The effect of different strains of *Helicobacter pylori* on platelet aggregation

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BACKGROUND AND AIMS: *Helicobacter pylori* is the major causative agent in peptic ulcer disease and is strongly implicated in the development of gastric cancer. It has also been linked, less strongly, to cardiovascular disease. The mechanisms by which certain strains of *H pylori* induce platelet aggregation through interactions with platelet glycoprotein Ib have been previously described.

METHODS: In the present study, 21 different strains of *H pylori*, varying in their vacuolating toxin gene, cytotoxic-associated gene A status and other pathogenicity factors, were tested for their ability to induce platelet aggregation.

RESULTS: Ten of the 21 strains induced platelet aggregation, a response that appeared to be independent of their vacuolating toxin gene and cytotoxic-associated gene A status.

CONCLUSIONS: Platelet aggregation has been suggested to be one of the possible mechanisms involved in the effects on the cardiovascular system induced by *H pylori*. Our results suggest that any putative role *H pylori* plays in cardiovascular disease may be strain dependent. Further work to identify the *H pylori* factors involved in induction of platelet aggregation may allow for identification of 'higher risk' strains for cardiovascular disease.

Key Words: Cardiovascular disease; *H pylori*; Platelet aggregation

Helicobacter pylori plays a significant role in the pathogenesis of peptic ulcer disease, gastric carcinoma and primary B cell gastric lymphoma. However, only a minority of individuals infected with *H pylori* develop a clinically significant outcome, such as peptic ulcer disease or gastric cancer. There is ongoing interest in identifying *H pylori* virulence factors that may predict the risk for symptomatic clinical outcomes. Experience with other bacterial pathogens suggest that *H pylori* strain-specific factors may influence the pathogenicity of different *H pylori* isolates. *H pylori* studies have primarily focused

L'effet de différentes souches de *Helicobacter pylori* sur l'agrégation plaquettaire

HISTORIQUE ET OBJECTIFS : Le *Helicobacter pylori* et le principal agent responsable de l'ulcère gastroduodénal, et il contribue fortement à l'apparition du cancer gastrique. Il est également lié, mais moins solidement, aux maladies cardiovasculaires. Les mécanismes par lesquels certaines souches de *H pylori* induisent l'agrégation plaquettaire par des interactions avec la glycoprotéine plaquettaire IB ont déjà été décrits.

MÉTHODOLOGIE : Dans la présente étude, 21 souches différentes de *H pylori*, comportant des variations du gène de toxine vacuolante, du statut de gène A associé à la cytotoxine et des facteurs de pathogénie, ont fait l'objet d'essais afin d'établir leur capacité à induire l'agrégation plaquettaire.

RÉSULTATS : Dix des 21 souches induisaient une agrégation plaquettaire, une réponse qui semblait indépendante du gène de toxine vacuolante et du statut du gène A associé à la cytotoxine.

CONCLUSIONS : On a postulé que l'agrégation plaquettaire pourrait être l'un des mécanismes contribuant aux effets du système cardiovasculaire induits par le *H pylori*. Nos résultats indiquent que le rôle putatif du *H pylori* dans la maladie cardiovasculaire dépendrait de la souche. D'autres recherches pour dépister les facteurs du *H pylori* qui contribuent à l'induction de l'agrégation plaquettaire pourraient permettre de repérer les souches comportant un plus haut risque de maladie cardiovasculaire.

on two groups of putative bacterial virulence factors, the *cag* pathogenicity island (for which cytotoxic-associated gene A [*cagA*] is a marker) and the vacuolating cytotoxin (1,2).

Some studies (3) have shown the formation of platelet aggregates in *H pylori*-infected patients, which may explain the association that has long been suggested between *H pylori* infection and coronary artery disease (CAD) (4-10) and stroke (11), although other studies (12-14) have failed to show any link. Recent work (15) has suggested that *H pylori* could function as a triggering factor in thrombotic thrombocytopenic

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purpura by inducing platelet aggregation through an interaction with von Willebrand factor (vWF). In addition, eradication of *H pylori* from the gastric mucosa has been associated with improvement of several systemic diseases, including immune thrombocytopenic purpura (ITP). A review of published studies of patients with ITP who underwent *H pylori* eradication revealed an overall response rate of 52% in 193 patients in whom *H pylori* was eradicated (16). One of the several suggested mechanisms for involvement of *H pylori* in ITP is induction of platelet aggregation.

