Alterations in Bovine Platelet Function and Acute Phase Proteins Induced by *Pasteurella haemolytica* A1

Lynn A. Cheryk, Kathleen E. Hooper-McGrevy, and Patricia A. Gentry

ABSTRACT

Platelet function was assessed by aggregometry in 10 Holstein calves before and after exposure to *Pasteurella haemolytica* (biotype A, serotype 1) by intrabronchial challenge. At 24 h after exposure the platelets had become more reactive to stimulation with known platelet agonists such as adenosine diphosphate (ADP) and platelet-activating factor (PAF) and the platelet aggregates that formed were more resistant to disaggregation. The activation of platelets was an early response in the challenged calves as platelet function had returned to pretreatment levels 72 h after exposure to the bacteria while the acute phase reactant proteins, haptoglobin and fibrinogen, were approaching their peak values and α_2-macroglobulin levels had also risen significantly ($P < 0.05$) at this time. The plasma levels of these proteins were still elevated and albumin levels were depressed 6 d post-treatment. At post-mortem all calves exhibited pneumonic tissue damage.

When *P. haemolytica* leukotoxin was added directly to bovine platelet suspensions both spontaneous aggregation and an increase in the aggregation response to ADP and PAF stimulation were observed. The morphological appearance of the platelet aggregates exhibited the typical pattern for bovine platelets with 2 distinct zones of cells being visible within each aggregate. One zone contained platelets in which the cytoplasmic granules were still evident and the other zone contained irregularly shaped platelets devoid of granular content. In the latter zone, discrete gaps, or pores, were evident in the plasma membrane of numerous platelets. This pore formation is characteristic of leukotoxin action and is not observed in ADP or PAF induced aggregates.

RÉSUMÉ

L'activité des plaquettes fut évaluée par étude de leur aggrégation chez 10 veaux de race Holstein avant et après une inoculation par voie intrabronchique de *Pasteurella haemolytica* (biotype A, sérotype 1). Vingt-quatre heures après l'exposition les plaquettes étaient plus réactives à une stimulation par des agonistes connus des plaquettes tel l'adénosine diphosphate (ADP) et le facteur d'activation des plaquettes (PAF), et les aggrégats de plaquettes formés étaient plus résistants à la désaggrégation. L'aggrégation plaquettaire était une réponse hâtive chez les veaux suite à l'inoculation étant donné que la fonctionalité des plaquettes revint aux niveaux prétraitement dans les 72 h suivant l'exposition à la bactérie. Les protéines réactives de la phase aigüe, l'haptoglobine et le fibrinogène, approchaient leurs valeurs maximales et les niveaux de macroglobulines-α_2 étaient également augmentés de façon significative ($P < 0,05$) après 72 h. Les niveaux plasmatiques de ces protéines étaient toujours élevés et les niveaux d'albumine étaient diminués au jour 6 post-exposition. Lors de l'examen post-mortem tous les veaux montraient des lésions de pneumonie. Lorsque la leucotoxine de *P. haemolytica* fut ajoutée directement à des suspensions de plaquettes bovines une aggrégation spontanée et une augmentation de la réponse d'aggrégation à une stimulation par l'ADP et au PAF furent observées. La morphologie des aggrégats de plaquettes avait l,apparence typique des plaquettes bovines avec 2 zones distinctes de cellules visibles à l'intérieur de chacun des aggrégats. Une zone contenant des plaquettes à l'intérieur desquelles les granules cytoplasmiques sont toujours évidentes, alors que l'autre zone contient des plaquettes de formes irrégulières dépourvues de contenu granulaire. Dans cette dernière zone, de petits trous ou pores étaient évidents dans la membrane plasmatique de nombreuses plaquettes. La présence de ces pores est caractéristique de l'action de leucotoxine et n'est pas observée lors d'aggrégation induite par l'ADP ou le PAF.

(Traduit par docteur Serge Messier)

INTRODUCTION

Bovine pneumonic pasteurellosis (shipping fever) is a respiratory disorder that occurs when the lungs of cattle are colonized by the bacterium *Pasteurella haemolytica* (biotype A, serotype 1) which is normally only found in low numbers in the nasal flora of healthy animals (1–4). The disease is characterized by alveolar edema, serofibrinous exudation into the alveoli with hemorrhage, microvascular thrombosis and endothelial cell swelling (5–9). *Pasteurella haemolytica* produces several virulence factors important for the induction of the disease including a polysaccharide capsule, endotoxin (lipopolysaccharide (LPS)) and a leukotoxin (10). Many of the studies on the etiology of the disease have

Department of Biomedical Sciences (Cheryk, Gentry); Veterinary Pathobiology (Hooper-McGrevy), Ontario Veterinary College, University of Guelph, Guelph, Ontario N1G 2W1.

