Clostridium perfringens phospholipase C-induced platelet/leukocyte interactions impede neutrophil diapedesis

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Clostridium perfringens gas gangrene is a fulminant necrotizing infection in which inflammatory cells are notably absent from infected tissues but are often massed within adjacent vessels. It has been shown that *C. perfringens* phospholipase C (PLC) stimulates formation of large intravascular platelet/leukocyte complexes and that PLC-induced activation of platelet gpIIb/IIIa plays a major role. *In vivo*, such aggregates contribute to microvascular thrombosis and ischaemic necrosis of tissue. However, the effects of adherent platelets on neutrophil diapedesis have not been established. The present work investigated (1) the contribution of platelet P-selectin (CD62P) to PLC-induced cellular complex formation and (2) the effects of platelet adhesion on neutrophil diapedesis. The effects of anti-gpIIb/IIIa and anti-CD62P strategies on PLC-induced complex formation were measured by flow cytometry and followed by light microscopy. Both platelet gpIIb/IIIa and CD62P contributed to the formation of platelet/leukocyte complexes. Specifically, gpIIb/IIIa mediated the formation of large platelet/platelet aggregates that were tethered to the leukocyte principally via CD62P. Neutrophil diapedesis, quantified by a transendothelial cell migration assay and visualized by electron microscopy, was significantly reduced (> 60 %) by the adherence of large platelet aggregates. It was concluded that the absence of a tissue inflammatory response in *C. perfringens* gas gangrene is due, in part, to impaired neutrophil mobility caused by large aggregates of adherent platelets induced by PLC. Further, an adjunctive immunotherapeutic strategy targeting both gpIIb/IIIa and CD62P may improve the tissue inflammatory response, prevent vascular occlusion, maintain tissue viability, and reduce the need for radical amputation in patients with clostridial gas gangrene.

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INTRODUCTION

Clostridium perfringens gas gangrene is one of the most fulminant necrotizing infections affecting humans. The infection can become well established in traumatized tissues in as little as 6–8 h and the destruction of adjacent healthy muscle can progress several inches per hour despite appropriate antibiotic coverage. Shock and organ failure are present in 50 % of patients, and among these 40 % die. Even with modern medical advances and intensive care regimens, the centuries-old practice of radical amputation on an emergent basis remains the single best treatment. Histologically, this infection is characterized by widespread destruction of muscle and the absence of polymorphonuclear leukocytes

(PMNL) at the site of infection. Instead, leukocytes accumulate within adjacent vessels. Classical studies have identified the alpha toxin, a phospholipase C (PLC), as the principal toxin involved in these pathologies (reviewed by Bryant & Stevens, 1997; MacLennan, 1962).

We have recently shown that intramuscular injection of PLC caused a rapid and irreversible decline in skeletal muscle blood flow due to toxin-induced intravascular aggregates of activated platelets, leukocytes and fibrin (Bryant *et al.*, 2000a, b). Neutrophils in these aggregates were often margined along the endothelium but all remained intravascular (Bryant *et al.*, 2000a). These findings suggested that the large heterotypic aggregates of platelets and leukocytes induced by PLC also contributed to impairment of the tissue inflammatory response.

Abbreviations: MFI, mean fluorescence intensity; PE, phycoerythrin; PLC, phospholipase C; PMNL, polymorphonuclear leukocytes.

In vitro, PLC-induced platelet/PMNL aggregate formation was largely gpIIb/IIIa dependent (Bryant *et al.*, 2003, 2000a). Yet blockade of gpIIb/IIIa did not completely prevent PLC-induced platelet/neutrophil binding, suggesting that other adherence molecules play a role in these critical heterotypic cellular interactions. For most physiologic agonists (e.g. thrombin, ADP), induction of platelet/leukocyte aggregates involves an initial P-selectin (CD62P)-dependent tethering step, followed by a gpIIb/IIIa-dependent stabilization step (Blockmans *et al.*, 1995).

