

Aggregation of Human Platelets by *Tannerella Forsythia*

Abstract

Context: Periodontitis is a persistent infection of the tissues surrounding the teeth characterized by inflamed microvasculature, and is associated with increased systemic platelet activation. **Aims:** The purpose of this study was to assess the *in vitro* platelet aggregating potential of the red-complex bacterium *Tannerella forsythia*. A second-related objective was to ascertain the *in vitro* effect of dual platelet inhibitors on *T. forsythia*-platelet interaction. **Settings and Design:** These *ex vivo* experiments were done in a basic science laboratory combining isolated human platelets with isolated bacterial cells. **Methods:** Dilutions of cells were counted by quantitative polymerase chain reaction. Aggregation was assayed in a platelet aggregometer after adding cells or sonic extracts to gel filtered platelets, some of which were preincubated with the dual platelet inhibitors aspirin plus clopidogrel. **Results:** Platelets aggregate *in vitro* when exposed to *T. forsythia* cells or sonic extracts and dilution results in increased lag times and decreased aggregation. Platelets preincubated with the combination of aspirin plus clopidogrel do not aggregate in response to *T. forsythia*. **Conclusions:** Within the limitations of this *in vitro* study, *T. forsythia* cells aggregate human platelets and the activity can be attenuated by diluting the cells and blocked by the combination of aspirin plus clopidogrel.

Keywords: Aggregation, bacteria, platelets

Introduction

Human periodontitis is associated with the presence of *Tannerella forsythia* and *Porphyromonas gingivalis*.^[1,2] Many studies have addressed the virulence factors of these red-complex bacteria.^[3,4]

Previously, we have characterized the platelet aggregating activity of *P. gingivalis*.^[5] This activity was attenuated by aspirin and essential oil but could be enhanced by soluble IgG immune complexes to RgpA.^[6-8] The purpose of this work is to investigate the platelet aggregating activity of *T. forsythia*. In addition, this study considered the effect of the dual platelet inhibitors acetylsalicylic acid plus clopidogrel on platelet aggregation induced by *T. forsythia*.

Methods

T. forsythia (43,037) was purchased from the American Type Culture Collection (Manassas, VA). *T. forsythia* was grown in (BHI) medium containing heat-inactivated calf serum (5%, vol/vol), yeast extract (5 g/L),

L-cysteine (1 g/L), N-acetylmuramic acid (10 mg/L), hemin (5 mg/L), and menadione (0.5 mg/L) (TF medium) for 5–14 days at 37°C under anaerobic conditions in jars using the Gas Pak system (Mitsubishi Gas Chemical Co., Tokyo, Japan).

Bacterial cells were enumerated by real-time quantitative polymerase chain reaction (PCR). Quantitative genomic DNA from *T. forsythia* (ATCC 43037DQ; 10⁵ copies/μl) was used to generate an absolute copy number standard curve and compare to target DNA extracted from *T. forsythia* by boiling cells in Instagene Matrix (Bio-Lab Inc, CA). The primer mix included the following oligonucleotides: Forward primer (5'-3', GCA ACC AAG ATT GCC AGA GA) (2 μM); backward primer (5'-3', AAC AGC GAC TGC AAC GAA) (2 μM).^[9] To 10 μl of each primer was added 20 μl Chai Green Master Mix and 10 μl standard or target DNA. Amplifications were performed in a single channel Chai Open (Qpcr) thermocycler (Chai Biotech, Santa Clara, Ca) with cycling as follows: Initial denaturation at 95°C for 3 min, followed by 40 cycles at 95°C for 30 s, 62°C for

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30 s, and at 72°C for 30 s. The terminal denaturation was performed at 72°C for 5 min. PCR products were detected by monitoring the increase in fluorescence. The most probable number of cells was estimated by comparing dilutions of DNA extracted from cells to standard copy number assuming one copy per cell.

The cells from 5 to 14-day cultures were dispersed in Hank's balanced salt solution (HBSS) and adjusted to 10^3 cells/ μ l. Aliquots (0.5–20 μ l) were used in the aggregation assay described below.