Address correspondence and reprint requests to Dr. P.A. Gentry.

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focused on the effect of these bacterial products on neutrophil and alveolar macrophage function (11–17). In vitro studies have demonstrated that the leukotoxin produced by *P. haemolytica* causes neutrophils to aggregate, to undergo the respiratory burst and to release chemotactic agents such as leukotrienes and endoperoxides (14–17). It has been suggested that the platelet aggregates observed in the pulmonary capillaries of cattle with *P. haemolytica* induced pneumonia are a secondary consequence of platelet activation induced by these agonists released from neutrophils or the arachidonic acid metabolite, thromboxane A_2 (TxA_2) released from alveolar macrophages (8,9). This is an unlikely explanation for platelet activation and aggregate formation because bovine platelets are insensitive to activation by arachidonic acid metabolites, such as TxA_2, and bovine platelet aggregation is independent of endoperoxide formation (18–20). In this regard the bovine platelet is distinct from many other types of mammalian platelets that are highly responsive to activation by TxA_2 (21).

The primary objective of this study was to determine whether the administration of *P. haemolytica* to calves by intrabronchial challenge would cause an alteration in platelet function in platelets collected from the peripheral circulation. Platelet function was assessed in an aggregometer by determining the extent of aggregation and the resistance of formed aggregates to dispersion following the addition of either ADP or PAF. Both ADP and PAF are known to be effective activators of bovine platelets (21). A decline in circulating platelet numbers is one of the characteristic hematological features in natural infections occurring under field conditions and thus platelet counts were also performed on samples before and after treatment (22). To ensure that any observed platelet changes could be correlated with other markers of infection, the circulating levels of a panel of acute phase proteins, including haptoglobin, α_2-macroglobulin, fibrinogen, albumin, fibronectin and antithrombin, were also part of this study. It has previously been reported that in cattle there is a temporal relationship between the increase in serum haptoglobin values and the onset of bacterial infection (23). Further, an increase in both haptoglobin and α_2-macroglobulin values has been reported to occur 3–4 d after challenge with *P. haemolytica* (24). The effect of challenge on the other acute phase proteins has not previously been reported.

Since alterations in platelet function were observed within 24 h of the intrabronchial challenge, the direct effect of *P. haemolytica* leukotoxin on both bovine platelet function and morphology was examined in vitro following the addition of the leukotoxin to platelet suspensions. The leukotoxin was selected as the most likely bacterial product that would affect bovine platelet function because previous studies had shown that bovine platelets are not affected by exposure to bacterial lipopolysaccharides (25). Also the effects of the leukotoxin on platelet morphology were assessed because of the suggestion that bovine platelets are lysed by this toxin (26). The results of this study indicate that *P. haemolytica* leukotoxin is capable of directly activating bovine platelets both in vivo and in vitro without inducing platelet lysis. Further, in animals developing bronchial pneumonia as a result of exposure to *P. haemolytica*, the alterations in platelet function precede the alterations in circulating levels of acute phase reactant proteins.

MATERIALS AND METHODS

ANIMALS

Ten 5 mo old Holstein bull calves were utilized for the in vivo challenge with *P. haemolytica* and 4 healthy female Holstein calves, 5–7 mo of age, were used in the in vitro study. The calves were housed at the Ontario Veterinary College, according to the guidelines of the Canadian Council on Animal Care and the experimental protocols were approved by the Animal Care Committee, University of Guelph.

COLLECTION OF BLOOD

Blood samples were collected prior to challenge and then every 24 h up to day 6 post-challenge. Blood was collected from healthy Holstein cows, by puncture of the external jugular vein, into a plastic syringe containing 0.13 M trisodium citrate to provide a 1:9 ratio of anticoagulant to blood. Following transfer to polypropylene tubes, the blood was centrifuged at $170 \times g$ for 15 min at 22°C and the platelet rich plasma (PRP) was removed. Platelet poor plasma (PPP) was obtained by recentrifuging the remaining blood at $2500 \times g$ for 15 min at 4°C. The Unoppette method (Becton-Dickinson Co., Rutherford, New Jersey, USA) was used to determine platelet counts in the PRP suspensions. For the aggregation studies, the PRP was adjusted to 2×10^8 platelets per mL with homologous PPP. The PPP was also used to evaluate fibrinogen, fibronectin, α_2-macroglobulin, antithrombin and albumin. Blood was collected into plain vacutainers and centrifuged at $2500 \times g$ for 15 min at 4°C to obtain serum for the haptoglobin assay.

