Bovine platelet aggregation by *Fusobacterium necrophorum*

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Summary. *Fusobacterium necrophorum* aggregated bovine platelets. The aggregation was paralleled by the haemagglutinating ability of the organism. Treatment of the bacterial cells with antiserum to the homologous purified haemagglutinin reduced the degree of platelet aggregation. Scanning electronmicroscopy revealed that little lysis of the affected platelets occurred during the 1-h incubation period. Purified haemagglutinin became bound to the surfaces of the platelet cells as shown by immunofluorescence microscopy. These observations suggest that platelet aggregation is mediated by the haemagglutinin and may be related to the pathogenicity of the bacterium.

Introduction

The ability to induce platelet aggregation is associated with virulence in some micro-organisms which induce thrombocytopenia, disseminated intravascular coagulation, and other coagulative effects (Kurpiewski *et al.*, 1983). Microbial components such as lipopolysaccharides (Morrison *et al.*, 1978), tuberculin (Rourke *et al.*, 1979), candida mannan (Maisch and Calderone, 1981), and peptidoglycan (Greenblatt *et al.*, 1978) are known to contribute to platelet aggregation.

Fusobacterium necrophorum, a pathogen commonly found in animals, adheres to bovine ruminal epithelial cells (Kanoë and Iwaki, 1987). It is suspected that the bacterium sometimes penetrates into the venous system and then infects the visceral organs (Kanoë *et al.*, 1984). A recent study (Forrester *et al.*, 1985) revealed that bovine isolates of *F. necrophorum* could aggregate human platelets in platelet-rich plasma. Little information, however, is available about whether the bacterium causes aggregation of bovine platelets and what bacterial component is responsible for the bacterium-platelet interaction. In this paper, we report aggregation of bovine platelets by *F. necrophorum* and the role played by the bacterial haemagglutinin (HG).

Materials and methods

Bacterial strains and culture

F. necrophorum strains employed in this study were VPI 2891, 118, 130, and 410, kindly supplied by Dr

Shinjo, Miyazaki University, Japan and S-45 from Dr Miwatani, Osaka University, Japan. As described previously (Nagai *et al.*, 1984), the organisms were cultured at 37°C for 24 h in modified heart infusion broth supplemented with cysteine monohydrochloride 0.1% w/v and ascorbic acid 0.1% w/v, collected by centrifugation at 8000 *g* for 20 min, washed three times with phosphate-buffered saline (PBS, pH 7.2) and resuspended in PBS to a density of 10⁷ cells/ml.

Platelets

Bovine platelets were prepared by the method of Dausset *et al.* (1960). Nine volumes of heparinised bovine fresh blood were mixed with one volume of ethylenediaminetetraacetic acid disodium salt (EDTA) 5% w/v. The mixture was centrifuged at 1000 *g* for 10 min at 4°C. The platelet-rich plasma was collected and centrifuged at 3000 *g* for 30 min. The pellet was washed three times with a solution containing four volumes of Na₂HPO₄ 0.94% w/v in saline, one volume of KH₂PO₄ 0.9% w/v in saline, 20 volumes of saline and EDTA at a final concentration of 0.33% w/v. The platelets were then resuspended in saline to a concentration of 10⁶ cells/ml.

Platelet aggregation

The platelet suspension (0.5 ml) and the bacterial suspension (0.5 ml) were mixed in a siliconised tube and incubated at 37°C for 1 h in a water bath. One drop of the mixture was smeared over a glass slide, covered with a cover glass, and examined by phase contrast microscopy (×600). Usually, three objective fields per slide were observed. Percentage aggregation was expressed as (number of aggregated platelets/total number of platelets) × 100.

