The aggregation of human platelets by *Lactobacillus* species

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The ability to aggregate human platelets was examined for five *Lactobacillus rhamnosus* strains and five *Lactobacillus paracasei* subsp. *paracasei* strains isolated from patients with infective endocarditis (IE), 25 laboratory isolates from the same two species, and 14 strains from five other oral species, namely *Lactobacillus acidophilus*, *Lactobacillus fermentum*, *Lactobacillus oris*, *Lactobacillus plantarum* and *Lactobacillus salivarius*. Amongst the *L. rhamnosus* strains, platelets were aggregated by all five IE strains and 8/16 laboratory strains. For the *L. paracasei* subsp. *paracasei* strains, the respective numbers were 2/5 and 2/9. Aggregation also occurred with 11/14 strains of the other five species; each species was represented. The optimal ratio of bacteria to platelets for aggregation was approximately 1:1, and there was considerable variation in the lag phase that preceded aggregation, depending on the source of the platelets. Overall, the lag phase varied between 0.25 ± 0.1 and 20.4 ± 3.2 min and the percentage aggregation ranged between 70 ± 2.6 and 104 ± 13.5 %. Confirmation that aggregation was being observed came from studies with five strains on the inhibitory effects of EDTA, dipyridamole, apyrase, imipramine, acetylsalicylic acid and quinacrine. Inhibition of aggregation by *L. rhamnosus* strains by the peptide arginine-glycine-aspartic acid-serine (RGDS) further indicated a role for fibronectin and/or fibrinogen. Pronase treatment of cells for 1 h and extraction of bacterial surface components with 0.1 M-Tris/HCl (pH 8.5) at 37 °C for 1 h stopped aggregation in 8/9 IE strains. Extracted surface proteins (200 µg) completely inhibited platelet aggregation by 8/9 of the homologous strains. A comparison of the platelets from four donors showed that an increase in the lag phase by 50% required 11.5, 14.8, 33.3 or 115 µg of extract, indicating a variability in donor response to aggregation by lactobacilli. The data indicate that platelet aggregation by lactobacilli may be an important contributory factor in IE. The potential to cause IE is present in the general population of oral lactobacilli and due regard should be taken when strain selection is made for probiotic purposes.

Introduction

Lactobacilli are often considered to be beneficial or non-pathogenic to man, with small numbers of human infections being reported, including septicaemia and infective endocarditis (IE) (Davies *et al.*, 1986; Naudé *et al.*, 1988; Sharpe *et al.*, 1973; Struve *et al.*, 1988; Sussman *et al.*, 1986). It has been observed recently, however, that lactobacilli should now be considered as

emerging pathogens, with an increase over the last 7 years of isolates from blood cultures (Maskell & Peard, 1992). Oral bacteria, including lactobacilli, may be introduced into the bloodstream by trauma during dental procedures or by poor dental hygiene (Axelrod *et al.*, 1973; Davies *et al.*, 1986; Naudé *et al.*, 1988). The aggregation of blood platelets by bacteria is thought to contribute to the progression of IE, which is the result of the colonization of a platelet-fibrin clot on the endothelial surface of the heart by bacteria (Knox & Hunter, 1991; Sheld, 1984; Herzberg *et al.*, 1983a, 1992). Platelet aggregation has been investigated for a wide range of micro-organisms, including streptococci (Douglas *et al.*, 1990; Isogai *et al.*, 1991; Herzberg *et al.*, 1983a; Sullam *et al.*, 1987), enterococci (Usui *et al.*, 1991), staphylococci (Yeaman *et al.*, 1992), *Listeria monocytogenes* (Czuprynski & Balish, 1981), *Fusobacterium necrophorum* (Forrester *et al.*, 1985; Horose *et al.*, 1992), *Yersinia pseudotuberculosis* (Simonet *et al.*, 1992), *Aspergillus fumigatus* (Sheth *et al.*, 1980) and

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Abbreviations: ASA, acetylsalicylic acid; IE, infective endocarditis; PPP, platelet-poor plasma; PRP, platelet-rich plasma; RGDS, peptide arginine-glycine-aspartic acid-serine.

