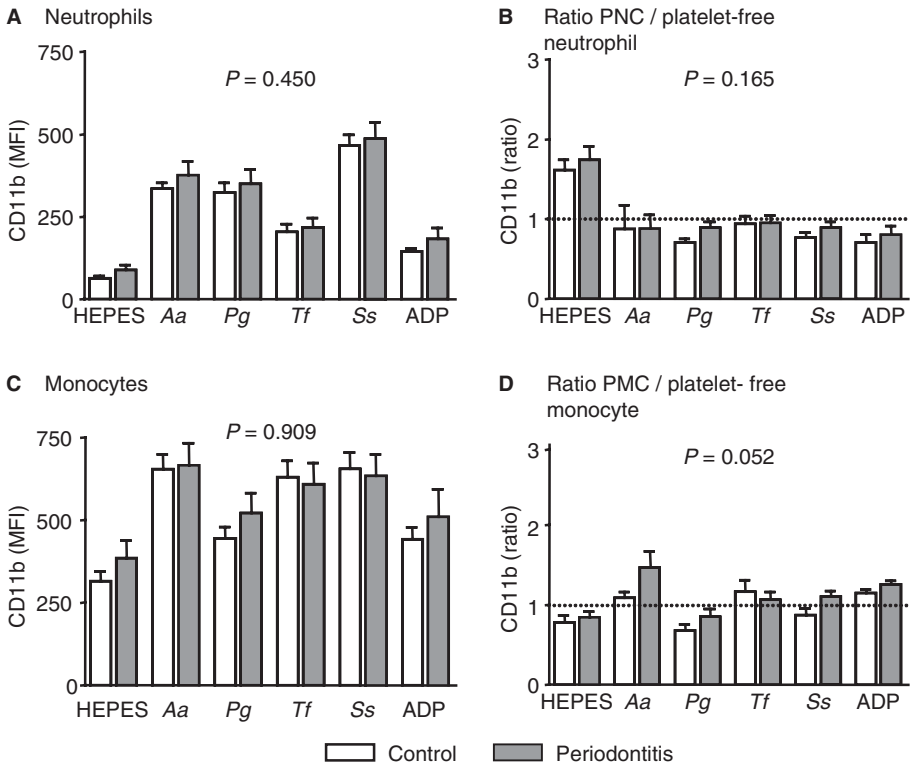

Fig. 3. Leukocyte activation. Graphs represent the MFI of (A) CD11b on neutrophils or (C) monocytes, (B) the ratio [CD11b MFI of PNCs]/[CD11b MFI of neutrophils] or (D) the ratio [CD11 of PMCs]/[CD11b MFI monocytes]. Abbreviations for stimulation conditions are as in Fig. 1. Data are means ± SEM. After transformation, data were analyzed by repeated-measures ANOVA and the overall *P*-values of the comparison between patients and controls are provided in the graphs; overall *P*-values of the different stimulations both within the patient group and within the control group were < 0.001.

complexes with neutrophils (PNCs) or with monocytes (PMCs) bound more oral bacteria than uncomplexed neutrophils/monocytes. Because more PMCs were formed in blood from periodontitis patients compared with healthy control subjects, the total inflammatory response in periodontitis is, on average, larger than in healthy individuals. These findings were not influenced by whether a given individual was colonized with *Aa*, *Pg* or *Tf* as determined by culture techniques (data not shown).

Previous studies showed that endotoxin-treated platelets activate neutrophils, which then release proteases capable of damaging the underlying endothelium, resulting in increased exposure of subendothelial tissues to platelets [24]. Platelet adhesion to the damaged vessel wall is known to contribute to the pathogenesis of atherosclerosis [25]. Similarly, such a mechanism is conceivable in periodontitis patients, where Gram-negative bacteria and their products enter the circulation directly, thereby activating platelets and leukocytes (Fig. 5). Platelet-monocyte complexes are of importance, not only in homing to inflammatory sites, but also for functional alterations of these cells. Formed PMCs produce monocyte chemotactic protein-1 (MCP-1) and IL-8 in larger amounts that uncomplexed monocytes, both these proinflammatory cytokines being related to progression of atherosclerosis [26,27]. Moreover, platelets provide cholesterol to monocytes for cholesteryl ester synthesis [28], and these monocytes may then differentiate into foam cells seen in atherosclerotic lesions [29].

