BACTERIAL PATHOGENICITY

Interaction of *Yersinia enterocolitica* and *Y. pseudotuberculosis* with platelets

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Yersinia enterocolitica is a bacterium capable of growth at 4°C in donated blood and has been responsible for many deaths following transfusion. Interaction of *Y. enterocolitica* with blood cells is of interest in understanding the mechanisms of survival and growth in blood. The closely related organism *Y. pseudotuberculosis* is known to invade platelets and cause platelet aggregation by a mechanism that involves expression of the chromosomal *inv* gene. *Yersinia* isolates were made to express green fluorescent protein (GFP) and their interaction with platelets was studied by flow cytometry. *Y. enterocolitica* did not cause platelet aggregation or activation, not even when grown at 22°C to maximise *inv* expression. Attachment of *Y. enterocolitica* O:9 to platelets occurred with virulence plasmid-bearing (pYV⁺) strains grown at 37°C but not with pYV[−] strains nor with strains grown at 22°C. *Y. pseudotuberculosis* containing *inv* did cause platelet activation and aggregation when grown at 22°C, as has been shown before, but also showed enhanced attachment to platelets when grown at 37°C. Electron microscopy studies confirmed that *inv*-expressing *Y. pseudotuberculosis* invaded platelets but *Y. enterocolitica* attached only to the outer surface of platelets. Interaction of *Y. enterocolitica* O:9 with platelets provided a modest protection against bacterial killing by human serum. Interaction of *Y. enterocolitica* O:9 with platelets does not lead to platelet invasion or activation, and is mediated through plasmid-coded factors, not *inv*.

Introduction

The enteric pathogen *Yersinia enterocolitica* has been the leading cause of bacterial growth in stored donated blood for the past 15 years [1–3]. At least 21 cases of *Y. enterocolitica* transfusion-related sepsis, of which 12 resulted in death, have occurred in the USA since 1982 [4] and a similar or greater number of cases has been reported from other developed countries [3]. Growth of *Y. enterocolitica* in donated blood is associated with mild gastrointestinal illness and serological evidence of acute infection in the donor. Therefore, *Y. enterocolitica* contamination is thought to be the result of asymptomatic bacteraemia in the donor. *Y. enterocolitica* serotypes involved have included O:3, O:9, O:1,2,3; O:5,27 and O:20, but not O:8 [5].

The ability of *Y. enterocolitica* to grow at 4°C is presumed to be a critical factor in its ability to multiply

in donated blood. In addition, *Y. enterocolitica* has several temperature-regulated virulence factors that may be relevant. YadA is an adhesin encoded by the *Y. enterocolitica* virulence plasmid pYV [6]. YadA is expressed at 37°C and plays a role in adhesion to leucocytes and prevention of phagocytosis and killing by those cells [7]. Complement resistance involves both the virulence plasmid (pYV) [8, 9] and chromosomal factors [10], and is also best expressed at 37°C. Invasin, encoded by the chromosomal *inv* gene, is best expressed *in vitro* at temperatures below 30°C [11]. Invasin expression by *Y. enterocolitica* leads to invasion of epithelial cells and invasins in the closely related *Y. pseudotuberculosis* has been shown to cause invasion and aggregation of platelets [12]. The potential role of invasins of *Y. enterocolitica* in promoting interactions with blood cells in whole blood is unclear [13].

The interaction of *Y. enterocolitica* with donated blood is likely to be complex. The bacteria must cope with complement and immune cells in the host and with the drop in temperature to 4°C over *c.* 8 h [14] after the blood is collected. *Y. enterocolitica* is expected to be extracellular in the infected donor, as in the tissues of

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animal models [15–17], so that expression of complement resistance would be essential for survival of the organism in the donor blood before donation. Expression of *yadA* is likely to be important in the donor blood before donation in preventing phagocytosis [7]. Complement resistance is likely to be lost in a few hours after blood donation as the blood cools [18]. Cooling will cause down-regulation of *yadA* expression, and up-regulation of *inv* expression. The activity of complement and phagocytes will also diminish as the temperature drops.