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Haemagglutinin (HG) and HG antiserum

F. necrophorum HG was prepared according to the method of Nagai *et al.* (1984). Briefly, 5 ml of the bacterial cell suspension (10^7 cells/ml) containing trypsin (Difco, Detroit) 0.1% w/v was held at 37°C for 1 h. The trypsin was removed by washing twice with a mixture of 0.15 M NaCl and 10 mM Tris-HCl buffer at pH 7.0 (TBS). The cells were resuspended in TBS supplemented with cysteine monohydrochloride 2% w/v and ascorbic acid 2% w/v, incubated at 37°C for 1 h, sonicated with a super-sonic vibrator (VR 150P, Tomy Seiko, Tokyo) and then centrifuged at 10 000 *g* for 30 min at 4°C. The supernate was filtered through a membrane filter (pore size, 0.6 µm) and purified by Sephadex G-100 (Pharmacia, Uppsala) column chromatography with TBS. Purity of the fraction was confirmed by polyacrylamide gel electrophoresis and agar gel double immunodiffusion tests. The HG preparation had a protein content of 140 µg/ml. HG antiserum was prepared in rabbits by four intramuscular injections, at weekly intervals, of 1 ml of a mixture containing the HG emulsified with an equal volume of Freund's complete adjuvant. Sera were collected one week after the last injection and stored at -20°C.

Haemagglutination test

As described previously (Nagai *et al.*, 1984), the bacterial suspension (50 µl) was diluted serially in doubling dilutions in the wells of a plastic microtitration tray. An equal volume of chicken red blood cell suspension, 1% v/v, was added to each well. After incubation at 37°C for 30 min and room temperature for 1 h, the test was read. Haemagglutination titre was estimated as the highest dilution that showed complete reaction.

Inhibition of platelet aggregation

Bacterial suspension (0.8 ml) was mixed with 0.2 ml of each of three dilutions of homologous HG antiserum (1 in 5, 1 in 10, and 1 in 50 in PBS), allowed to stand at 37°C for 1 h and then washed twice with PBS at 8000 *g* for 20 min. The bacterial cells were resuspended in PBS to a density of 10^7 cells/ml. Subsequently the bacterial suspension was mixed with an equal volume of the platelet suspension and incubated at 37°C for 1 h. One drop of the mixture was smeared over a glass slide and examined by phase contrast microscopy. Percentage inhibition was calculated from the following formula:

$$\left[\frac{\text{Number of platelets aggregated by antiserum-treated bacteria} - \text{control}}{\text{Number of platelets aggregated by untreated bacteria (control)}} \right] \times 100$$

Immunofluorescence

The platelet suspension (0.5 ml) was mixed with the HG (0.5 ml; protein content, 576 µg/ml), left to stand at

37°C for 1 h, washed twice with PBS, resuspended in a small volume of PBS, and then smeared on a glass slide. The smear was dried, fixed with acetone for 15 min and exposed to a 1 in 10 dilution of homologous HG antiserum at 37°C for 30 min in a moist chamber. After washing with PBS it was exposed to a 1 in 20 dilution of fluorescein isothiocyanate-conjugated goat antiserum to rabbit IgG at 37°C for 1 h. After washing, the platelets were examined by fluorescence microscopy. As a control, PBS was employed instead of the HG.

Scanning electron microscopy

Bacterial suspension (0.5 ml, 10^7 cells/ml) and platelet suspension (0.5 ml, 10^6 cells/ml) were mixed and incubated at 37°C for 1 h. One drop of the mixture was smeared on a glass slide, fixed in glutaraldehyde 2% w/v in 0.1 M cacodylate buffer, pH 7.2, dehydrated for 10-min intervals in a series of ethanol preparations (50, 70, 80, 90, 95, 99 and 100%), and dried by a critical point drying apparatus (JEOL IFC-1100, Japan Electron Optics, Tokyo). After a gold evaporation step, specimens were examined with a scanning electron microscope (JSM-25S III, Japan Electron Optics, Tokyo) at an operating voltage of 25 kV.

Statistical analysis

Statistical significance between means was estimated by Student's *t*-test.

Results

Platelet aggregation and haemagglutination

All the five strains of *F. necrophorum* aggregated bovine platelets and three of them possessed ability to agglutinate chicken red cells. The aggregation of platelets usually began within 10 min and required about 40–60 min to reach a maximum value. As shown in fig. 1a and table I, strain VPI 2891 had high platelet aggregating and high haemagglutinating activities and this strain was used in subsequent tests.

Scanning electron microscopy

F. necrophorum aggregated bovine platelets with little lysis (fig. 2a). No differences were observed between the cell surfaces of the aggregated platelets and those of the unaggregated cells (fig. 2b).