CHALLENGE

A 12 h brain heart infusion broth (BHIB) culture of *P. haemolytica* type I was centrifuged at $4000 \times g$ for 15 min and resuspended in sterile PBS to an optical density of 1.0 at 525 nm. The respective viable bacterial counts for these experiments ranged from 2.2×10^{10} to 9.2×10^{14} colony forming units (CFU)/mL. Calves were anesthetized by xylazine injection and 25 mL of bacterial suspension, followed by 50 mL of sterile PBS, were delivered to the lung by means of an intratracheal catheter (27). Calves were euthanized with intravenous barbiturate either on day 3 or day 6 after challenge. The lungs were removed at necroscopy, macroscopic lesions were assessed and the percentage of pneumonic tissue was recorded (28). This experimental design is consistent with previous studies which evaluated the efficacy of vaccination procedures and the effects of the leukotoxin on white cell function (14,27,29,30).

LEUKOTOXIN PREPARATION

The leukotoxin used for the in vitro studies was a lyophilized culture supernatant from a 1 h culture of *P. haemolytica* serotype 1 prepared as described previously (29). The material was resuspended in Hanks' balanced salt solution (HBSS) to give a stock solution of 200 μg/mL which

was further diluted with HBSS before use. The cytotoxic dose of the preparation was 2.6 μg/mL. As controls, the leukotoxin preparation was heat inactivated by incubation at 56°C for 30 min, and a sample of culture medium, devoid of bacteria, was prepared and used to assess the effect of the medium constituents alone on platelet activation in vitro. Lipopolysaccharide contamination of the leukotoxic culture supernatant was measured by the *Limulus* amoebocyte lysate assay and indicated that the leukotoxin preparation contained less than 5 μg/mL LPS.

PLATELET AGGREGATION

Aggregation of bovine platelets was initiated by the addition of 10 μM ADP (Sigma Chemical Co., St. Louis, Missouri, USA) 0.5 μM synthetic platelet-activating factor (PAF) (Calbiochem, La Jolla, California, USA).

Platelet aggregation was monitored in a dual channel aggregometer (Chrono-log Corp., Havertown, Pennsylvania, USA) which monitors aggregate formation by the change in light transmission through the sample as single platelets coalesce to form large aggregates. For each experiment, the aggregometer was calibrated using PRP to establish the 0% aggregation limit (corresponding to 0% transmittance through PRP) and PPP to establish the 100% aggregation limit (corresponding to 100% transmittance through PPP) according to the method originally devised by Born (31). Platelet function was assessed in the presence and absence of agonist using aggregometry to evaluate the extent of aggregation and the time between the onset of aggregate formation and the onset of dispersion of the aggregates, i.e. disaggregation.

ELECTRON MICROSCOPY

Samples were prepared according to a modification of the method of Tablin and Castro (32). Platelet rich plasma samples, collected before and after the addition of leukotoxin, were layered onto solidified agarose (1%) contained in a Wintrope tube and centrifuged at 1000 × *g* for 15 min to form a pellet. The pellet was covered with a fixative solution containing 2.5% glutaraldehyde and 2.5% paraformaldehyde in a 0.1 M phosphate buffer and left in fixative overnight at room temperature. The pellet was placed in 1% tannic acid for 1 h and then fixed with 1% osmium tetroxide buffer for 1 h. The samples were dehydrated with an ascending series of ethanol and embedded in epon LX112, and allowed to polymerize for 24 to 36 h at 60°C. Ultrathin sections were prepared and stained with uranyl acetate and lead citrate.