Experimental studies of inoculation of *Y. enterocolitica* into fresh donated blood have been conducted to try to understand the mechanisms involved in bacterial survival and growth. These experiments have consistently shown that most of the organisms inoculated are killed rapidly by the action of complement [18, 19]. The few inoculated bacteria that survive may be protected from complement by being inside a cell. These bacteria then multiply to high numbers after *c.* 2 weeks. It has been suggested that bacteria survive inside leucocytes [20], but this has not been demonstrated directly, and platelets might also provide some protection.

The present study aimed to explore the possibility that platelets might be a site of survival of *Y. enterocolitica* in donated blood. *Y. pseudotuberculosis* has been found

to be associated with platelets in experimental infection [16]. *Y. enterocolitica* has also been observed to be associated with platelets in the peripheral blood of a patient with overwhelming *Y. enterocolitica* sepsis (A. P. Gibb, unpublished observation). *Y. pseudotuberculosis* can invade platelets through a mechanism that requires invasins expression [12]. As invasins expression and function are very similar in *Y. enterocolitica* and *Y. pseudotuberculosis* [21] it seemed likely that *Y. enterocolitica* might also invade platelets. This might occur as donated blood cooled and invasins expression increased. In these circumstances *Y. enterocolitica* might be protected from complement by the platelets. Therefore, the present study aimed to investigate the interaction of yersiniae with platelets, with green fluorescent protein (GFP)-expressing bacteria and flow cytometry as the primary tools.

Materials and methods

Bacterial strains and plasmids

All bacteria and plasmids used in this work are listed in Table 1 [12, 18, 22–27]. Plasmids were prepared and constructed by the alkaline lysis method [28] and standard recombinant DNA techniques [29]. Enzymes were purchased either from Bethesda Research Laboratories (Gaithersburg, MD, USA), or from New England Biolabs (Beverly, MA, USA). Bacteria were

Table 1. Bacterial strains and plasmids

Designation	Description or genotype	Reference or source
Bacteria		
3169	<i>Y. enterocolitica</i> serotype O:9, pYV ⁺ , from a case of transfusion-associated sepsis	18
3169L	Virulence plasmid ⁻ derivative of strain 3169	18
WA-314	<i>Y. enterocolitica</i> serotype O:8, with virulence plasmid (pYVO8) ⁺	23
YPIII	<i>Y. pseudotuberculosis</i> pYV ⁺	12
YPIIIc	<i>Y. pseudotuberculosis</i> pYV ⁻	12
YP202	<i>Y. pseudotuberculosis</i> pYV ⁻ , <i>inv</i> ⁻	12
UC310	Laboratory strain of <i>Y. enterocolitica</i> O:8	24
32815	Human enteric isolate of <i>Y. enterocolitica</i> serotype O:7,8, biotype 1A	*
21553	Human enteric isolate of <i>Y. enterocolitica</i> serotype O:7,8, biotype 1A	*
56167	Human enteric isolate of <i>Y. enterocolitica</i> serotype O:7,8, biotype 1A	*
9347	Human enteric isolate of <i>Y. enterocolitica</i> serotype not typable biotype 1A	*
7515	Human enteric isolate of <i>Y. enterocolitica</i> serotype O:9, biotype 1B	*
14175	Human enteric isolate of <i>Y. enterocolitica</i> serotype O:9, biotype 1B	*
66364	Human enteric isolate of <i>Y. enterocolitica</i> serotype O:3, biotype 4	*
64225	Human enteric isolate of <i>Y. enterocolitica</i> serotype O:3, biotype 4	*
61318	Human enteric isolate of <i>Y. enterocolitica</i> serotype O:3, biotype 4	*
116731	Human blood culture isolate of <i>Y. enterocolitica</i> , serotype O:3, biotype 4	*
3327	<i>Y. enterocolitica</i> from transfusion-associated sepsis, serotype O:5,27	25
ER1325	<i>Y. enterocolitica</i> , serotype O:8, pYV ⁺	†
ER2601	<i>Y. enterocolitica</i> , serotype O:8, pYV ⁺	†
JM109	<i>Escherichia coli</i> endA1, gyrA96, hsdR17(rk ⁻ mk ⁺), mcrB ⁺ , recA1, relA1, supE44, thi-1, Δ(lac-proAB), F'[traD36, proAB, lacIqZΔM15]	26
Plasmids		
GFP plasmids	pGFPM1, pGFPM2, pGFPM3	22
pUCP22	Broad host-range vector, lac promoter Gent ^R , Amp ^R	27
pYG1	pUCP22 with gfpM1	This study
pYG2	pUCP22 with gfpM2	This study
pYG3	pUCP22 with gfpM3	This study
pACYC 184-GFP	Broad host-range plasmid expressing GFP	‡