It is very interesting to interpret the behavior of activated platelets in the platelet-leukocyte interaction assays. Activated platelets preferentially adhere to monocytes, rather than to neutrophils [30]. This was also observed in our assays. The platelet agonist ADP induced increased formation of PMCs and essentially did not induce formation of PNCs. Moreover, platelet activation by ADP induced monocyte activation rather than neutrophil activation. Although activated platelets bind to leukocytes, this seems not the only mechanism for platelet-leukocyte complex formation in our experiments. Stimulation with oral bacteria induced increased formation of PNCs preferentially via activation of neutrophils rather than platelets. This phenomenon is similar to PNC formation in response to stimulation with the bacterial peptide fMLP, which also induces formation of PNCs more efficiently than ADP [31].

In addition to *Pg* and *Ss* that were shown to promote platelet aggregation *in vitro* and in animal studies [18,32], *Aa* emerged for the first time as a potent platelet activator in a human *ex vivo* study. The different species of oral bacteria, however, induced distinct activation patterns, which are most likely related to activation of different intracellular signaling pathways [33]. At this point it is unclear which bacterial components are responsible for the platelet activation. This requires additional experimentation to reach a mechanistical

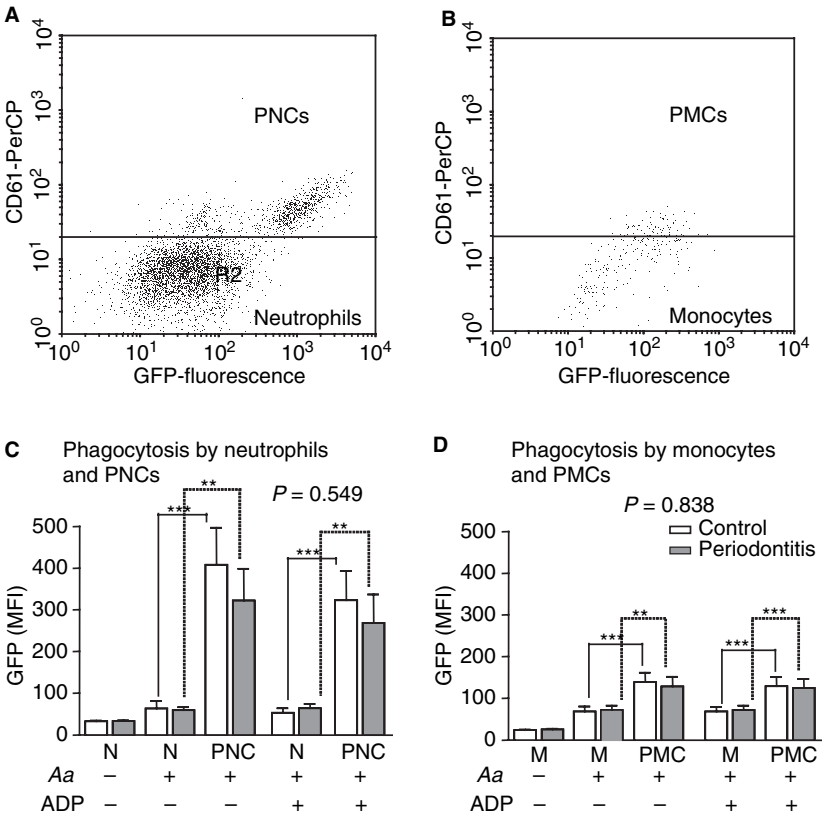

Fig. 4. Binding and/or phagocytosis of green fluorescent protein-labeled (GFP)-*Aa* in PNCs and neutrophils (N) or in PMCs and monocytes (M). (A, B) Extent of binding and/or phagocytosis of GFP-Aa to PNCs/PMCs (above fluorescence threshold on the Y-axis) and neutrophils/monocytes (below threshold). (C, D) MFI of the GFP-associated fluorescence of (C) PNCs and neutrophils or (D) PMCs and monocytes from controls and patients after stimulation with GFP-*Aa* alone or in combination with ADP. Data are means ± SEM. After transformation, data were analyzed by repeated-measures ANOVA and the overall *P*-values of the comparison between patients and controls are provided in the graphs; overall *P*-values of the different stimulations both within the patient group and within the control group were <0.001. $^{**}P < 0.01$, $^{***}P < 0.001$ after Bonferroni correction in *post-hoc* testing.