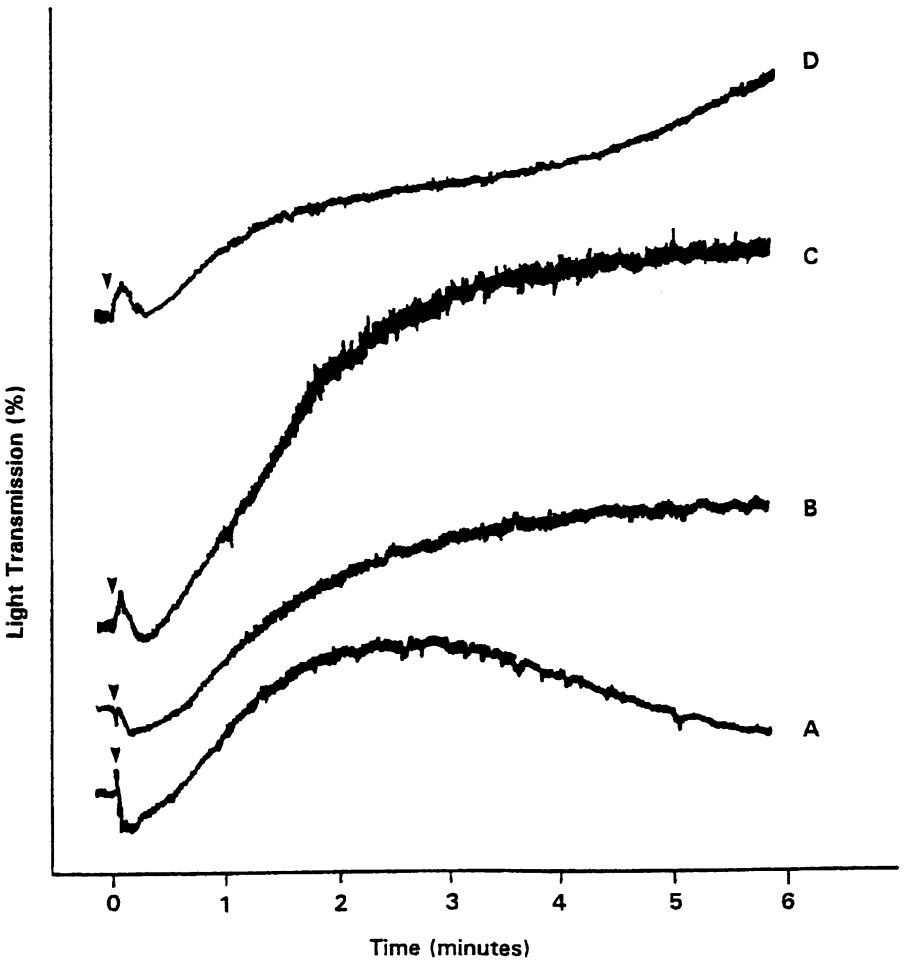

Figure 1. The aggregation responses of platelet rich plasma suspensions obtained from calves before and after intrabronchial challenge with *P. haemolytica* bacterial suspensions. Tracing (A) represents the typical aggregation response curve of bovine platelets stimulated with 10 μM ADP prior to the animal being challenged with *P. haemolytica*. By 24 h post-challenge (day 1), the aggregation response to 10 μM ADP has become irreversible (B). Aggregation tracings of bovine platelet rich plasma suspensions exposed to (C) *P. haemolytica* leukotoxin (20 mg/mL) and (D) heat-inactivated leukotoxin (20 mg/mL) in vitro. The addition of agonist is denoted by the arrow.

EVALUATION OF ACUTE PHASE REACTANTS

Haptoglobin was determined according to the chromogenic cyanmethemoglobin method (Stanbio Laboratories, San Antonio, Texas, USA). Plates were read on an ELISA reader at 405 nm and experimental values were calculated from a haptoglobin standard curve. Fibrinogen determinations were performed with a thrombin time method using the Fibrosystem (BBL, Division of Becton Dickenson, Franklin Lakes, New Jersey, USA) as previously described (33). Albumin was measured using the bromcresol green (BCG) dye method (Sigma Chemical Co., St. Louis, Missouri, USA) and antithrombin (AT) was determined using the chromogenic substrate, S-2238 (Helena Lab, Mississauga, Ontario) as described (34). Fibronectin and α_2-macroglobulin were determined by immunoelectrophoresis as previously described (34).

STATISTICAL ANALYSIS

The Student's paired *t*-test was used to compare groups before and after

TABLE I. Platelet aggregation response, circulating platelet numbers and percentage of lung tissue damage in calves exposed to *P. haemolytica* leukotoxin