*Human isolates of *Y. enterocolitica* provided by the Provincial Laboratory for Public Health of Southern Alberta.

†Human isolates of *Y. enterocolitica* O:8 were provided by Dr Neal Denhollander, *Yersinia* Reference Laboratory, Toronto.

‡pACYC 184-GFP was a gift from Dr J. Mescas, Stanford University School of Medicine.

grown in low salt Luria Broth (LLB, Gibco BRL, Gaithersburg, MD, USA). Antibiotics were used at the following concentrations: *Escherichia coli* – ampicillin 100 mg/L, kanamycin 25 mg/L; for *Y. enterocolitica* – gentamicin 60 mg/L, kanamycin 25 mg/L. Congo red-magnesium oxalate agar medium [30] was used to confirm the presence of pYV⁺ in *Y. enterocolitica* and *Y. pseudotuberculosis* strains before each experiment.

GFP constructs

Three red-shifted *gfp* clones used in this study, M1, M2 and M3, were a gift from Brendan Cormack [22]. All three genes were cloned into the broad host range vector pUCP22 and expressed from the constitutive *lac* promoter. These three constructs, pYG1, pYG2, pYG3, and the vector control were moved into *Y. enterocolitica* by electroporation [31].

Bacterial preparation

After an initial incubation for 18 h at either 22°C or 37°C, bacteria were washed once and resuspended in calcium- and magnesium-free phosphate-buffered saline (PBS; Gibco BRL). Bacteria were counted with Flow-Count™ Fluorospheres (Beckman-Coulter, FL, USA) and adjusted to 1×10^{11} bacteria/L. To maximise expression of genes on the virulence plasmid, strains were cultured overnight at 22°C with appropriate antibiotics, diluted 1 in 50 in LLB containing 20 mM sodium oxalate and 20 mM magnesium chloride solution, incubated for 2 h at 37°C, washed and resuspended in PBS [18].

Preparation of human platelets

Citrated whole blood was collected from one of five subjects. All were tested and found to be negative for antibodies to *Y. enterocolitica* and *Y. pseudotuberculosis* (tests performed by Dr Neal Denhollander, *Yersinia* Reference Laboratory, Toronto, Canada). Platelet-rich plasma (PRP) was obtained from citrated whole blood by centrifugation at 300 g for 10 min (4°C). The PRP was washed and resuspended in calcium- and magnesium-free PBS, and platelets were counted with Flow-Count™ Fluorospheres and adjusted to 1×10^8 platelets/L. Platelet preparations were used within 2 h of collection.

Preparation of human serum

Whole blood samples from the five subjects described above were collected and allowed to coagulate for 15 min at 22°C. Serum was obtained by centrifugation of the coagulated blood at 500 g for 10 min (4°C) and was immediately frozen and stored at -70°C.

Flow cytometry

Unlabelled bacteria, GFP-labelled bacteria, unlabelled

platelets and platelets stained with CD42b-PC5 antibody (Beckman-Coulter) were analysed with a Coulter XL-MCL (Beckman-Coulter) flow cytometer to determine appropriate voltages and compensation settings for size (log forward scatter), granularity (log side scatter), green fluorescence and red fluorescence.