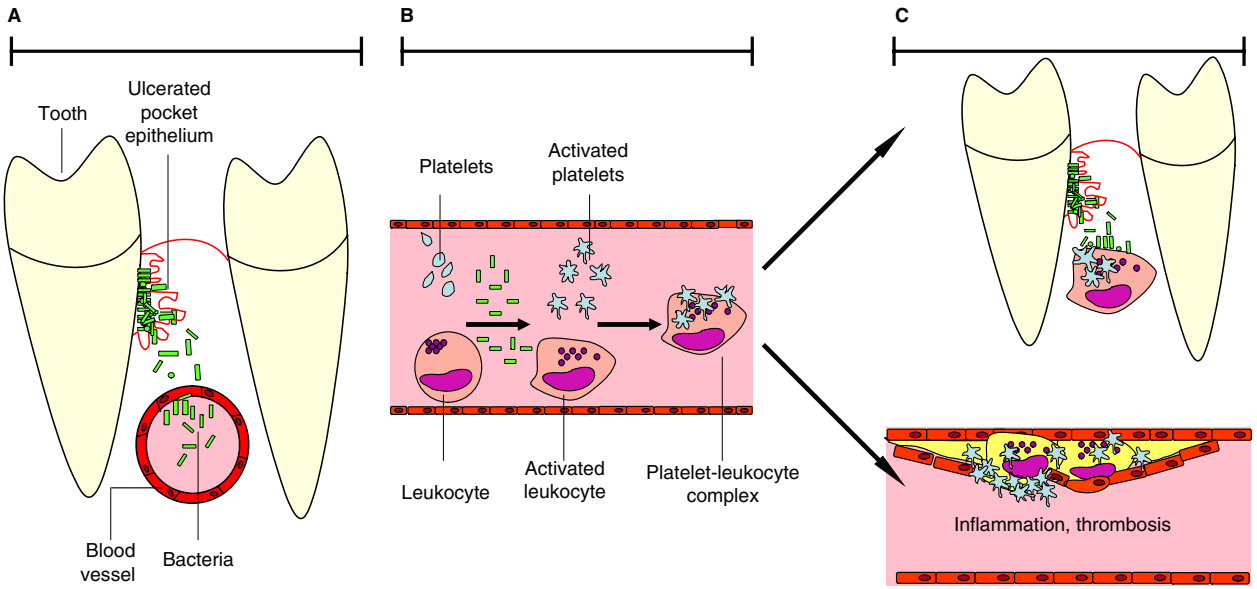

Fig. 5. Bacteremias with oral pathogens as a link between periodontitis, platelet and leukocyte activation. (A) Periodontitis is associated with regularly occurring bacteremias with oral pathogens. (B) Enhanced activation of platelets and leukocytes (neutrophils and monocytes) in response to these bacteria is concurrent with enhanced formation of platelet-leukocyte complexes in patients with periodontitis. (C) On the one hand platelet-leukocyte complexes are capable of better bacterial clearance; on the other hand they contain activated platelets and leukocytes that may contribute to atherothrombosis.

explanation. Especially with regard to *Aa*, a marked increase in the exposure of CD63 and binding of PAC-1 were observed on activated platelets, whereas exposure of P-selectin was hardly affected. This seemingly discrepant finding is explained by the rapid binding of P-selectin-exposing platelets to monocytes. Indeed, concurrently an increased number of PMCs was observed in *Aa*-treated blood samples.

Randomized controlled clinical trials studying the effects of treatment of periodontitis on platelet activation may provide further insight into the contribution of this chronic infectious condition to atherothrombosis. One intervention study showed that treatment of patients with periodontitis is followed by an improvement of endothelial function [34]. Although the biological basis of this improvement is unknown, one may speculate that reduced platelet activation and its concurrent procoagulant phenotype may be part of the explanation. Within our department, a study has been initiated to evaluate whether periodontal therapy indeed affects platelet activation and prothrombotic phenotype in periodontitis patients.

In summary, our data show that not only monocytes but also platelets from periodontitis patients are more sensitive to stimulation with oral bacteria than cells from controls. Because oral bacteria regularly get disseminated in the blood of periodontitis patients, this increased cellular sensitivity may contribute to more inflammation and thrombosis at atherosclerotic sites (Fig. 5). Because both processes are involved in the development of CVD, our findings may in part explain the increased relative risk for cardiovascular events in periodontitis patients.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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