Candida albicans (Skerl *et al.*, 1981). Certain strains of streptococci have been shown to possess cell-surface proteins which mediate interactions with platelets (Herzberg *et al.*, 1983b, 1990), while the binding of IgG to the platelet Fc receptor, and the presence of protein A on the cell wall of *Staphylococcus aureus* will also initiate aggregation (Hawiger *et al.*, 1979). Strains deficient in these cell surface proteins have been shown to produce significantly smaller vegetations in animal models (Herzberg *et al.*, 1992; Manning *et al.*, 1993), indicating an important role for platelet aggregation in the progression of the disease.

The emergence of lactobacilli as potential pathogens has been linked to the use of broad-spectrum antibiotics, immunosuppression and increased life expectancy due to modern medical practices (Maskell & Pead, 1992; Chomarat & Espinouse, 1991). Lactobacilli are also increasingly being investigated for their potential beneficial effects. The use of lactobacilli, particularly as microbial feed supplements (probiotics), is attracting great interest and claims are being made for their use in the amelioration of mucosal dysfunction caused by enteric bacteria, in the production of antimicrobial substances (bacteriocins), in the inhibition of tumour formation, in the treatment of irritable bowel syndrome and in the protection of the gut microflora during antibiotic therapy (Abstracts of the XVII International Congress on Microbial Ecology and Disease). These proposed uses of lactobacilli as probiotics may increase human exposure to any potentially pathogenic strains.

In this study, we examine the interaction of platelets with lactobacilli isolated from IE patients and also the frequency of occurrence of the ability to aggregate platelets amongst oral *Lactobacillus* species.

Methods

Bacterial strains and culture conditions. Ten IE strains were available, nine isolated by the Public Health Laboratory Service (PHLS), UK and provided by Dr L. R. Hill, NCTC Colindale, UK and one strain, YS-88-6-6441, provided by Professor Y. Chong, Yonsei University College of Medicine, Seoul, Korea (Chong *et al.*, 1991; Harty *et al.*, 1993). All other strains were either from the Institute's own culture collection (LC and LR strains were isolated from saliva samples and freeze-dried) or freshly isolated from saliva samples by plating on MRS agar (Oxoid) (see Tables 3 and 4). No strain from the Institute's culture collection had been subcultured more than nine times (the majority only three times) since isolation. All strains were identified using the API 50CHL test kit and the 'identify' computer program (Cox & Thomsen, 1990) as previously described (Harty & Knox, 1991; Harty *et al.*, 1993). Bacteria were grown in 10 ml MRS broth (Oxoid) at 37 °C for 18 h in an anaerobic jar with an atmosphere of 5% carbon dioxide in nitrogen. Larger cultures (400 ml) were grown as static batch cultures in a 37 °C incubator. Purity was checked by plating on MRS agar. Cultures for platelet aggregation were harvested by centrifugation, and washed three times in phosphate-buffered saline (PBS) pH 7.3, containing (g l⁻¹): NaCl, 8.0; KCl, 0.2; Na₂HPO₄ · 12H₂O, 2.9;

KH₂PO₄, 0.2. The cells were resuspended to an OD₆₆₀ of 1.0 ± 0.02 (5 × 10⁸ c.f.u. ml⁻¹) and then concentrated fourfold to give a suspension containing 2 × 10⁹ c.f.u. ml⁻¹.

Platelet aggregometry. Platelets were provided by the Red Cross Blood Bank from fresh blood mixed immediately with 0.1 M-trisodium citrate (9:1 v/v). Platelet-rich plasma (PRP) was produced by centrifugation of the citrated blood sample at 100 g for 10 min at 22 °C; platelet-poor plasma (PPP) was produced by further centrifugation at 2350 g for 10 min at 22 °C. The PRP was then adjusted with the PPP to an OD₆₆₀ of 0.5 ± 0.01, giving a platelet concentration of 2.27 × 10⁸ platelets ml⁻¹. Platelet aggregation was carried out in a recording aggregometer (either a model 430 single-channel aggregometer or a model 540 dual-channel aggregometer; Chrono-Log Corp.), with light transmission through PPP representing 100% aggregation and that through PRP representing 0% aggregation. Platelet viability was confirmed by addition to PRP of the agonist ADP to a final concentration of 20 µM. The optimal ratio of bacteria to platelets was examined by varying the c.f.u. ml⁻¹ added to PRP and determining the ratio that gave the maximum percentage aggregation. Lactobacilli were examined for their ability to induce platelet aggregation by the addition of 25 µl of a bacterial suspension (2 × 10⁹ c.f.u. ml⁻¹) to 0.25 ml PRP and PPP, preincubated at 37 °C for 5 min. A lag phase of greater than 25 min was assumed to be a negative aggregation. ADP was added to negatively aggregating strains to confirm platelet function. All aggregations were carried out in triplicate, utilizing different batches of platelets, and the results given are the mean and standard deviation.