Platelet adhesion assay

Bacteria were grown overnight at either 22°C or 37°C in LLB with appropriate antibiotics, washed and resuspended in PBS. Then 1×10^6 platelets and 5×10^6 bacteria were combined in PBS to a total volume of 100 µl. Tests were incubated at either 22°C or 37°C for 1 h. After incubation, platelets were stained with CD42b-PC5 for 10 min at 22°C and then diluted to a total volume of 1 ml with PBS for immediate analysis by flow cytometry. A minimum of 5000 platelet events was analysed per sample. Two parameter fluorescent histograms (CD42b-PC5 versus GFP) were generated for analysis, gated on either GFP-positive or CD42b-positive events. The percentage of platelets co-staining with GFP and the percentage of bacteria co-staining with CD42b were recorded.

Platelet aggregation assay

Platelet preparation and aggregation assay followed standard methods [32] with 230 µl of PRP incubated with a 60-µl suspension containing 2×10^7 bacteria (resulting in a platelet to bacteria ratio of 1:3) in a John's Scientific Platelet Aggregation Profiler, Model PAP-4 (BIO/DATA Corporation, Hatboro, PA, USA).

Platelet activation assay by flow cytometry

Bacterial strains were grown overnight at 22°C or 37°C, then 5×10^6 bacteria and 1×10^6 platelets were incubated in 100 µl of PBS for 1 h at either 22°C or 37°C. Platelets were stained for 15 min at 22°C with CD42b-PC5 and either CD62P-PE, a marker for platelet activation [33], or mouse immunoglobulin-PE (Beckman-Coulter), an isotype control antibody. At least 5000 platelet events were analysed per sample. Two parameter fluorescent histograms gated on CD42b-positive events were generated for analysis (CD42b-PC5 versus mouse immunoglobulin and CD42b-PC5 versus CD62P-PE). Platelet activation was determined by subtracting the percentage of platelets staining with mouse immunoglobulin from the percentage of platelets staining with CD62P.

Platelet protection assay by culture

Platelet and bacterial preparations were performed as described in the flow cytometry experiments. Tests were performed in triplicate; 10×10^6 bacteria and 2×10^6 platelets were used in each test. A control was made with 10×10^6 bacteria without platelets. Bacteria with or without platelets were incubated for 1 h at

37°C. Half of the sample was removed, treated with Triton X-100 1% for 15 min at 22°C, serially diluted and triplicate 100- μ l samples were spread on LLB agar and incubated for 36 h at 28°C. Serum was added to the remaining sample to a concentration of 30% and incubated at 37°C for 2 h. The samples were then treated with Triton X-100 1% for 15 min at 22°C and plate counts were performed as above. Survival in serum was determined by comparing the number of colonies that survived the serum treatment with the number of colonies in the same samples before serum treatment. A platelet protection ratio was calculated as (survival of bacteria pre-incubated with platelets)/(survival of bacteria without platelets).

Platelet protection assay by flow cytometry

Tests were performed in triplicate with platelets and serum from three subjects. Each test consisted of 30×10^6 washed platelets and 3×10^6 washed bacteria with PBS added to a total volume of 300 μ l. Washed bacteria without platelets were used as a control. After incubation at 37°C for 1 h, 100 μ l were removed and stained for 10 min at 22°C with CD42b, diluted with 1 ml of PBS and 100 μ l of Flow-Count™ Fluorospheres and analysed on the flow cytometer as described in the platelet adhesion assay. The percentage of bacteria co-staining with CD42b and the total number of GFP events were recorded. The remaining 200 μ l were added to 300 μ l of thawed fresh-frozen serum from the platelet donor and 500 μ l of PBS with Mg^{2+} and Ca^{2+} for a total volume of 1 ml containing serum 30%. After incubation for 2 h at 37°C, 500 μ l were diluted in PBS with Triton X-100 1% to a volume of 2 ml and incubated for 15 min at 22°C. Flow-Count™ Fluorospheres were added and the number of GFP events was counted on the flow cytometer. Bacterial survival was defined as the number of GFP events found divided by the number in a serum-free control.