Thus, the present study investigated (1) the contribution of CD62P to PLC-induced platelet/platelet and platelet/neutrophil aggregate formation, and (2) the functional ability of PMNL, with and without adherent platelets, to migrate across an endothelial cell layer. Results demonstrate that P-selectin and gpIIb/IIIa each uniquely contribute to PLC-induced platelet/leukocyte complex formation and that the adherence of large platelet aggregates significantly reduces PMNL diapedesis.

METHODS

C. *perfringens* PLC and anti-PLC mAb. Recombinant PLC was prepared as previously described (Titball *et al.*, 1991). Phospholipase activity of the stock toxin preparation, determined by *p*-nitrophenylphosphorylcholine hydrolysis (NPPC) assay (Stevens *et al.*, 1987), was 31 100 units ml⁻¹, specific activity 19 438 units mg⁻¹. Neutralizing monoclonal anti-PLC antibody (clone 1C6, murine IgG₁) was provided by Dr Hiroko Sato, National Institute of Health, Tokyo, Japan (Sato *et al.*, 1989).

Cellular complex formation in whole blood. PLC-induced platelet/platelet and platelet/neutrophil complex formation was followed by flow cytometry as previously described (Bryant *et al.*, 2000a). Briefly, heparinized whole blood was obtained from healthy human volunteers who gave signed informed consent and who denied taking any medication for the previous 10 days. Whole blood (100 µl) was mixed with 10 µl each of FITC-conjugated anti-human CD42b (a pan platelet marker, green; Coulter) and phycoerythrin (PE)-conjugated anti-human CD11b (a neutrophil marker, red; Coulter). Stock PLC was diluted in PBS containing 2.0 mM CaCl₂ and 100 µM ZnCl₂. These ions are necessary for PLC binding and enzymic activities, respectively. Blood was stimulated with either PLC (0.03–1 unit) or PBS vehicle for 5 min at 37 °C. Red blood cells were removed by formic acid lysis and remaining cells were fixed in 2% (v/v) paraformaldehyde in PBS for flow cytometric analysis as described below.

A modification of this protocol was followed to prepare aggregates for use in transendothelial cell migration assays (Fig. 1). In this case, 100 µl heparinized whole blood was stimulated with either 1 unit of PLC or PBS vehicle for 10 min at 37 °C (Fig. 1, step 1). To stop the reaction, anti-PLC mAb (20 µg ml⁻¹) was added to all samples and red blood cells were removed by formic acid lysis. Cells were sedimented by gentle centrifugation (300 g, 5 min) in the presence of anti-P-selectin antibody (10 µg ml⁻¹; Pharmingen) to inhibit spontaneous platelet binding induced by soft pelleting of the cells (Fig. 1, step 2). This treatment had no effect on complexes formed prior to centrifugation. Cells were resuspended in cell isolation buffer (CIB) (10 mM HEPES, 129 mM NaCl, 9.9 mM NaHCO₃, 2.8 mM KCl, 0.8 mM KH₂PO₄, 0.8 mM MgCl₂, 5.6 mM D-glucose, pH 7.4 supplemented with 1.5% human serum albumin) to the original 100 µl volume and used in the transendothelial cell migration assay (see next section). Using this

isolation procedure, the number of leukocytes obtained from 100 µl whole blood and subsequently added to the inserts was routinely 4–5 × 10⁵. These values did not change with PLC treatment.

Flow cytometric analysis of cellular aggregates. Flow cytometric analysis was performed as previously described (Bryant *et al.*, 2000a, b) using an Epics XL flow cytometer (Coulter). Neutrophils were gated by characteristic forward and side scatter, and purity was assessed by red fluorescent staining (PE-conjugated anti-CD11b). The percentage of neutrophils bearing platelets (i.e. dual-colour positive events) and the mean fluorescence intensity (MFI) of the green platelet marker (FITC-conjugated CD42b) were evaluated on a mean of 10 000 events.

