Summary

There is increasing evidence for a relationship between bacterial infections and several cardiovascular disorders. Although the precise mechanism(s) underlying this association is unknown, the direct activation of platelets by bacteria is one possibility. Individual strains of \textit{S. sanguis} activate platelets in a non-uniform, donor-dependent manner. In the current study, platelet aggregation profiles were obtained for fourteen donors in response to four strains of \textit{S. sanguis} (2017–78, 133–79, SK112, SK108a) and one of \textit{S. gordonii} (SK8). The platelets from all donors responded to strains 2017–78 and 133–79, whereas strains SK112, SK8 and SK108a caused aggregation in one, five and twelve donors, respectively. Immunoglobulin G (IgG) binding to strains 2017–78, 133–79 and SK108a were significantly greater than to strains SK112 and SK8. Absorption of IgG by strain 2017–78 caused significant decreases in IgG binding, and platelet aggregation in response, to all strains. Single-strand conformational polymorphisms were observed in the FcγRIIA gene from four donors. Sequencing revealed two known and two novel point mutations, none of which correlated with the aggregation profile. Thus, platelet activation to the various strains depends on a common IgG and, while in most cases the level of IgG binding to \textit{S. sanguis} determines platelet responsiveness, neither the levels of IgG nor FcγRIIA polymorphisms can fully account for donor variability.

Keywords

Platelet, FcγRIIA, polymorphisms, IgG, \textit{Streptococcus sanguis}

Introduction

There is some evidence for a relationship between bacterial infections and the pathogenesis of several cardiovascular or cerebrovascular disorders (1). Epidemiological and aetiological studies support a link between infection with \textit{Chlamydia pneumoniae} and the incidence of coronary heart disease, including atherosclerosis and myocardial infarction (2, 3). For example, \textit{C. pneumoniae} has been identified in atherosclerotic plaques (4, 5), and a high proportion of patients with coronary heart disease have circulating \textit{C. pneumoniae}-specific immune complexes (5, 6). However, these observations, and their implications, are somewhat contentious (7, 8).

Similarly an association between \textit{Helicobacter pylori} infections and cardiovascular disorders has been proposed (9, 10), though again this has been widely challenged (11). Finally relationships, although not necessarily causal, have also been proposed between bronchial (12) and, particularly, oral (13, 14) infections with the development of cardiovascular disease.

The mechanisms underlying the relationship between bacterial infections and cardiovascular disorders are unknown, although a variety of possibilities have been postulated. Infectious organisms, such as \textit{C. pneumoniae}, are potential initiators of the early endothelial dysfunction which serves as the initial focus for atherosclerotic lesions (15). Similarly infecting organisms may induce cytokine-mediated inflammatory responses recognized to play a role in both the development and progression of cardiovascular disorders such as atherosclerosis and angina (15, 16). Finally the direct activation of platelets by bacteria provides a plausible link between infection and cardiovascular disorders, including thrombosis, angina and atherosclerosis (6).

A range of bacterial species, including \textit{C. pneumoniae}, \textit{H. pylori}, \textit{Staphylococcus aureus}, \textit{Streptococcus faecalis}, \textit{Streptococcus pyogenes}, \textit{Escherichia coli}, \textit{Streptococcus pneumoniae}, Por-
phyromonas gingivalis and Streptococcus sanguis, have been shown to induce platelet activation in vitro (17–20). However, the mechanisms of platelet activation appear diverse. For example, C. pneumoniae-induced platelet activation involves a role for LPS (17), whereas gram positive organisms, which do not contain LPS, can induce platelet activation. Various groups have proposed that individual species of bacteria induce platelet activation by collagen-, ADP-, PAF- or immunoglobulin G-(IgG) like mechanisms (21–24). Furthermore, S. pyogenes and S. aureus activate platelets by a dual mechanism involving the bacterial adhesion to platelets and an independent activation step involving common, specific IgG (24). It was subsequently hypothesised that antibody titre influenced the variability of individual donor’s platelet response in response to S. pyogenes and S. aureus (24).