Electron microscopy

Human cells and bacteria were centrifuged at 800 g and the supernate was removed. The pellet was fixed for 2 h at 20°C in Karnovsky's fixative with the addition of ruthenium hexamine trichloride (Polysciences) 0.7%. After fixation, the pellet was rinsed in cacodylate buffer 0.1% containing 5 mM calcium (pH 7.4). The pellet was centrifuged and post-fixed in osmium tetroxide 1% for 1 h at 20°C. The fixative was removed and the pellet was dehydrated in graded alcohols, infiltrated and embedded in Spurr's resin. Thin sections (80–100 nm) were cut with a diamond knife on a Reichert OM-U3 ultra-microtome, mounted on grids, stained with uranyl acetate and lead nitrate, and examined with a Hitachi H-7000 electron microscope operated at 75 kV.

Results

pYG plasmid as an effective bacterial label for flow cytometry

Y. enterocolitica strains UC310, 3169 and 3169L carrying pUCP22, pYG1, pYG2 or pYG3 were analysed with a flow cytometer. pYG3 was determined to have the brightest fluorescence and was chosen for subsequent experiments. Evaluation by flow cytometry demonstrated that GFP-labelled bacteria could be resolved from CD42b-PC5-stained platelets (Fig. 1) and Flow-Count™ Fluorospheres by fluorescence and size parameters. Furthermore, it was shown that the GFP signal was stable in the presence of heat-treated serum but was lost in fresh serum at a concentration similar to that which caused bacterial killing and could, therefore, be used as a measure of complement action (Fig. 2). The loss of GFP signal was interpreted as leakage of GFP protein following membrane disruption.

Effect of temperature and virulence plasmid (pYV) on the interaction of *Y. enterocolitica* with purified human platelets

When pYG3⁺, pYV⁺ *Y. enterocolitica* strain 3169 was grown at 37°C and incubated with platelets at 37°C, 93% of platelets were associated with GFP signal (Fig. 3). In contrast, when pYG3⁺ strain 3169 was grown and incubated at 22°C, only 11.5% of platelets were associated with bacteria. The pYV⁻ *Y. enterocolitica* strain 3169L associated with <10% of platelets at either 22°C or 37°C and similar low results were seen with the *E. coli* control strain. This clearly shows that

Fig. 1. Flow cytometric analysis of the interaction of *Y. enterocolitica* 3169 (pYG3) with platelets at 37°C. Ungated histogram showing interaction of *Y. enterocolitica* 3169 (pYG3) with platelets at 37°C. Region 1 contains fluorescent bacteria (GFP events). Region 2 contains CD42b-PC5-labelled platelets co-staining with GFP. Region 3 contains RBC debris and non-fluorescent bacteria. Region 4 contains CD42b-PC5-labelled platelets.

Fig. 2. Complement activity assay by flow cytometry. *Y. enterocolitica* 3169L expressing GFP was grown overnight at 37°C; 1×10^6 bacteria were added to serial dilutions of normal (□) or heat-treated (■) serum and incubated for 2 h at 37°C. Heat-treated serum was incubated at 56°C for 30 min. After incubation, 100 μ l of Flow-Count™ Fluorospheres were added and the number of GFP events was counted.

Fig. 3. Association of GFP-expressing bacteria with platelets. Strains were grown and incubated at 22°C (□) and 37°C (▨) to assess the role of plasmid or chromosomally encoded genes in platelet association. *E. coli* strain JM109 was used as a negative control for platelet association. Association was determined by dual staining of platelets with CD42b-PC5 and GFP. Results are an average of five replicates conducted on three different blood donors. Platelets were incubated with bacteria for 60 min at a bacteria to platelet ratio of 5:1. Representative results for *Y. enterocolitica* serotypes O:3, O:8 and O:9 are shown, together with *Y. pseudotuberculosis* (YP) strains.

one or more genes carried by the plasmid and induced at 37°C is responsible for the platelet association.