T. forsythia cells (1 ml, 10^6 cells/ml) were suspended in HBSS (pH 7.0) at 4°C and sonicated for 3 min at 45 kHz. Cells debris was pelleted for 2 min at 1500 rpm and the supernatant was used in aggregation assays. For these assays, the sonic extract was adjusted to 1 mg/ml and 10 μ l was used in the aggregation assays described below. Protein was measured by the BCSA protein assay reagent (Pierce Biochemicals, Rockford, IL).

Anticoagulated whole blood was obtained from healthy donors by the addition of 7 volumes of freshly drawn blood to 3 volumes of 3.8% sodium citrate. Platelet-rich plasma (PRP) was prepared by centrifugation of the anticoagulated blood at 180 x g for 10 min. For aggregation assays, gel filtered platelets (GFP) were used rather than PRP. The method of Tangen was used to prepare GFP to rid the platelets of plasma proteins.^[10] PRP (5 ml) was applied to a column (2.5 cm x 25 cm) of Sepharose 2B equilibrated with Tyrodes buffer (pH 7.4). Platelets eluted in the void volume were pooled and used in aggregation experiments. Platelet separation from plasma proteins was confirmed by centrifuging a sample of GFP and noting a zero absorbance of the supernatant at 280 nm. Platelet counts were adjusted to 275,000–300,000 platelets/ μ l as measured on a Petroff-Hausser counting chamber (Hausser Scientific, Horaham, PA). Platelet-poor plasma (PPP) was prepared from PRP by centrifuging at 7120 x g for 5 min. GFP (cloudy) and PPP (clear) were set at 100 and 0% absorbance, respectively.

The standard assay mixture consisted of 450 μ l of GFP to which was added 0.5–20 μ l of the cell suspension or sonic extract to be tested. The duration of the assay was up to 25 min or until aggregation was noted. After 20 min, 10 μ l of Adenosine diphosphate (ADP) (1.0 mg/ml) was added to the reaction mixtures, in which little or no platelet aggregation had occurred to confirm the ability of the platelets to aggregate (positive control). Buffer (20 μ l) added to 450 μ l of PRP served as the negative control.

For some assays, gel filtered platelets were preincubated for 30 min at 37°C with acetylsalicylic acid (90 μ M) plus clopidogrel bisulfate (final concentration 10 μ M).

ADP was purchased from Chronolog Corp (Havertown, PA). Acetylsalicylic acid (aspirin) and clopidogrel bisulfate were purchased from Sigma Chemical Co (St. Louis, MO). All other chemicals were reagent grade.

Results

Bacterial load was adjusted to (10^3 cells/ μ l) as determined by real-time quantitative PCR [Figure 1]. Platelet aggregation induced by 0.1–20 μ l of *T. forsythia* cells (10^3 cells/ μ l) demonstrates that serial dilution of bacterial cells results in progressively increased lag times until platelet shape change and decreased platelet aggregation (increased absorbance) [Figure 2]. Further dilutions of bacteria did cause platelets to aggregate.

A standard assay, i.e., 20 μ l *T. forsythia* cells (10^3 cells/ μ l) added to 450 μ l PRP, was repeated every 10 min for cells that were incubated at 4°C and 37°C. The results indicated that the activity was labile at 37°C, losing activity within an hour, and could be preserved for up to 3 h at 4°C, progressively losing activity (data not shown). Sonic extracts (1 mg/ml), used in the same assay showed similar lability. Similarly, sonic extract activity was inhibited by boiling for 1 min (data not shown). The robust aggregation of GFP elicited by *T. forsythia* was not seen when platelets were pretreated with a combination of acetylsalicylic acid (90 μ M) plus clopidogrel (10 μ M) [Figure 2].