The mechanism of S. sanguis-induced aggregation remains unclear. The expression of the platelet αIIbβ3 integrin, in an “open conformation” with subsequent fibrinogen binding (25), is required. Thus this is a true activation process, rather than a passive cross-linking action. Indeed roles for the assembly of complement proteins on the bacterial surface (26), a variety of platelet receptors (e.g. α3β1 and the GPIb/V/IX complex) (27), IgG (25, 28), platelet cyclo-oxygenase activity and ADP production (21, 29) have all been suggested.

The role of IgG and its low affinity platelet receptor, FcyRIIA, in platelet activation by S. sanguis is of particular interest. The removal of IgG from plasma or blockade of IgG binding to FcyRIIA, prevents platelet aggregation in response to some S. sanguis strains (25), consistent with a requirement for FcyRIIA in the activation process. Polymorphic variation in IgG receptors, including FcyRIIA, have been shown to critically affect their ability to interact with IgG, leading to a possible role for IgG receptor allotypes in various autoimmune and infectious diseases (30). Interestingly, polymorphic variation of platelet receptors, such as α3β1, glycoprotein Ib and the αIIbβ3 integrin, have been shown to impact upon an individual’s platelet responsiveness and susceptibility to thrombosis (31, 32).

The objective of the current study was to determine the potential roles played by IgG titre, IgG binding and FcγRIIA allotype in the inter-strain and inter-donor variation observed in S. sanguis-induced platelet aggregation.

**Materials and methods**

**Materials**

Four strains of S. sanguis and one strain of S. gordonii were employed in this study. S. sanguis strains 2017–78 and 133–79 were gifts from Dr. M. Herzberg (University of Minnesota); S. sanguis strains SK112 and SK108a, and S. gordonii strain SK8 were originally isolated by Dr. M. Kilian (University of Aarhus). Pure human IgG and anti-serum IgG (γ-chains) were gifts from Dr. M. Cole (Georgetown University). Rabbit antibody to human IgG was obtained from DakoCytomation (Mississauga, Ontario), Blood Agar Base No. 2 from Oxoid Inc. (Nepean, Ontario), sheep’s blood from Atlas Laboratories (Winnipeg, Manitoba), IgG ELISA kits from VWR International (Mississauga, Ontario), QAE-sephadex from Amersham Pharmacia Biotech (Baie d’Urfé, Québec) and QIAquick Gel Extraction Kits from QIAGEN Inc. (Mississauga, Ontario). All other chemicals and materials were of the highest grade available.

**Blood collection**

The study was approved by the Research Ethics Board of the University of Manitoba and informed consent was obtained from all volunteers. Blood was collected by venipuncture of human volunteers and platelet rich plasma prepared as previously reported (28). An aliquot was centrifuged at 8500 g for 30 s to yield platelet-poor plasma (PPP) which was stored at −20°C until use.

**Bacteria and culture conditions**

Bacterial cells were prepared as previously reported (28). The absorbance at 600 nm was recorded and the number of bacteria in the suspension was calculated according to a standard curve of absorbance versus bacteria/ml, the latter obtained using a Petroff-Hauser counting chamber (28).

**Platelet aggregation**

Aggregation of platelets in plasma was measured in a Payton dual channel aggregometer as previously described (28). Platelets were challenged for 20 mins. with each strain at a platelet:bacteria ratio of 1:2. If there was no change in light transmission by 20 mins., the donor/strain combination was defined as non-responding. All aggregations were carried out in duplicate on two separate occasions.