Further platelet association tests were performed with other pYV⁺ *Y. enterocolitica* strains of serotypes O:3, O:8 and O:9. Both of two additional O:9 strains tested associated with platelets at 37°C but not at 22°C. The O:8 strain and the O:3 strains did not associate with platelets at either temperature (Fig. 3 and Table 2).

The observation of a pYV-dependent interaction between *Y. enterocolitica* O:9 and platelets in flow

cytometry was very different from the interaction of *Y. pseudotuberculosis* with platelets in the aggregometer system in which invasins expression plays a key role [12]. Therefore, the interaction of platelets with *Y. pseudotuberculosis* in the flow system and the interaction of platelets with *Y. enterocolitica* in the aggregometer were investigated to determine whether the differences observed were related to methodology or bacterial factors.

Interaction between Y. pseudotuberculosis and platelets in the flow system

Y. pseudotuberculosis strains were made to express GFP by insertion of the pACYC 184-GFP, and their interaction with platelets was analysed by flow cytometry (Fig. 3). A more complex picture was observed than had been seen with *Y. enterocolitica*. The pYV⁻ strains YPIIIc and YP202 both associated with c. 20% of platelets at 37°C. Association was reduced to background levels at 22°C in the *inv*⁻ strain YP202, but increased to c. 50% in the isogenic *inv*⁺ strain. The pYV⁺ strain YPIII showed greater platelet association than strain YPIIIc at 37°C but less association at 22°C. These results suggest a role for a chromosomal adhesin expressed at 37°C such as *psa* [34], as well as invasins and a plasmid factor expressed at 37°C.

Platelet aggregation by Y. pseudotuberculosis and Y. enterocolitica

The present study was able to replicate the key observations obtained by Simonet *et al.* [12] in the aggregometer system. Platelet aggregation by *Y. pseudotuberculosis* occurred only when the organisms were grown at low temperature, was independent of the presence of the virulence plasmid and did not occur with the *inv*⁻ strain (Table 2). Platelet aggregation by *Y. enterocolitica* strains 3169 and 3169L was then examined under identical conditions. No platelet aggregation was observed at any of the growth temperatures. As it was possible that strain 3169 was defective in some aspect of invasins expression or function, 12 other unrelated *Y. enterocolitica* strains of various serotypes (O:3, O:9, O:7,8 and O:8) were tested and none of them produced platelet aggregation in the aggregometer system.

Platelet activation by Yersinia

The findings that *Y. enterocolitica* O:9 associated with platelets in the flow system but did not cause aggregation and that *Y. pseudotuberculosis* YPIII adhered to platelets at 37°C but did not cause aggregation at that temperature suggest that adherence can occur without platelet activation. Platelet activation was examined by flow cytometry with an antibody to the platelet protein CD62P, a marker of platelet activation [33]. When grown at 22°C, strains YPIII and YPIIIc

Table 2. Summary of interactions of *Yersinia* with platelets

Strain no.	Serotype	Temperature	Platelet association	Platelet aggregation	CD62P expression
3169	O:9	37°C	+++	—	—
		22°C	—	—	—
3169L	O:9	37°C	—	—	—
		22°C	—	—	—
7515	O:9	37°C	+++	—	ND
		22°C	—	—	ND
11475	O:9	37°C	+++	—	ND
		22°C	—	—	ND
YPIII		37°C	++	—	—
		22°C	+	+++	+++
YPIIIc		37°C	+	—	—
		22°C	++	+++	+++
YP202		37°C	+	—	—
		22°C	—	—	—
WA-314	O:8	37°C	—	ND	ND
		22°C	—	ND	ND
ER1325	O:8	37°C	—	ND	ND
		22°C	—	ND	ND
ER2601	O:8	37°C	—	ND	ND
		22°C	—	ND	ND
61318	O:3	37°C	—	—	ND
		22°C	—	—	ND
64225	O:3	37°C	—	—	ND
		22°C	—	—	ND
66364	O:3	37°C	—	—	ND
		22°C	—	—	ND
116731	O:3	37°C	—	—	ND
		22°C	—	—	ND
3327	O:5,27	37°C	ND	—	ND
		22°C	ND	—	ND
32815	O:7,8	37°C	ND	—	ND
		22°C	ND	—	ND
21553	O:7,8	37°C	ND	—	ND
		22°C	ND	—	ND
56167	O:7,8	37°C	ND	—	ND
		22°C	ND	—	ND
9347	NT	37°C	ND	—	ND
		22°C	ND	—	ND