Inhibition of platelet aggregation. The effect of various inhibitors of platelet aggregation was tested with five representative strains, namely *L. rhamnosus* A64/87 and LR19, and *L. paracasei* subsp. *paracasei* LC4, LC16 and A198/89. All studies were carried out on the dual-channel recorder with one channel for a control aggregation without inhibitor. Addition of 25 µl PBS to PRP and PPP as a control to monitor the dilution effect of addition of inhibitors caused no change in the length of the lag phase or final percentage aggregation. The potential inhibitors were dissolved to the required concentration in PBS; EDTA (40 mM), apyrase (Grade V, 10 mg ml⁻¹), imipramine (2 mM), quinacrine (1 mM) and acetylsalicylic acid (ASA, 20 mM), all obtained from Sigma. Dipyridamole (5 mM) was dissolved in PBS containing 0.4% (v/v) conc. HCl; a control aggregation of PBS plus 0.4% (v/v) conc. HCl was carried out at the same time. The peptide arginine-glycine-aspartic acid-serine (RGDS, Sigma), known to inhibit binding of fibrinogen to platelets (Gartner & Bennett, 1985), was dissolved in PBS (11.5 mM). In all cases 25 µl of the inhibitor at the required concentration was added to PRP and PPP, incubated at room temperature for 10 min and then brought up to 37 °C for 5 min before the addition of bacteria. Platelet aggregation was followed until aggregation was completed or until 25 min had passed.

Pronase treatment. Bacterial suspensions (10 ml, OD₆₆₀ 1.0) were incubated with an equal vol. 5 mg pronase ml⁻¹ (Sigma) at 37 °C for the required time, then cooled on ice before centrifugation and washing three times with PBS. The cells were then resuspended to the correct optical density required for use in platelet aggregation.

Heat treatment. Bacterial suspensions (10 ml, OD₆₆₀ 1.0) were heated for 1 h at either 37 °C or 60 °C, cooled on ice and the optical density checked before use in aggregation.

Extraction of bacterial surface components. Cultures (400 ml) were harvested and washed once in distilled water. The cells were then resuspended in 10 ml 0.1 M-Tris/HCl (pH 8.5) and incubated with shaking at 37 °C for 1 h (Reniero *et al.*, 1990). The suspension was centrifuged and the extract immediately dialysed against distilled water at 4 °C for 18 h. After determination of the protein concentration of the extract by the method of Bradford (1976) (Pierce Biochemicals kit), the

extract was lyophilized and redissolved in PBS to give a protein concentration of 800 µg ml⁻¹. Inhibition of aggregation was tested by the addition of 25 µl extracted material to PRP and incubation with stirring for 5 min before the addition of bacteria.

SDS-PAGE. Lyophilized protein extracts (50 µg) were dissolved in 50 µl sample buffer containing 0.125 M-Tris (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 0.001% (w/v) bromophenol blue and 5% (v/v) 2-mercaptoethanol. The dissolved extracts were then boiled for 2 min and electrophoresed through 12.5% (w/v) acrylamide gels, containing 0.1% SDS, by the method of Laemmli & Favre (1973). Gels were stained with Coomassie Blue R250, destained and photographed. Molecular masses of proteins were estimated with reference to the following standards (kDa): α -lactalbumin, 14; trypsin inhibitor, 20; carbonic anhydrase, 30; ovalbumin, 43; albumin, 67; phosphorylase b, 94.