Day of treatment	Platelet Function: Time to disaggregation (min) ADP (10 μM)	Time to disaggregation (min) PAF (0.5 μM)	Maximum aggregation (%) ADP (10 μM)	Maximum aggregation (%) PAF (05 μM)	Platelet count (10^{11} cells/L)	% Pneumonic Tissue
0	3.8 ± 0.4	4.1 ± 0.6	37.3 ± 1.8	39.5 ± 8.9	4.8 ± 0.4	—
1	9.7 ± 1.1*	4.9 ± 0.6	62.5 ± 6.4*	49.1 ± 7.4	3.9 ± 0.3*	—
2	9.4 ± 0.5*	5.2 ± 0.5	54.0 ± 6.2*	40.1 ± 7.1	4.0 ± 0.4	—
3	8.0 ± 1.0*	5.1 ± 0.8	36.1 ± 3.4	42.9 ± 7.6	4.5 ± 0.6	35.5 ± 9.3[a]
6	—	—	—	—	—	30.0 ± 7.0[b]

Results are expressed as mean ± SE, $n = 10$ expect for [a]$n = 3$, [b]$n = 5$
*$P < 0.05$ compared to day 0

treatment (SigmaStat, Jandel Scientific). For the in vivo study, all samples were assayed at least in duplicate and for the in vitro study, all experiments consisted of a minimum of at least 4 separate trials using different individuals with all samples done in duplicate.

RESULTS

IN VIVO STUDY

The platelet aggregation response in the 10 treated calves pre- and post-challenge was monitored for 72 h post-treatment using aggregometry with ADP (10 μM) or PAF (0.5 μM) as the platelet activating agent. In the pre-challenge samples, the aggregation response to ADP and PAF was similar and a typical aggregation tracing to ADP is illustrated in Figure 1, trace A. Before the addition of the agonist, the non-activated single platelets impede the transmission of light through the sample as the platelet suspension is being stirred in the aggregometer cuvette. Following the addition of the agonist, the light transmission through the sample is further transiently depressed as the platelets undergo shape change. As the single platelets begin to coalesce and form large aggregates the light transmission increases to a maximum value. To increase the sensitivity of the assay system, submaximal concentrations of ADP and PAF were used in this study so that a reversible aggregation response was consistently observed in the control pre-treatment platelet samples. The reversible aggregation response is reflected by the gradual decrease in light transmission through the sample post-aggregation as the large platelet aggregates begin to disperse (Figure 1, trace A). The most obvious change in the aggregation response to ADP in the calves post-challenge was the resistance of the formed aggregates to disperse, i.e. the coalesced platelets were slow to disaggregate. A typical aggregation response in a calf 24 h post-challenge is illustrated in Figure 1, trace B. At this observation period, the time to disaggregation had increased by 144% from a mean (± standard error, SE) pre-treatment value of 3.8 ± 0.4 min to 9.7 ± 1.1 min (Table I). This resistance to dispersion following ADP activation of the platelets persisted throughout the 72 h observation period. The extent of platelet aggregate formation in response to ADP stimulation was also more pronounced in the samples examined post-challenge (Table I). For example, 24 h after treatment, the extent of aggregation had increased by 68% from a pre-challenge mean (± SE) value of 37.3 ± 1.8 % to 62.5 ± 6.4%. However, unlike the disaggregation response, the alteration in the extent of aggregation was not sustained and the mean value for the 10 animals in the study had returned to baseline values by 72 h post-treatment. Although a similar type of response was observed for both the extent of aggregate formation and the dispersion of the aggregates after activation with PAF, as shown in Table I, the effect of challenge was less pronounced with this agonist than with ADP.

As shown in Table I, only a transient decline in platelet counts recovered in the PRP samples was observed following challenge. In all treated animals a decrease in platelet counts was observed 24 h after challenge with the mean values declining by 19% compared to pre-treatment. By 72 h post-treatment the circulating numbers had essentially returned to pre-challenge levels (Table I). However, by this time, all the treated animals were beginning to exhibit signs of respiratory distress. Two of the calves died before further sampling could be done and of the remaining calves, 3 were euthanized after day 3 and their lung tissue scored for the percentage of pneumonic tissue present. The percentage of pneumonic tissue ranged from 24.5% to 54.1% with an average value of 35.5 ± 9.3% (Table I). This response was similar to that observed for the remaining 5 calves that were euthanized at day 6 post-challenge. In this group the percentage of pneumonic tissue ranged from 12.8% to 54.4% with an average value of 30.0 ± 7.0% (Table I).