ND, not done.

were able to activate platelets readily. In contrast, strain YP202 and *Y. enterocolitica* strains 3169 and 3169L did not activate platelets (Fig. 4). No platelet activation was observed if bacteria were grown overnight at 37°C despite association of the bacteria with platelets (Table 2 and Fig. 4).

Physical interaction of Y. pseudotuberculosis and Y. enterocolitica with platelets

EM studies with ruthenium red staining to distinguish vesicles from invaginations confirmed that invasively expressing *Y. pseudotuberculosis* invaded platelets and was found inside vesicles which were not accessible to ruthenium red staining [12] (Fig. 5a). *Y. enterocolitica* did not invade platelets in this way, but was seen tightly associated with them, often appearing completely 'wrapped up' by a number of platelets (Fig. 5b).

Platelets and complement survival

The tight association of *Y. enterocolitica* O:9 with platelets might offer protection against complement, increasing the survival of *Y. enterocolitica* in blood

products. This possibility was examined in conventional culture and by flow cytometry. In conventional culture assay in individual experiments with wild-type pYG⁻ strain 3169, platelet protection ratios of 3.2, 14.5 and 27.5, respectively, were obtained in platelets from three different donors. In the flow cytometry assay, association with platelets increased the survival of *Y. enterocolitica* (as determined by preservation of GFP signal) by approximately two-fold (Fig. 6).

Discussion

Flow cytometry with GFP-expressing bacteria is a useful way of investigating the interaction of bacteria with cells. The method is particularly useful for platelets because their small size and instability would make analysis in other systems very difficult. The method has the advantage that large numbers of observations can be made quickly and the results can be captured in electronic form with minimal manipulations.

Y. enterocolitica and *Y. pseudotuberculosis* interact

Fig. 4. Platelet activation by *Y. enterocolitica* and *Y. pseudotuberculosis*. Platelets were incubated with bacteria for 60 min at a bacteria to platelet ratio of 5:1. Activation was determined by dual staining of platelets with CD42b-PC5 and CD62P. (a) *Y. enterocolitica* strains grown and incubated at 37°C, conditions which promote platelet association. No increased CD62P expression was observed in platelets interacting with *Y. enterocolitica* strains. (b) *Y. pseudotuberculosis* strains grown and incubated at 22°C, conditions which promote platelet association and aggregation. High levels of CD62P expression were observed with platelets interacting with the invasin-positive strains YPIII and YPIIIc; no increase was observed in the invasin-negative strain YP202.

Fig. 5. Electron microscopy of platelets and *Yersinia* spp. (a) *Y. enterocolitica* 3169 grown at 37°C (Ye) or (b) *Y. pseudotuberculosis* YPIII grown 22°C (Yp) were incubated with platelets (P) at growth temperature for 1 h then fixed and stained with ruthenium red as described in *Materials and methods*. Ruthenium red (RR) stains surface membranes and invaginations but does not stain vesicles cut off from the cell surface. Bar, 1 μ m.

differently with platelets. Association of *Y. enterocolitica* O:9 with platelets, as seen in flow cytometry, appeared to be greatly enhanced by the expression of virulence plasmid genes at 37°C but did not lead to

platelet activation. *Y. pseudotuberculosis* had a more complex pattern of association with platelets in the flow cytometry system with a possible role for one or more thermo-inducible chromosomal factors (perhaps