Discussion

There is evidence indicating that periodontal disease causes recurrent bacteremia by way of the inflamed tissues surrounding the teeth, following dental procedures, simple toothbrushing, or chewing.^[11-13] Due to the proximity and relationship of the bloodstream to the periodontal pocket, the host responds in predictive ways to periodontitis. Epithelial cavitations, ulcerations, and increased number of blood vessels result in increased exposure of the bloodstream to infection and explains how periopathogenic cells might become involved systemically. Bacteria enter into the circulation through dilated, inflamed endothelium and encounter blood platelets, whose primary role is to circulate close to the endothelium and defend against breaks in the circulatory system.^[14] Platelets have a striking tendency to gather at inflamed sites due to their small size and numbers in the trillions.^[15]

Platelets aggregate in response to vascular injury, but they also aggregate in response to some pathogens.^[16] Aggregation follows platelet activation, in which platelets respond to certain stimulants by first changing shape and secreting cytoplasmic granules containing ATP, ADP, and serotonin.^[17] Periodontal disease is associated with increased subgingival levels of *P. gingivalis*, *T. forsythia*, and *T. denticola*, designated as red-complex bacteria.^[18,19] *P. gingivalis* secretes small membrane vesicles which are proteolytic packages containing high concentrations of gingipain-R (Rgp) that causes platelets to aggregate with the efficiency of thrombin.^[3] Since *T. forsythia* has similar proteolytic activity, it is logical to study the *in vitro* activation of platelets by this periopathogen.

This study indicates that platelets aggregate rapidly when exposed to *T. forsythia* cells (10^3 cells/ μ l) and the

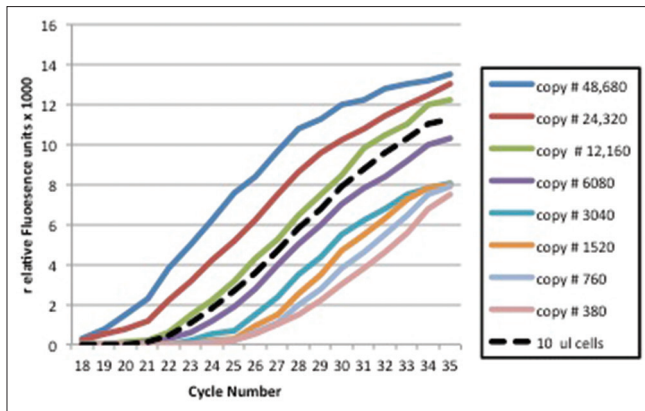


Figure 1: Quantitative real-time polymerase chain reaction Determination of Cell Number: Dilutons of *T. forsythia* suspensions were used to quantitate cells. The illustrated sample (----) corresponds to 10^6 cells/ $10 \mu\text{l}$

response can be attenuated by diluting the cells. As we have demonstrated previously, this is similar and of the same order of magnitude as evoked by *P. gingivalis* cells.^[3] *T. forsythia* activation of platelets *in vitro* requires that at least 100 cells be stirred with 1.35×10^8 platelets (i.e., $450 \mu\text{l}$ of $300,000$ platelets/ μl), causing shape change after lag times that vary according to cell concentration, followed by aggregation. The use of gel filtered platelets indicates that there is a direct interaction between bacteria and platelets, rather than an indirect interaction mediated by plasma proteins. This direct interaction was confirmed by the findings that *T. forsythia* sonic extract also causes platelet aggregation of gel filtered platelets. Liability studies indicate that the aggregation may be due to an enzymatic reaction.

Although our *ex vivo* studies have been confirmed, the clinical relevance of platelet aggregation by *T. forsythia* is not yet known.^[20] This is because platelet aggregation by *T. forsythia* is a new finding, happens at local sites, and circulating aggregates are fleeting and routinely filtered out in the spleen or liver or attached to roughened plaques. Nevertheless, platelet activation occurs in patients with periodontal disease and the level correlates with the severity of the disease.^[21] Likewise, the platelets of periodontal patients form platelet-leucocyte complexes when exposed to *T. forsythia* and other red-complex bacteria as compared to controls.^[22] This is not to imply that antibiotic prophylaxis is needed for the periodontal treatment of patients with *T. forsythia*. First of all, for periodontal patients who culture positive for *T. forsythia*, it only makes up 0.1%–4% of subgingival plaque obtained from deep pockets.^[23] In addition, periodontal treatment does not result in detectable platelet activation *per se*.^[24] Rather, more important is the experience of low-grade recurring episodes of bacteremia caused by daily activities such as mastication and tooth brushing in patients with chronic periodontitis.^[25] Thus, despite the low bacterial load, *T. forsythia* is frequently detected in atheromatous plaque and is particularly associated with hemorrhagic