Following the bronchial challenge, the circulating levels of all the acute phase proteins exhibited significant ($P < 0.05$) changes. The mean (± SE) values for the individual proteins are shown in Table II for the 5 calves from which blood samples were collected daily for the entire 6 day observation period. Both haptoglobin and fibrinogen values increased significantly ($P < 0.05$) from pre-challenge values within 24 h with the peak values being observed at 72 h post-challenge when haptoglobin levels had reached 2.7 ± 0.4 g/L and fibrinogen values had reached 10.4 ± 0.9 g/L compared to pre-challenge values of 1.1 ± 0.1 g/L and 4.7 ± 0.5 g/L respectively. The plasma level of each of these proteins was still elevated at day 6 post-challenge. A more gradual increase in α_2-macroglobulin values was observed with a peak value of 5.3 ± 0.2 g/L being observed on day 6 post-challenge. In contrast, the plasma values for albumin, fibronectin and antithrombin values all declined after challenge. The albumin values decreased gradually reaching a nadir on day 3 when the value was 26.2 ± 0.5 g/L compared to the

pre-challenge value of 31.7 ± 0.7 g/L and the values were still significantly depressed ($P < 0.05$) on day 6 post-challenge. In comparison, both fibronectin and antithrombin values exhibited a more rapid decline with the lowest values being recorded on days 2 and 3 post-challenge when their values had decreased by 60 and 37% respectively. For each of these proteins the values subsequently began to increase and approached pre-treatment values by day 6.

TABLE II. Alterations in acute phase protein levels in calves after exposure to *P. haemolytica* leukotoxin

Day of treatment	Analytes[a]					
	Hp	Fib	α_2-M	Alb	Fn	AT[b]
0.00	1.1 ± 0.1	4.7 ± 0.5	4.3 ± 0.2	31.7 ± 0.7	0.5 ± 0.1	11.7 ± 0.4
1	1.8 ± 0.2*	6.6 ± 0.6*	4.5 ± 0.1	31.1 ± 0.4	0.3 ± 0.0*	9.4 ± 0.4*
2	2.6 ± 0.3*	9.0 ± 0.9*	4.7 ± 0.2	29.2 ± 0.4	0.2 ± 0.0*	7.5 ± 0.5*
3	2.7 ± 0.4*	10.4 ± 0.9*	4.6 ± 0.1	26.2 ± 0.5*	0.2 ± 0.0*	7.4 ± 0.9*
4	2.5 ± 0.1*	10.2 ± 0.4*	4.9 ± 0.1*	27.4 ± 0.7*	0.4 ± 0.1	9.3 ± 2.4
5	2.1 ± 0.3	9.9 ± 0.6*	5.2 ± 0.1*	27.2 ± 0.0*	0.5 ± 0.1	10.5 ± 1.9
6	2.1 ± 0.2	8.1 ± 1.2	5.3 ± 0.2*	26.4 ± 0.6*	0.5 ± 0.0	10.6 ± 0.3

[a] Values expressed in g/L as mean ± SE; [b] Values expressed as 10^{-2} g/L; $n = 5$
* Significantly different ($P < 0.05$) from untreated day 0 value
Hp = haptoglobin; Fib = fibrinogen; α_2-M = α_2-macroglobulin; Alb = albumin; Fn = fibronectin; AT = antithrombin

IN VITRO STUDY

Since the results of the in vivo study indicated that platelets might be directly affected by *P. haemolytica*, the effect of adding *P. haemolytica* leukotoxin to platelet suspensions was investigated. The addition of the leukotoxin, in the absence of any additional agonists, frequently resulted in an irreversible platelet aggregation response. A typical aggregation tracing exhibiting this response is illustrated in Figure 1, trace C. The aggregation responses following treatment with heat-inactivated leukotoxin (Figure 1, trace D) or culture medium alone were similar to one another and were lower than induced by unheated leukotoxin. When samples of the platelets aggregated by the leukotoxin were collected and examined by transmission electron microscopy, the morphological appearance of the platelets contained within the aggregates was generally similar to that previously reported for platelet aggregates formed in response to ADP (35) or PAF (20,25). The major difference in the appearance of the leukotoxin aggregated platelets was the appearance of small pores, or gaps, in the plasma membranes. Figure 2A illustrates the typical ultrastructural appearance of an unstimulated bovine platelet. Note the regular shape which is maintained by the circumferential band of microtubules and the appearance of numerous granules that are randomly dispersed through the cell. In comparison, Figure 2 B depicts a section through an aggregate formed by the exposure of platelets to the leukotoxin (20 mg/mL). Two zones of platelets are visible in the electronmicrograph. In 1 zone, platelets have retained their granules but have become irregularly shaped; in the other zone, platelets are devoid of defined structural cytoplasmic elements with many platelets exhibiting breaks or gaps in the plasma membrane. In addition to the spontaneous aggregation, the leukotoxin, but not