**Anti-S. sanguis IgG titre**

The levels of IgG in plasma binding to the bacteria were measured by enzyme-linked immunosorbent assay (ELISA). ELISA plates were coated with bacteria (100 μg/ml dry weight) overnight at 4°C. The plates were blocked (0.1% BSA in PBS-Tween; 1 hr.; room temperature), washed with PBS-Tween, then incubated with the PPP samples, serially diluted (1:50 – 1:1600) in PBS-Tween containing 0.1% globulin-free albumin (3 hr.; room temperature). After washing, the plates were incubated with anti-human IgG HRP (1:1000 in PBS-Tween with 0.1% globulin-free albumin; 1 hr.; room temperature) and washed in PBS-Tween. O-phenylenediamine substrate (0.4 mg in 10 ml citrate-phosphate buffer, pH 5.0 and 40 μL hydrogen peroxide) was added and subsequent colour development read at 450 nm in a BioRAD Microplate Reader Model 550. IgG binding to individual strains of S. sanguis was quantified on three separate occasions. Data presented from 1:400 dilution.

**Deletion of S. sanguis 2017–78-specific antibody**

An aliquot of PPP (3.0 ml) was incubated with S. sanguis 2017–78 (300 μL) during gentle stirring at 4°C for 2 hr. IgG-bound bacteria were removed by centrifugation at (three x 1 min at 8500 x g). The above procedure was repeated with a fresh 300 μL of S. sanguis 2017–78 to maximize depletion. The level of IgG binding following absorption with 2017–78 was measured by ELISA, as described above.

**IgG isolation by QAE-sephadex chromatography**

QAE-sephadex was swollen and packed in buffer (ethylene diamine/acetic acid; pH 7.0). Following column equilibration with the same buffer, PPP (1.0 ml) was applied to the column and
allowed to pass through. Double diffusion of the eluate was performed on agarose gel using anti-human IgG to determine which fractions contained IgG. Those fractions containing IgG were combined and stored at –20°C until use.

**Single-strand conformational polymorphism analysis**
Exons 1–7 of FcγRIIA were amplified by polymerase chain reaction (PCR) using intronic primers flanking each exon (Table 1) and genomic DNA as template. Subsequent to amplification, all samples were treated as previously described (33).

**DNA sequence analysis**
DNA was amplified by PCR using primers for exons 3, 4, 5 and 7 (Table 1) and amplified products isolated using a QIAquick Gel Extraction Kit according to manufacturer’s instructions. DNA sequencing using forward and reverse primers (Table 1) was conducted at the Centre for Applied Genomics, Toronto, Canada. The differences reported were observed in both the forward and reverse sequences.

### Results

#### Platelet aggregation in response to *S. sanguis*

The platelets of all donors (n=14) aggregated when challenged with *S. sanguis* strains 2017–78, SK112, 133–79 and SK108a, and to *S. gordonii* strain SK8, were determined, and the corresponding IgG binding quantified. The presence of aggregation, measured by light transmittance, is noted by filled bars and the absence of aggregation by open bars. IgG binding was quantified by ELISA and is expressed as µg/mg bacteria (mean±SEM, n=3). Aggregation status was determined twice for each donor and each strain. Dotted line corresponds to calculated theoretical threshold level of 13.63 µg IgG/mg bacteria.

#### IgG binding to *S. sanguis*

The levels of each donors’ IgG binding to the respective strains were determined by ELISA (Fig. 2). The mean level of IgG binding to strain 2017–78 was 20.32±1.49 µg IgG/mg bacteria (mean±SEM; n=14), which did not differ statistically to the binding to strains 133–79 (19.92±1.31 µg IgG/mg bacteria; mean±SEM; n=14) or SK108a (20.35±1.40 µg IgG/mg bacteria; mean±SEM; n=14). In contrast, the mean level of IgG binding to strains SK112 (11.88±1.17 µg IgG/mg bacteria; mean±SEM; n=14; p<0.001) and SK8 (17.43±1.11 µg IgG/mg bacteria;
mean±SEM; n=14; p<0.05) were significantly lower than the IgG binding to strains 2017–78 and SK108a (Fig. 2).