Results

Bacteria to platelet ratio

The optimal ratio of bacteria to platelets was determined for three strains, *L. rhamnosus* A64/87 and LR19, and *L. paracasei* subsp. *paracasei* A198/89. Variation in the ratio had little effect on the length of the lag phase but the highest percentage aggregation was observed at a ratio of approximately 1:1, which was used in all further experiments.

Effects of batch to batch variation in platelets

When tested with *L. rhamnosus* A64/87, platelets from different donors showed differences in response for both the duration of the lag phase, namely 6.3 ± 1.9 min, and for the final percentage aggregation, namely $89.0 \pm 17.3\%$ (Table 1, Fig. 1). Accordingly, all aggregations in the survey of strains were carried out with platelets from three different donors.

Effects of platelet aggregation inhibitors

The effects of various inhibitors of platelet aggregation were tested with two strains of *L. rhamnosus* and three of *L. paracasei* subsp. *paracasei* (Table 2). Aggregation by *L. rhamnosus* strains A64/87 and LR19 was inhibited completely by the addition of 3.6 mM-EDTA, and varying degrees of inhibition were also achieved by dipyridamole (460 µM), which may have a similar effect to EDTA in reducing the availability of calcium ions, apyrase (0.9 mg ml⁻¹), which is an ADP hydrolase, quinacrine (90 µM) and ASA (1.8 mM), both of which inhibit the arachidonate cascade, and imipramine (180 µM), which is reported to be active on membrane stability (Mills & Roberts, 1967). The addition of RGDS (139 µM), an inhibitor of fibronectin and fibrinogen binding to platelets (Gartner & Bennett, 1985), also inhibited aggregation by both strains.

Aggregation of platelets by *L. paracasei* subsp.

paracasei strains LC4 and LC16 was significantly affected by the addition of EDTA (3.6 mM), dipyridamole (460 µM), apyrase (0.9 mg ml⁻¹) and imipramine (180 µM),

Table 1. Platelet aggregation by *L. rhamnosus* strains

Values in parentheses represent the standard deviations.

Strain	Lag phase (min)	Final aggregation (%)*
<i>Endocarditis isolates</i>		
A103/70	9.7 (1.6)	70.0 (2.6)
A544/84	9.4 (2.7)	103.5 (13.5)
A64/87	6.3 (1.9)	89.0 (17.3)
A65/87	14.0 (6.5)	83.1 (2.5)
A77/87	8.1 (1.3)	95.9 (12.2)
<i>Laboratory isolates</i>		
NCTC 10302	> 25	0
ATCC 7469	> 25	0
GV-261-R	9.6 (3.7)	97.5 (5.9)
GV-261-F	20.4 (3.2)	92.0 (12.2)
324M	> 25	0
LR9	> 25	0
LR10	> 25	0
LR12	> 25	0
LR13	10.7 (2.2)	77.9 (6.9)
LR15	> 25	0
LR19	9.0 (3.1)	77.1 (9.7)
LR33	19.4 (1.9)	76.9 (1.6)
LR36	18.6 (1.2)	87.9 (1.0)
F1†	13.9 (6.6)	92.8 (9.9)
K1†	7.3 (0.9)	85.2 (22.4)
K2†	> 25	0

* 100% aggregation represents light transmission through PPP.

† Freshly isolated from saliva.

Fig. 1. Effect of increasing concentration of protein extract on the length of the lag phase of *L. rhamnosus* A64/87 with platelets from different donors (●, ▽, ○, ▼). Each point is a single representative value.