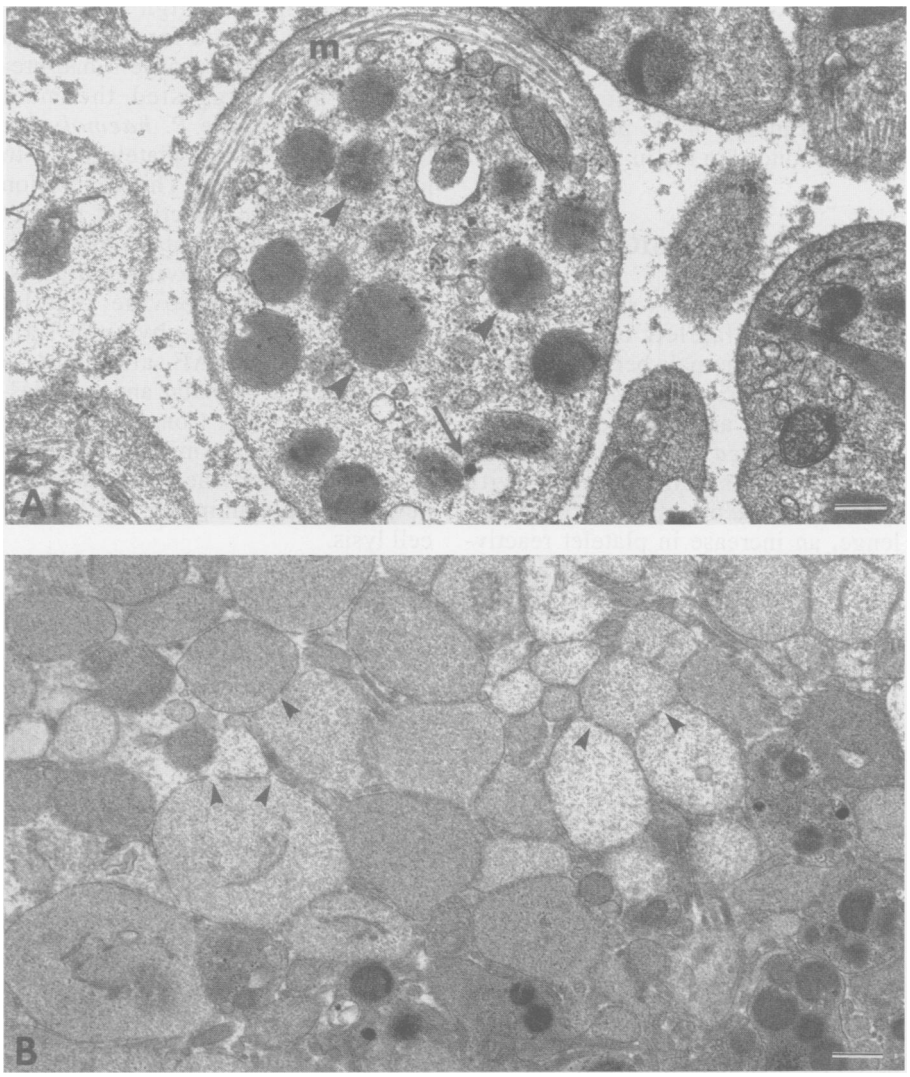

Figure 2. Electron micrograph of bovine platelets before and after exposure of platelet rich plasma suspension to *P. haemolytica* leukotoxin (20 mg/mL). (A) Prior to stimulation with agonist, note the large α-granules (arrowhead) characteristic of the bovine platelet and the occasional dense granule (arrow) and microtubules (m). Bar = 0.22 μm. (B) After exposure to the leukotoxin, aggregates appear similar to those formed in the presence of PAF, some of the platelets contain granules while others are devoid of granules. Note the appearance of pores or "gaps" present in the plasma membrane of these platelets (arrowhead). Bar = 0.65 μm.

the heat inactivated product or the culture medium, consistently produced an increase in the extent of the platelet aggregation induced by the addition of either of the agonists, ADP or PAF. For example, the extent of aggregation in response to activation with 10 μM ADP increased significantly ($P < 0.05$) from a control value of 53.3 ± 5.5 % to 77.7 ± 2.7% following the addition of leukotoxin (20 mg/mL) to the platelet suspension while the equivalent values for the aggregation response induced by 0.5 μM PAF were 77.2 ± 5.4 % and 84.4 ± 3.8% for the control and leukotoxin treated samples respectively. No disaggregation, or dispersion of the platelet aggregates, was observed in any of the platelet aggregates produced in the in vitro study.