None of these studies have shown any mechanisms by which *H pylori* might induce platelet aggregation. We have previously described the mechanism by which certain strains of *H pylori* interact with the main platelet receptor glycoprotein Ib/IX as well as plasma factors such as vWf and immunoglobulin (IgG) to induce platelet aggregation (17). To explore the potential clinical implications of our molecular findings, we investigated 21 different strains of *H pylori* varying in their pathogenicity factors and their ability to induce platelet aggregation. These results were compared with the disease presentation of the patient from which the bacterium was isolated.

METHODS

Bacterial growth

H pylori strains were grown and maintained on *H pylori* agar plates (bioMérieux, France) in a microaerobic environment (Campy pak plus, Becton Dickinson, USA) at 37°C. After 48 h growth, *H pylori* strains were harvested directly from the plates, with sterile cotton buds, into phosphate buffered saline and resuspended to yield an optical density of 1.6 at 450 nm (approximately 4×10^9 bacteria/mL). Stock cultures were stored at -70°C in broth supplemented with 15% glycerol.

Bacterial strains

Helicobacter strains used in the present study were a kind gift from Dr John Atherton (University of Nottingham, UK) and Dr Torkel Wadstrom (Lund University, Sweden). Several strains of *H pylori* were used because the pathogenicity of *H pylori* varies depending on the presence or absence, among other factors, of the *cag* pathogenicity island (for which the gene *cagA* is a marker) and genotype of the vacuolating cytotoxin gene, *vacA*. Twenty-one strains of *H pylori* were assessed in the present study. Bacterial characteristics are as follows:

A109, J258, J178, J254, J99 (*cagA*⁺, *vacA* s1/m1);
J87, 25, 66, 1139, 17874, 17875, 915 (*cagA*⁺, *vacA*⁺);
J223, J226, J174, J182, J128 (*cagA*⁺, *vacA* s1/m2);
9366, J190, J63, J150 (*cagA*⁻, *vacA*, s2/m2).

Bacterial strains were taken from patients who underwent routine upper gastrointestinal endoscopy for a variety of indications. An active peptic ulcer was defined as a circumscribed break in the mucosa, with apparent depth, measuring more than 1 cm in any dimension; an erosion was defined as a definite circumscribed break in the mucosa not fulfilling the criteria for an active ulcer.

Platelet preparation

Whole blood was drawn from healthy human volunteers who had abstained from nonsteroidal anti-inflammatory drugs and acetylsalicylic acid for 10 days. Nine volumes of blood were collected by clean venipuncture using a 19-gauge needle and added to one volume of 3.8% sodium citrate. Platelet-rich plasma (PRP) was prepared through centrifugation of anticoagulated whole blood at room temperature at 150 g for 10 min. The uppermost layer rich in

platelets, PRP, was transferred into a 15 mL Greiner tube. The remaining blood was centrifuged at room temperature at 630 g for 10 min to yield platelet-poor plasma.

Platelet aggregation

Platelet aggregation was assayed by light transmission at 37°C using a platelet aggregometer (Bio/Data Corporation, USA). Platelets (suspensions of 500 µL) were warmed to 37°C, and the aggregation response was measured in siliconized cuvettes with continuous stirring at 1200 rpm. All experiments were repeated on at least three occasions using platelets from three different donors and were completed within 4 h of blood sampling. Platelets were tested for normal responses using arachidonic acid (0.5 mg/mL), and/or ADP (2 µM) before each experiment. Fifty microlitres of *H pylori* bacterial suspension (4×10^9 bacteria/mL of phosphate buffered saline) were added to 450 µL of human PRP.

Platelets respond immediately (within 10 s) to soluble agonists such as ADP. However, with bacteria, there is a lag time to aggregation that is dependent on the type of bacterium. *Staphylococcus aureus* has a rapid lag time of 2 min to 3 min, while *Streptococcus sanguis* can have a lag time of approximately 18 min. Not aggregating is usually defined as having a lag time of greater than 30 min.

RESULTS

Of the 21 strains tested, 10 strains induced platelet aggregation. There is no normal value in platelet aggregation; some bacteria induce platelet aggregation, while others do not. For aggregating strains, platelet aggregation took place within 5 min to 6 min.