Immunofluorescence

Fluorescence specific for the HG was observed on the surface of bovine platelets exposed to a purified HG preparation (fig. 3a). Little fluores-

Fig. 1. Phase contrast micrographs of bovine platelets with (a) and without (b) *F. necrophorum* VPI 2891. Bar = 10 μ m.

cence was evident with control platelets, exposed to PBS alone and then treated with fluorescein-labelled antiserum (fig. 3b).

Aggregation inhibition

Platelet aggregation by the *F. necrophorum* cell suspension was strongly inhibited by pretreatment of the bacteria with the homologous HG antiserum

in a dose-dependent manner. It was also inhibited slightly by normal rabbit serum, but the inhibitory effect was weaker than that of the HG antiserum (table II).

Discussion

Recently, attention has been paid to the pathogenic role of platelet aggregation by *F. necrophorum* in the establishment of human and animal necrobacillosis (Forrester *et al.*, 1985). In this study we observed that bovine platelets were aggregated by *F. necrophorum* *in vitro* but aggregation was slightly slower than that reported for human platelets. It is well known that biovar A of the species demonstrates a high haemagglutinating ability with chicken red blood cells and is considered to be more virulent for laboratory animals, whereas biovar B shows little or no haemagglutinating ability and is thought to be less virulent (Langworth, 1977). Moreover, a recent study revealed that the HG of the biovar A strain (VPI 2891) mediates bacterial adherence to bovine ruminal epithelial cells (Kanoë and Iwaki, 1987). In this study, the biovar A strain (VPI 2891) showed strong platelet-aggregating

Table I. Platelet aggregation and haemagglutinating ability of *F. necrophorum* strains

<i>F. necrophorum</i> strain	Biovar	Percentage aggregation (SD)*	Haemagglutination titre
VPI 2891	A	73.2 (3.0)	128
S-45	B	1.0 (0.1)†	0
410	B	7.5 (2.4)†	0
130	B	19.1 (2.1)†	8
118	B	20.6 (5.5)†	8

* SD = standard deviation of triplicate determinations.

† Significantly different ($p < 0.01$) from the value for strain VPI 2891.

Fig. 2. Scanning electronmicrographs of bovine platelets with (a) and without (b) *F. necrophorum* VPI 2891. Bar = 10 μ m.

Fig. 3. Indirect immunofluorescence staining of bovine platelets treated with (a) the HG of *F. necrophorum*, and (b) PBS. Bar = 20 μ m.

Table II. Inhibition of fusobacterial platelet aggregation by homologous haemagglutinin antiserum

Antiserum	Antiserum dilution factor	Number of aggregated platelets (SD)*	Percentage inhibition of aggregation by HG antiserum
Control (none)	...	75.1 (4.4)	0
<i>F. necrophorum</i> haemagglutinin antiserum	5	26.9 (2.5)†	64
	10	28.8 (7.5)†	62
	50	40.6 (2.5)†	46
Normal rabbit serum	5	53.8 (3.1)†	28
	10	61.2 (5.0)†	19

* SD, standard deviation of triplicate determinations.

† Significantly different ($p < 0.01$) from the control.

activity, whereas the other strains were less effective. These results suggest that *F. necrophorum*-

stimulated platelet aggregation may also be associated with the virulence of the strain.

Scanning electronmicroscopy revealed that platelet lysis did not occur. Platelet aggregation without lysis has also been observed with *Listeria monocytogenes* (Czuprynski and Balish, 1981) and *Streptococcus pyogenes* (Kurpiewski *et al.*, 1983). Fusobacterial platelet aggregation was reduced when the bacteria were pretreated with the HG antiserum. Moreover, immunofluorescence studies demonstrated that the HG readily became bound to the platelet cell membranes. These observations suggest that bovine platelet aggregation by *F. necrophorum* is mediated by the bacterial HG.

It is thought that the bacteria colonise bovine ruminal epithelium, sometimes penetrating into the venous system and then infecting the abdominal tissues (Kanoe *et al.*, 1984). The data presented here indicate that *F. necrophorum*-induced platelet aggregation could probably participate in the initial stage of the abscess formation during an infection with the bacterium.

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