Fig. 6. Effect of platelets on bacterial survival in human serum. Bacterial strains containing green fluorescent protein (GFP) were incubated with (■) or without (□) platelets for 2 h in serum 30% at 37°C. After incubation, Triton X-100 was added and the tests were analysed by flow cytometry. Survival was defined as the number of GFP events found after lysis with Triton X-100 divided by the number of GFP events in a control with no serum.

psa [34]) as well as *inv* and virulence plasmid genes. Activation of platelets, as seen in the aggregometer and by CD62P expression in flow cytometry, was seen only with *Y. pseudotuberculosis* and was seen only in the strains containing *inv* and grown at low temperature.

It has been demonstrated clearly by Simonet *et al.* [12] that activation of platelets by *Y. pseudotuberculosis* is due to interaction of invasins with β 1-integrin on the platelet membrane. As *Y. enterocolitica* also has an *inv* gene, which is very similar in its binding domains to that of *Y. pseudotuberculosis* [21], it is surprising that an equivalent effect was not seen with any of the *Y. enterocolitica* strains tested. However, recent work has shown that the invasins of *Y. pseudotuberculosis* can self-associate and that the region responsible for this is missing from the *Y. enterocolitica* *inv* gene [35]. Deletion of this self-associating region reduces uptake of *Y. pseudotuberculosis* into cultured cells, probably because self-association is required for clustering of the integrin receptor in the mammalian cells. This absence of the self-associating region in *Y. enterocolitica* and the consequent failure to cluster integrin receptors may explain the failure of *Y. enterocolitica* strains to activate platelets.

The association of *Y. enterocolitica* serotype O:9 with platelets is dependent on the presence of the virulence plasmid and growth at 37°C. This indicates the involvement of a temperature-dependent plasmid-encoded protein. YadA, the best studied adhesin on the *Y. enterocolitica* virulence plasmid [6], seems the most likely candidate. However, pYV⁺ O:3 strains and the well characterised pYV⁺ O:8 strain 314 did not bind to platelets. YadA from serotypes O:3 [36] and O:9 (M. Iriarte, C. Kerbouch and G. R. Cornelis, GenBank accession no. AF056092) are only 97% identical in amino acid sequence, whereas YadA of serotype O:8 has two deletions compared to O:3 or O:9 [36]. Thus, the difference in behaviour may be explained by

differences on YadA. Alternatively, the adhesin may be another protein that is found on the virulence plasmid of *Y. enterocolitica* serotype O:9 but not O:8 or O:3. The virulence plasmids are known to be conserved in size within serotypes but show differences in size and sequence homology between serotypes [37], so they may possess distinct adhesins.

These findings are also consistent with the observation that pYV⁺ *Y. pseudotuberculosis* in mouse tissues is extracellular and associated with platelets [16]. There was an inconsistency between the latter observation and the subsequent in-vitro demonstration of aggregation and invasion of platelets by a mechanism that involved invasins [12] that can now be easily explained. The failure of the in-vitro study to detect a pYV-dependent mechanism of interaction between *Y. pseudotuberculosis* and platelets occurred because the aggregometer assay used was not able to detect interaction without activation.

The findings of the present study are significant for blood transfusion because they help define the possible mechanism by which *Y. enterocolitica* survives and multiplies in blood and they allow some optimistic predictions to be made about the effect of leukodepletion by filtration on *Y. enterocolitica* O:9. Previous experiments with *Y. enterocolitica* O:9 came to the conclusion that there must be a protected site in blood where the bacterium can survive until sufficient cooling has occurred to stop complement activity [18]. The present findings support the possibility that platelets may provide that protected site. The degree of protection that the study was able to demonstrate was not dramatic, but it is not known how much protection is required to allow *Y. enterocolitica* to survive in some donations, nor is it known how the natural history may differ from these experiments. Further experiments are required to elucidate the relative roles of platelets and leucocytes. Future studies should also address the finding of differences in interaction with blood elements by different serotypes of *Y. enterocolitica*.

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