The aggregation percentages are as follows: A109 (24%±5%), J258 (44%±18%), J178 (25%±4%), J254 (2%±2%), J87 (2%±1%), J223 (0%±0%), J226 (5%±4%), J174 (5%±4%), 25 (32%±11%), 66 (0%±0%), 1139 (27%±3%), 17874 (30%±7%), 17875 (0%±0%), J99 (1%±1%), 915 (19%±5%), J182 (21%±5%), J128 (20%±1%), 9366 (41%±0%), J190 (1%±1%), J63 (1%±0%), J150 (24%±12%).

The ability to induce platelet aggregation is summarized for each of the 21 strains tested in Table 1. There are no normal values in platelet aggregation. Unstimulated PRP generates an aggregation response of 0%; although a small amount of drift can take place especially over a long period. In the present study, we defined aggregation as a 10% change in light transmission. Thus, when bacteria are added to PRP some have no effect and others induce aggregation. In the present case, approximately 50% of the clinical strains induced aggregation.

Data are presented as mean ± SD from experiments performed in triplicate. Figure 1 represents a sample tracing showing induction of platelet aggregation for four of the 21 strains tested.

There was no correlation between the ability to induce aggregation and the type of gastroduodenal disease present in the patient (Table 2).

DISCUSSION

The injury induced by *H pylori* in the gastroduodenal tract may be due in part to inflammation and thrombosis within the gastric microvasculature. Numerous studies (18,19) have shown platelet activation in animal and human models. In vivo studies in the rat gastric mucosa have characterized the response of microcirculation to extracts of *H pylori*, including upregulation of neutrophil adhesion molecules, subsequent

TABLE 1

Twenty-one strains of *Helicobacter pylori* were tested for their ability to induce platelet aggregation

<i>H. pylori</i> strain	Virulence factors	Disease status	Aggregation
A109	<i>vacA</i> ⁺ <i>cagA</i> ⁺	DU	Yes
J258	<i>vacA</i> ⁺ <i>cagA</i> ⁺	DU	Yes
J178	<i>vacA</i> ⁺ <i>cagA</i> ⁺	DU	Yes
J254	<i>vacA</i> ⁺ <i>cagA</i> ⁺	Gastritis only	No
J87	<i>vacA</i> ⁺ <i>cagA</i> ⁺	DU	No
J223	<i>vacA</i> ⁺ <i>cagA</i> ⁺	DU	No
J226	<i>vacA</i> ⁺ <i>cagA</i> ⁺	DU/GU	No
J174	<i>vacA</i> ⁺ <i>cagA</i> ⁺	Gastritis only	No
25	<i>vacA</i> ⁺ <i>cagA</i> ⁺	Gastritis only	Yes
66	<i>vacA</i> ⁺ <i>cagA</i> ⁺	Gastritis only	No
1139	<i>vacA</i> ⁺ <i>cagA</i> ⁺	Gastritis only	Yes
17874	<i>vacA</i> ⁺ <i>cagA</i> ⁺	Gastritis only	Yes
17875	<i>vacA</i> ⁺ <i>cagA</i> ⁺	Gastritis only	No
J99	<i>vacA</i> ⁺ <i>cagA</i> ⁺	DU	No
915	<i>vacA</i> ⁺ <i>cagA</i> ⁺	DU	Yes
J182	<i>vacA</i> ⁺ <i>cagA</i> ⁺	DU	Yes
J128	<i>vacA</i> ⁺ <i>cagA</i> ⁺	DU/GU	Yes
9366	<i>vacA</i> ⁺ <i>cagA</i> ⁻	Gastritis only	No
J190	<i>vacA</i> ⁺ <i>cagA</i> ⁻	Gastritis only	No
J63	<i>vacA</i> ⁺ <i>cagA</i> ⁻	Gastritis only	No
J150	<i>vacA</i> ⁺ <i>cagA</i> ⁻	Gastritis only	Yes

The strains were derived from patients with benign infection or disease pathologies such as gastric ulcer (GU) and duodenal ulcer (DU). *cagA* Cytotoxic-associated gene A; *vacA* Vacuolating toxin gene

neutrophil adhesion and extravasation (20,21), and platelet aggregation (18). Platelets can further act on the microcirculation by releasing compounds such as serotonin, platelet-activating factor, thromboxane B₂ and P-selectin, recruiting more neutrophils (19). The occlusion of microvessels by platelet thrombi may lead to mucosal infarction and necrosis, as well as the release of bactericidal peptides from the platelet. These activated platelets would lead to local tissue occlusion, ischemia, generation of cytotoxic products and, eventually, ulceration. Indeed, circulating platelet aggregates and activated platelets have been detected in patients infected with *H. pylori* (18). Whether this is a direct effect of *H. pylori* on platelets or secondary to vascular injury is unknown.