DISCUSSION

The results of this study indicate that bovine platelets become more reactive after animals become infected with the bacterium *P. haemolytica* or after platelets are exposed directly to *P. haemolytica* leukotoxin. Within 24 h of calves being infected with *P. haemolytica* by intrabronchial challenge, an increase in platelet reactivity was observed. The data indicate that the platelet aggregates observed in the pulmonary capillaries of cattle with fibrinous pneumonia caused by *P. haemolytica* may, at least in part, be due to a direct action of this bacterium or its products on bovine platelets.

The morphological appearance of platelet aggregates formed by the exposure of platelets to leukotoxin in the absence of another activator was similar to the ultrastructural appearance of aggregates formed in response to activation by established agonists such as ADP and PAF (25,35). There was, however, a greater frequency of activated platelets in which pores, or gaps, in the plasma membranes were distinctly visible when the platelets were treated with the leukotoxin. This observation is consistent with the fact that *P. haemolytica* leukotoxin belongs to a family of Gram-negative bacterial cytolysins that act on target cells by forming transmembrane pores, resulting in osmotic lysis (36–39). The *P. haemolytica* leukotoxin is characterized by its specificity of action compared to other cytolysins. For example, the leukotoxin is cytotoxic towards bovine leukocytes but not non-ruminant leukocytes (29,40,41), consistent with the observation that human platelets, unlike bovine platelets, are unaffected by exposure to *P. haemolytica* leukotoxin (20).

Although the leukotoxin caused pores to form in the membranes of activated platelets, it did not induce platelet lysis as indicated by the discrete platelets that were visible within the aggregates. A high dosage of leukotoxin was used for the in vitro morphology study because it had previously been suggested the main effect of the crude *P. haemolytica* leukotoxin on bovine platelets was to induce cell lysis (26). This conclusion was based on the detection of platelet cytoplasmic constituents in the supernatant of treated bovine platelets. However, normal bovine platelet aggregates formed by non-lytic agents, such as PAF, also contain cells devoid of granular and cytoplasmic elements (25). Consequently, the release of cytoplasmic constituents from activated bovine platelets cannot be used as presumptive evidence of cell lysis.

It is well recognized that in the cardiovascular system platelet activation plays a critical role in thrombin formation. The results of this study indicate that the activation of bovine platelets may have an important role in initiating the formation of pulmonary thrombi in cattle with *P. haemolytica* induced pneumonia. Preliminary results suggest that a glycoprotease enzyme, produced by the bacteria, can directly affect bovine platelet adhesion and may be one of the contributing factors to the enhancement of bovine platelet adhesion induced by *P. haemolytica* (42,43). Part of the reason for the severity of the thrombotic complications in the lungs of affected cattle may be the result of the combined action of the leukotoxin on platelet function, noted in this study, and the stimulation of procoagulant activity in alveolar macrophages induced by *P. haemolytica* endotoxin (44,45).

Although endotoxin released from *P. haemolytica* has been implicated in activation of white cells, complement and coagulation proteins (45), it is not likely to be a major factor in bovine platelet activation since no alteration in platelet function or morphology has been observed in platelets isolated from endotoxin treated calves (F. Tablin, personal communication). It is possible, however, that endotoxin released from the bacteria could contribute to the alteration in acute phase proteins observed in this study. Acute phase reactants are liver-derived proteins whose synthesis is influenced by interleukins produced during infection and inflammatory reactions (46). In the challenged calves the maximal alterations in the acute phase reactants occurred 3–4 d post-treatment when the platelet counts and platelet function had essentially returned to pre-treatment levels. This temporal difference in response supports the concept that different agents may influence the platelet and acute phase protein responses.

The increase in circulating levels of haptoglobin and α_2-macroglobulin observed in this study agree with previous reports that the serum levels of these proteins rise in cattle with pulmonary disease (23,47,48). Plasma albumin values showed the characteristic decline associated with this negative acute phase reactant (47). Whether fibronectin and antithrombin are also negative acute phase reactants in the bovine remains to be determined because the decrease in plasma levels of these proteins may be a function of their increased consumption during alveolar fibrin formation in the *P. haemolytica* treated calves. For example, not only is fibronectin incorporated into fibrin clots but it has also been reported to bind with a variety of bacteria present in bovine mastitis (50). Thus, it is possible that fibronectin may interact with the *P. haemolytica* bacteria present in the lungs during the development of pasteurellosis.

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