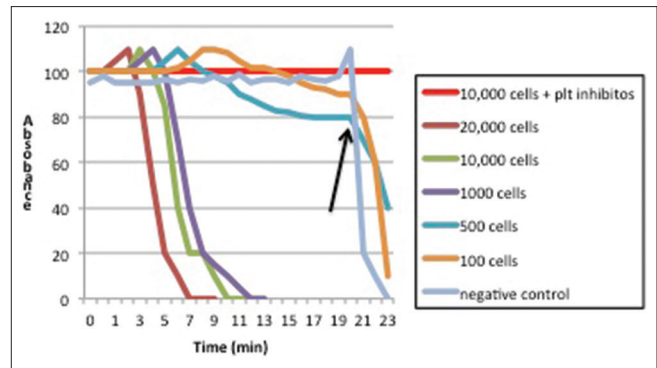


Figure 2: Aggregation of Platelets by *Tannerella forsythia*: *Tannerella forsythia* cells ($0.1\text{--}20 \mu\text{l}$; 10^3 cells/ μl) were added to $450 \mu\text{l}$ Platelet-rich plasma ($300,000$ plts/ μl). After various lag times, platelet shape change was observed by increase in absorbance, followed by platelet aggregation (a rapid decrease in absorbance). Adenosine diphosphate ($10 \mu\text{l}$, 1.0 mg/ml) was added at 20 min (arrow) to confirm the ability of the platelets to aggregate

atherosclerotic carotid plaques, which are made up of aggregated platelets and leucocytes^[26,27] It is a keystone pathogen with many virulence factors that promote not only biofilm dysbiosis but also host immune evasion.^[28] In addition, the pathogenic potential of *T. forsythia* is enhanced in the presence of other bacteria, especially *P. gingivalis*, including entry into host cells.^[29] Besides contributing to atherosclerotic plaque, platelet aggregation and release of platelet antimicrobial peptides may be the first step in host defense, and *T. forsythia* may exploit this mechanism, becoming entrapped within platelet aggregates that deposit in atherosclerotic plaque.^[30,31]

Previously, we have shown that activation of platelets by *P. gingivalis* cells could be partially attenuated, but not completely inhibited, by aspirin.^[7] Acetylsalicylic acid (aspirin, ASA) alters platelet aggregation by irreversible inhibition of cyclo-oxygenase.^[32] However, aspirin does not completely inhibit platelet aggregation induced by ADP, collagen, and high levels of thrombin. As a result, aspirin is often used in combination with other antiplatelet drugs such as clopidogrel, which inhibits platelet activation via ADP.^[33] Patients routinely ingest 81 mg ASA and 75 mg clopidogrel bisulfite that becomes diluted to 5 L in the human body ($90 \mu\text{M}$ ASA, $10 \mu\text{M}$ clopidogrel). Thus, in the *in vitro* studies reported here, the combination of acetylsalicylic acid and clopidogrel was incubated with gel filtered platelets at corresponding concentrations ($90 \mu\text{M}$ and $10 \mu\text{M}$ respectively). The robust aggregation elicited by *T. forsythia* cells was inhibited by aspirin plus clopidogrel. Although clopidogrel is metabolized in the liver to an active metabolite, clopidogrel is effective *in vitro* if used with washed or gel filtered platelets.^[34,35]

Bacteria cause platelet aggregation utilizing a variety of mechanisms.^[36,37] Some agonists, for example, the gingipains, activate platelets through G-protein-coupled receptors.^[38] The final common pathway for all agonists is the activation of the platelet integrin glycoprotein

IIB/IIIa (α IIB β 3), the main receptor for adhesion and aggregation.^[39] Although the mechanism of *T. forsythia* platelet activation is yet unknown, its lability may indicate that it acts similarly to *P. gingivalis*. The elucidation of this mechanism awaits further study. This is important since there is increasing evidence that these pathogens play a role in the development and exacerbation of atherosclerosis, which may involve direct activation of platelets by bacteria.^[40]

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Conflicts of interest

The views expressed in this article reflect the results of research conducted by the authors and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, nor the United States Government.

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