Table 2. Effects of the addition of potential inhibitors of platelet aggregation

Inhibitor	Concn	<i>L. rhamnosus</i> LR19		<i>L. rhamnosus</i> A64/87		<i>L. paracasei</i> subsp. <i>paracasei</i> LC4		<i>L. paracasei</i> subsp. <i>paracasei</i> LC16		<i>L. paracasei</i> subsp. <i>paracasei</i> A198/89	
		Lag (min)	Aggn (%)	Lag (min)	Aggn (%)	Lag (min)	Aggn (%)	Lag (min)	Aggn (%)	Lag (min)	Aggn (%)
EDTA	0	5.5	82	6.7	100	6.7	100	9.2	85	0.4	100
	0.9 mM	5.5	82	8.3	100	ND	ND	9.2	76	0.6	100
	3.6 mM	> 25*	0	> 25	*0	> 25*	0	> 25*	0	> 25*	19
Dipyridamole	0	9.0	100	6.3	109	4.5	100	8.0	90	0.4	100
	90 µM	11.5	100	9.8	86.8	4.0	100	9.3	73	0.4	100
	460 µM	> 25*	14.4	11.0	36.9	9.5	35	> 25*	0	0.4	76.6
Apyrase	0	7.0	94	6.6	100	6.3	100	8.0	88	0.4	100
	0.46 mg ml ⁻¹	7.9	108	10	38	6.8	33	8.9	94	0.6	100
	0.9 mg ml ⁻¹	9.7	49	13.5	18	ND	ND	12.4	64	0.7	86
ASA	0	6.8	83	8.5	86	6.0	78	8.5	82	0.4	100
	0.9 mM	12	87	9.8	67	ND	ND	7.5	92	0.8	84
	1.8 mM	> 25*	20	9.8	64	6.0	78	9.0	98	0.8	64
Quinacrine	0	5.5	82	6.1	91.9	5.5	95	7.5	98	0.4	100
	9 µM	13.5	83	7.4	100	5.5	95	7.3	100	0.6	100
	90 µM	> 25*	11	11.1	68.1	8.5	60	> 25*	0	0.6	100
Imipramine	0	7.5	100	6.2	103	5.5	100	10.5	95	0.5	100
	90 µM	17.0	85	9.0	87.5	7.5	100	13.0	79	0.5	100
	180 µM	> 25*	18	12.0	54.4	> 25*	16	12.5	28	0.5	100
RGDS	0	8.5	100	7.3	100	7.0	88	8.0	92	0.4	100
	139 µM	15.5	50.6	9.5	71.3	11	84	9.6	100	0.4	100

ND, Not determined.

* No discernible lag phase.

Table 3. Platelet aggregation by *L. paracasei* subsp. *paracasei* isolates

Values in parentheses represent the standard deviations.

Strain	Lag phase (min)	Final aggregation (%)*
<i>Endocarditis isolates</i>		
A59/70	> 25	0
A22/73	18.0 (5.4)	88.0 (20.7)
A357/84	> 25	0
A198/89	0.25 (0.1)	101 (9.1)
YS 88-6-6441	> 25	0
<i>Laboratory isolates</i>		
NCFB 151	> 25	0
ATCC 393	> 25	0
ATCC 25302 (RO94)	> 25	0
ATCC 25303 (H831)	> 25	0
ATCC 29599 (DECP)	> 25	0
LC4	8.8 (1.0)	94.6 (21.9)
LC11	> 25	0
LC16	8.6 (2.2)	82.5 (7.6)
LC17	> 25	0

* 100% aggregation represents light transmission through PPP.

but ASA and RGDS in contrast had little effect on these two strains. EDTA (3.6 mM) completely inhibited aggregation by strain A198/89. Dipyridamole, apyrase and ASA caused slight inhibition while imipramine, quinacrine and RGDS had no effect.

Platelet aggregation by *L. rhamnosus* strains

The ability of *L. rhamnosus* strains to aggregate human platelets was tested for five strains isolated from IE patients and 16 laboratory strains (Table 1). All IE isolates aggregated platelets, with lag phases ranging from 6.3 to 14.0 min and final percentage aggregations ranging from 70% to 104%. Only 8/16 laboratory strains were active, with lag phases between 7.3–20.4 min and final percentage aggregations of 77–98%. It is noteworthy that two of the latter strains, GV-261-R and GV-261-F, which were reisolated from gnotobiotic rats infected with *L. rhamnosus* ATCC 7469 (R. J. Fitzgerald, personal communication), were able to aggregate platelets (Table 1), whereas the parent strain did not.

Subculturing strain A64/87 40 times in MRS broth had no observable effect on either the lag phase or the final percentage aggregation.

Platelet aggregation by *L. paracasei* subsp. *paracasei* strains

Two of the five IE isolates (strains A198/89 and A22/73) aggregated platelets compared with only 2/9 laboratory strains (Table 3). *L. paracasei* subsp. *paracasei* A198/89 also aggregated in PPP, indicating another interaction, possibly agglutination, apart from that with platelets.