Recent studies have shown both negative and positive associations between *H. pylori* and CAD. Although there are recent studies that suggest that the eradication of *H. pylori* does not have any influence in the treatment of ITP (22), others have shown that eradicating *H. pylori* is associated with improvement of several systemic diseases, including ITP (16). The role of bacterial infection in cardiovascular disease is, at present, an area of much debate. Recent work suggests that pathogen burden is a good predictor of outcome in cardiovascular disease patients. In animal models, exposure to certain pathogens is more effective at inducing atherosclerosis than a high cholesterol diet. The evidence for specific pathogens such as *H. pylori* is weaker, but some studies have shown an association. Equally, the data supporting a role for *H. pylori* in thrombocytopenia are intriguing but far from conclusive. One difficulty is that studies have failed to take into consideration the importance of a pro-thrombotic phenotype, especially because we have shown that this may only have a 50% prevalence.

Figure 1 Percentages of platelet aggregation for four different strains of *Helicobacter pylori*. Numerical lines (1-4) represent strains J179, J226, 6320 and J254, respectively. The percentages of induced platelet aggregations were 1%, 8%, 2% and 29%, respectively. The time was measured in 1 min intervals with the baseline starting at 4 min; overall aggregation took place approximately within 5 min to 6 min. Lag time is defined as the time taken from the addition of bacteria to platelet-rich plasma until the first recognizable signs of aggregation. A range of lag times was observed with the strains used, but the lag time was consistent for each strain

TABLE 2

Statistical analysis of correlation between the ability to induce aggregation and gastroduodenal disease presentation

Aggregation	Ulcer, n	Gastritis, n
Yes	6	4
No	4	7

The relative risk is 1.6 (95% CI 0.6 to 4.2) and is nonsignificant (Fisher's exact test)

Platelet activation is known to contribute to the development of acute phases of myocardial infarction in patients with CAD (23). Some have suggested that *H. pylori* infection may contribute to the development of thrombocytopenia and other cardiovascular diseases by promoting platelet consumption (24). Several theories have attempted to explain how a noninvasive organism can affect platelets, including molecular mimicry and platelet aggregation, but none have been proven. We have previously described a clear molecular mechanism by which certain strains induce platelet aggregation, namely, by binding vWf and inducing glycoprotein Ib- and FcγRIIa-dependent platelet aggregation in the presence of *H. pylori* antibodies (17). Our present study of 21 strains confirmed that this is potentially a clinically important interaction because almost one-half of the strains studied induced platelet aggregation. We found that 10 of the 21 strains of *H. pylori* induced platelet aggregation. We speculate strongly that the mechanism for platelet-induced aggregation is uniform for all strains of *H. pylori*, because anti-IIb/IIIa and anti-vWf antibodies inhibited a random test of one of the *H. pylori* strains used in the present study (data not shown). We also speculate that this was the uniform mechanism for all five strains in our previous mechanistic study (17), but we accept the valid criticism that

not all strains were tested in this way in the present study. The variable results in studies of ITP and other vascular associations of *H pylori* may be explained, to some degree, by the finding in our present study that not all strains of *H pylori* activate platelets. For some unknown reason, *H pylori* tends to induce an aggregation response that is weaker than that seen with certain strains of *S aureus* or *S sanguis*, possibly because a different activation method is involved.

Our study findings have some potentially important clinical implications. For example, the response of ITP to *H pylori* eradication may depend on the infecting strain (12,25). The phenotype of the infecting strain may be important in the outcome of the disease process of ITP. The difference in the clinical response to eradication therapy warrants further investigation into differences in the aggregating phenotype of *H pylori*. There are similar implications for the other vascular associations of *H pylori*, such as CAD. Whether *H pylori* plays a role in CAD, either via platelet aggregation or other methods, may be very strain dependent, further confounding the current confusion.

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SUMMARY

Ten of 21 strains of *H pylori* used in the present study induced platelet aggregation. The clinical relevance of this interaction remains to be clarified, but an increasing number of studies are suggesting a real association with changes in platelet function. The strain dependency in our in vitro model may explain some of the conflicting results with *H pylori* infection and extraintestinal diseases such as ITP and CAD, disease processes that may be partially dependent on the ability of the infecting strain(s) to induce platelet aggregation.

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