The mean level of IgG binding to all strains in donors where aggregation was induced was 19.78±0.79 µg IgG/mg bacteria (mean±SEM; n=46) which was significantly higher than that bound in donors where aggregation did not occur (14.52±1.02 µg IgG/mg bacteria; mean±SEM; n=24; p<0.001). A logistic regression was used to quantify the relationship between IgG binding and the likelihood of aggregation (χ² = 13.88, with 1 df, p=0.000195). This confirmed the existence of a highly significant, concentration-dependent, relationship between IgG binding and the induction of aggregation. Of the 70 donor/strain combinations, 54 were consistent with an IgG binding level of 13.63 µg IgG/mg bacteria approximating a threshold level required to produce aggregation. The platelets from one donor aggregated in response to 2017–78, two in response to SK8 and one in response to 133–79 with IgG binding to the respective strain of less than 13.63 µg IgG/mg. In contrast, the platelets from four donors failed to aggregate in response to SK112, seven in response to SK8 and one in response to SK108a, although in each IgG binding to the strain was above the threshold level.

**Depletion of IgG binding to S. sanguis strain 2017–78**

Absorption of plasma by strain 2017–78 caused significant decreases in IgG binding to 2017–78 (53.3±5.4%; n=3), SK112 (55.0±5.6%; n=3), SK8 (27.3±7.9%; n=3), SK108a (34.3±4.9%; n=3) and 133–79 (32.3±11.0%; n=3). Interestingly, the depletion of plasma IgG by binding to strain 2017–78 inhibited platelet aggregation in response to all five strains of bacteria tested (Fig. 3), although aggregation in response to collagen remained intact (data not shown).

**Supplementation of plasma of SK8 non-responders by IgG**

IgG was isolated from the donor (donor 7) whose platelets aggregated in response to all five strains. This isolated IgG was added to the plasma of the four donors whose platelets had failed to respond to strain SK8. The subsequent addition of SK8 failed to induce platelet aggregation in this IgG-supplemented plasma (Fig. 4).

**Single-strand conformational polymorphism and DNA sequence analyses**

Single-strand conformational polymorphism (SSCP) analysis was conducted on each exon of the FcγRIIA gene. SSCP patterns were observed in exons 3, 4, 5 and 7 in the amplified DNA products from donors 5, 7, 13 and 14. DNA sequencing revealed two point mutations in exon 3, resulting in a GLN63TRP substitution and a single point mutation in exon 4, resulting in a HIS167ARG substitution in all four donors. Each of these polymorphisms has been previously reported (34). Two novel polymorphisms, in exon 5 and exon 7, were identified in all four donors, but these did not alter the amino acid sequence (PRO215PRO and PRO293PRO) of FcγRIIA. The presence of none of these polymorphisms correlated with the platelet aggregation profile (Fig. 1).

**Discussion**

The relationship between bacterial infection and cardiovascular disorders has been the subject of some controversy. In particular, the potential role played by oral pathogens in atherosclerosis and thrombosis is unclear. Several extensive prospective cohort studies have demonstrated that periodontal disease is a risk factor for cardiovascular disease, independent of other accepted risk factors (35–38), although the significance of the correlation has been questioned (39). Most studies have focused on the consequences of bacteraemias which occur several times daily.
These low-level bacteraemias may initiate host responses altering coagulability, endothelial and vessel wall integrity, accentuating inflammatory responses and stimulating platelet function, thereby resulting in atherosclerotic and thrombotic events (40).

S. sanguis is frequently isolated from dental plaque and, while not considered a periodontal pathogen, it has been linked to the progression of various cardiovascular diseases (20). Several groups have reported the activation of platelets by S. sanguis in a strain- and donor-dependent manner (20, 28, 41, 42). The mechanism of S. sanguis–induced platelet activation is unclear although a role for IgG binding to FcγRIIA, the low affinity IgG receptor on platelets, has been suggested (25, 28, 43).