Platelet aggregation by other oral species

Studies on strains representing five species found in the oral cavity, namely *L. acidophilus* (1), *L. fermentum* (2), *L. oris* (3), *L. plantarum* (5) and *L. salivarius* (3), showed that most (11/14) were able to aggregate platelets, with all the species being represented (Table 4).

The role of bacterial surface components in platelet aggregation

All the IE strains that aggregated platelets lost this ability on incubation with pronase for 1–1.5 h. The lag phase of *L. rhamnosus* A544/84 was linearly dependent on the length of treatment with pronase, increasing from 6.7 min with no treatment to 11.8 min after 60 min with pronase (not shown). Heating *L. rhamnosus* A64/87 for 1 h at 60 °C destroyed the aggregating ability. With *L. paracasei* subsp. *paracasei* A198/89 the effect of heating was less, 1 h at 60 °C increasing the lag period (0.3–5.9 min) but having no effect on the percentage aggregation (100–99%), and 2 h at 60 °C increasing the lag phase to 9.3 min and reducing the percentage aggregation to 82%.

Treatment of the IE-aggregating strains with 10 ml 0.1 M-Tris/HCl (pH 8.5) with shaking at 37 °C for 1 h also caused the loss of aggregating ability. Incubation of platelets with 200 µg of soluble protein extract from the

Table 4. Platelet aggregation by other oral *Lactobacillus* species

Values in parentheses represent the standard deviations.

Species	Strain	Lag phase (min)	Final aggregation (%)*
<i>L. acidophilus</i>	ATCC 4356	12.3 (1.9)	97.0 (18.6)
<i>L. fermentum</i>	LAM1†	> 25	0
	NCIB 6991	8.8 (2.4)	84.4 (14.2)
<i>L. oris</i>	NCFB 2160	10.2 (4.4)	87.6 (17.0)
	NCFB 2161	10.4 (4.7)	86.5 (11.0)
	NCFB 2164	7.1 (1.5)	103.1 (5.1)
<i>L. plantarum</i>	TH1†	9.1 (1.7)	89.6 (9.9)
	TH3†	9.9 (3.6)	100.0 (9.2)
	A1/68	> 25	0
	A17/74	6.2 (1.8)	102.5 (8.7)
	ATCC 14917	8.7 (2.8)	93.8 (4.8)
<i>L. salivarius</i>	CC1†	> 25	0
	ATCC 11741	6.7 (1.5)	90.0 (17.4)
	ATCC 11742	8.4 (3.2)	76.3 (13.3)

* 100 % aggregation represents light transmission through PPP.

† Freshly isolated from saliva.

homologous Tris extract completely inhibited aggregation by 8/9 strains, the exception being *L. paracasei* subsp. *paracasei* A198/89 (the lag period increased to 1.5 min and percentage aggregation decreased to 49%).

Examination of the effect of the Tris extract on aggregation by *L. rhamnosus* A64/87 showed that a

Fig. 2. SDS-PAGE of 0.1 M-Tris/HCl extracts. Each lane contained 50 µg protein extract. Lane 1, *L. paracasei* subsp. *paracasei* NCFB 151; lane 2, RO94; lane 3, H831; lane 4, DECP; lane 5, A357/84; lane 6, A198/89; lane 7, YS-88-6-641; lane 8, LC4; lane 9, LC11; lane 10, LC16; lane 11, LC17; lane 12, *L. rhamnosus* LR12; lane 13, LR13; lane 14, LR15; lane 15, A103/70; lane 16, A544/84; lane 17, A65/87; lane 18, A77/87; lane 19, molecular mass markers.

linear increase in the length of the lag phase before onset of aggregation occurred as the amount of extract was increased. The inhibitory effect of the extract also depended on the source of the platelets. The results for four donors tested showed that an increase in the lag phase by 50% required 11.5, 14.8, 33.3 or 115 µg of extract. However, the final percentage aggregation was not affected by the amount of extract added to the platelets.

Additional analysis of the extracts by SDS-PAGE confirmed that protein was removed from the bacterial cell surface but it was not possible to distinguish any individual protein bands that were absent from the non-aggregating strains (Fig. 2).

Discussion

The present study is the first report of the ability of lactobacilli to aggregate human platelets. This ability is indicated by the length of the initial lag phase and by the final percentage aggregation. The lag phase has been interpreted as representing either the time for platelet-bacteria binding to occur (Clawson & White, 1971; Herzberg & Brintzenhofe, 1983; Herzberg *et al.*, 1983*a, b*) or the time for the steps required for platelet activation following bacterial adhesion (Sullam *et al.*, 1987). It is not surprising, therefore, that in this study and those of others (Herzberg *et al.*, 1983*a*) the results for different donors show a wide divergence in the length of the lag phase. Platelet aggregation by Gram-positive bacteria has been studied in detail with strains of *Staphylococcus aureus*, *Streptococcus sanguis* and group B streptococci. The ratio of bacteria to platelets has mainly been shown to be optimal at 1:1, as was also shown in this study, although group B streptococci have variously been shown to aggregate optimally at ratios of 1.5:1 (Wood & Gray, 1986) and 4.3:1 (Usui *et al.*, 1987). Observation of the lag phase for 25 min has become standard for most studies, and the variations in the lag phase in this study are comparable to those found in a large study of different strains and donors (Herzberg *et al.*, 1983*a*).

The preliminary studies also confirmed that a true platelet aggregation was being observed as shown by the effect of inhibitors that either remove cations (EDTA and dipyrindamole), affect the availability of ADP (apyrase), inhibit cyclooxygenase (ASA and quinacrine) or affect membrane stability (imipramine) (Forrester *et al.*, 1985). The results were particularly pronounced for the *L. rhamnosus* strains where the inhibition of strains by the tetrapeptide RGDS also indicated the involvement of fibrinogen and/or fibronectin. This peptide is an analogue of the attachment site for fibronectin and has previously been reported to inhibit both platelet ag-

gregation (Gartner & Bennett, 1985) and fibrinogen binding to platelets. It is also relevant that fibronectin is bound significantly better by IE strains than laboratory strains in a low pH environment (Harty *et al.*, 1993), as is found in the pits, fissures and carious dentine of the oral cavity where lactobacilli are normally isolated.

Aggregation of platelets appears to be a common characteristic amongst the oral lactobacilli, with representatives amongst all of the seven species that were tested. Although the numbers tested for some of the species were small, it does seem that the property is not very common amongst strains of *L. paracasei* subsp. *paracasei*. Again, though numbers were small, the ability to aggregate platelets was found at a higher frequency among IE isolates, particularly *L. rhamnosus* strains. This is consistent with previous studies (Harty *et al.*, 1993) that have shown significant differences between IE and laboratory *L. rhamnosus* strains for other cell-surface properties, particularly hydrophobicity, hydroxyapatite adhesion, salivary aggregation and fibronectin-binding under acidic conditions.

Subculturing can lead to loss of a particular characteristic but all strains had only been minimally subcultured. Subculturing *L. rhamnosus* A64/87 40 times in MRS broth produced no change in aggregating ability or other surface properties (Harty *et al.*, 1993). It is interesting, however, that the two morphological mutants of *L. rhamnosus* ATCC 7469 which had been reisolated after passaging through gnotobiotic rats (R. J. Fitzgerald, personal communication) were able to aggregate platelets while their parent showed no such activity.

The component that mediates aggregation would seem to involve a heat-sensitive protein on the surface of the lactobacilli. The variation in the amount of protein extract needed to inhibit the aggregation of platelets from different donors further emphasizes the response of platelets from different donors when challenged with lactobacilli (Fig. 1). However, examination of SDS-PAGE gels of the proteins contained in the Tris extracts from aggregating and non-aggregating strains failed to indicate any bands that would be candidates for the platelet aggregation protein (Fig. 2).

In conclusion, the data indicate that the ability to aggregate human platelets is a normal attribute of the lactobacilli in the oral cavity. The relative scarcity of *Lactobacillus* isolates from endocarditis cases, however, must indicate that other factors are involved in pathogenesis; for example, it is well-documented that lactobacilli do not grow well in blood culture. However, the recent increase in bacteraemias caused by lactobacilli in cases where there has been long-term prescription of antibiotics or immunosuppression points to their potential pathogenicity.

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