In the present study four strains of S. sanguis and one of S. gordonii were used to examine both strain and donor dependency with respect to IgG binding. Platelet aggregation was observed in all donors in response to both strains 2017–78, widely studied as an “aggregating strain” of S. sanguis, and 133–79. In contrast, the platelets from only one donor responded to SK112, a strain previously reported as a “non-aggregating strain”. Intermediate donor responses were observed with strains SK108a and SK8. These data confirm the strain and donor variability of the platelet response to S. sanguis and show that S. gordonii can cause platelet aggregation in a donor-dependent manner. Depletion of IgG, by binding to 2017–78, abolished both IgG binding, and aggregation in response, to not only 2017–78 but also to the other strains. Thus, platelet activation to the various strains would appear to be dependent upon the presence of a common IgG, and thus it is unlikely that the strain dependency of aggregation occurs as a result of sub-type variation in IgG. Sjöbring et al. similarly concluded that specific IgG was required for platelet activation by S. pyogenes and S. aureus (24). However, it is unclear whether a common epitope on all three bacterial species is responsible for IgG-mediated platelet activation.

The relationship between plasma IgG and donor variation in platelet aggregation was assessed by determining the levels of IgG binding to individual strains and correlating these levels with aggregation. In general, platelet aggregation occurred when levels of IgG binding above a threshold level of 13.63 µg IgG/mg bacteria were observed. This level accounted for 54 of the 70 donor/strain combinations and suggests that the level of IgG binding determines platelet responsiveness to individual strains in approximately 70% of the situations. Such a relationship was suggested, but not demonstrated, for S. pyogenes and S. aureus (24).

The platelets from five donors aggregated at IgG binding levels below the threshold, whereas the platelets from twelve donors failed to respond at levels of IgG binding above the predicted threshold. Consequently, in these cases the donor variation cannot be explained by levels of IgG binding alone. In general, comparison of the induction of platelet aggregation by the individual strains for any single donor suggests that increased IgG binding is more likely to induce aggregation. However there were situations where aggregation was observed in response to one strain at binding levels equal to, or below, those observed where aggregation was not observed in another strain. For example, in donor 6 aggregation was observed in response to strains 2017–78 (19.8±3.2 µg IgG/mg bacteria), SK108a (23.4±3.4 µg IgG/mg bacteria) and 133–79 (22.0±3.4 µg IgG/mg bacteria) but not to strain SK8 (23.4±1.8 µg IgG/mg bacteria).

The addition of IgG isolated from the plasma of a “responder” failed to support aggregation in a “non-responder”, confirming that the (sub)-type of IgG does not confer variability. Overall, these data suggest that, while in most cases the level of IgG binding to S. sanguis determines platelet responsiveness, in some cases an inherent property of platelets confers donor plasma specificity.

S. sanguis-induced, IgG-mediated platelet activation is an ill-defined process. Although the initial platelet receptor for adhesion to bacteria is unknown, roles for both α3β1, and the GPIb/VIIX complex during both the adhesive and activation phases have been suggested. Subsequently S. sanguis-associated IgG binds to its high affinity receptor on platelets, FcγRIIA, leading to a cascade of signaling events and ultimately platelet aggregation (28). It is plausible that polymorphic variation in any of the platelet receptors (either adhesive or activation) for bacterial components may account for donor variability.

Polymorphisms in platelet receptors have been widely reported and, in some cases, implicated in a variety of thrombotic situations. For example several polymorphisms in the GPIb gene have been reported (44, 45) including at least one which influences the pathogenesis of ischemic stroke, even after adjusting for conventional cardiovascular risk factors (46). Similarly a polymorphism in the α2 gene has been correlated with early age stroke (47).

Of significance for the current work, the HIS131ARG polymorphism in FcγRIIA has been reported not to affect platelet function (48). Similarly in the present study this polymorphism did not impact on the platelet responses to the bacterial species examined. Thus, in the current study, polymorphic variation of FcγRIIA could not account for the variability of platelet aggregation observed amongst the donors.

Taken together, the data indicates that IgG levels partially, excluding FcγRIIA polymorphisms, can account for donor variability in platelet responsiveness to S. sanguis. Other factors involved may include polymorphism variation in other receptors, or a variation in complement assembly on the platelet surface.

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