Transendothelial cell migration assays. The functional status of neutrophils when complexed with platelets was evaluated using a transendothelial cell migration assay. Human umbilical vein endothelial cells (HUVEC; Cambrex Bio Science) were maintained in tissue culture using complete endothelial cell growth medium (cEGM-2; Cambrex) following the manufacturer's instructions. Cells of passage 3–4 were plated on gelatin and fibronectin-coated Transwell polycarbonate filters with 5 µm pore size (Corning) at 2.5 × 10⁴ cells/insert in 100 µl cEGM. Filter inserts were suspended in the wells of a 24-well tissue culture plate containing 600 µl cEGM and HUVEC were cultured to confluence (~6 days). At the end of this period, visual light microscopic inspection of the insert revealed the presence of a bilayer of endothelial cells – one monolayer on the apical surface and one on the basal surface of the insert. This finding has been reported by others (Ebisawa *et al.*, 1992). The integrity of the endothelial cell barrier in each insert was verified before conducting an experiment by a transendothelial electrical resistance (TEER) volt/ohm meter (EndOhm-6; World Precision Instruments). Intact layers routinely gave a mean total unit area resistance value of 39.6 Ω cm² compared to 16.5 Ω cm² for an insert without cells.

One hundred microlitres of prepared platelet/neutrophil aggregates was placed into the top chamber of the insert (Fig. 1, step 3). A chemoattractant, *n*-formylmethionylleucylphenylalanine (5 × 10⁻⁷ M final concentration), was then added to the lower chamber, and the plate returned to the tissue culture incubator. After 2 h, the plate was removed and endothelial cell integrity was again evaluated by electrical resistance as described above. The upper and lower layers of the inserts were gently washed with PBS, then the insert was processed for electron microscopy as described below (Fig. 1, step 4). Leukocytes that had crossed the endothelial cell layer into the lower chamber were collected and stained with fluorochrome-conjugated anti-CD11b and anti-CD42b antibodies, as described above, for 20 min at 37 °C, then fixed in 1% paraformaldehyde in PBS (Fig. 1, step 5) for flow cytometry analysis.

The transmigration experiment was performed in replicates of three to six on four separate occasions using blood from different donors.

Electron microscopy. Transwell inserts were prepared for electron microscopy using standard procedures. Briefly, after the 2 h incubation period, the upper and lower faces of the inserts were gently washed with prewarmed EGM-2. The filter was left attached to the plastic insert throughout all steps, and reagents exchanged by passing the insert among the wells of a 24-well tissue culture plate containing the appropriate solutions. After washing, the cells were fixed in two changes of 4°C 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2–7.4 (Electron Microscopy Sciences), for 2 h each, then post-fixed in 1% osmium tetroxide (Ted Pella), stained with uranyl acetate (Ernst Fullam) and dehydrated in a series of ethanol dilutions. Inserts were infiltrated stepwise with EMBED (Electron Microscopy Sciences) to 100%, following the manufacturer's instructions for the hard resin. The inserts were then suspended in a small flexible embedding cup containing 100% resin, the upper

Fig. 1. Schematic of methodology used to generate PLC-induced platelet/neutrophil complexes. Step 1: heparinized whole blood (100 μ l) was treated for 10 min with either PBS vehicle or 1 unit PLC, followed by addition of anti-PLC mAb to stop the reaction. Step 2: PLC caused platelets in whole blood to become positive for P-selectin (depicted here by a small dark rectangle on the platelet surface), and to form large aggregates mediated by fibrin(ogen) cross-bridging between platelets (indicated by wavy lines). Platelet aggregates stably adhered to neutrophils resulting in heterotypic cellular complexes. Red blood cells were removed from these platelet/neutrophil complexes by formic acid lysis and complexes were collected by centrifugation in the presence of anti-P-selectin antibody to inhibit spontaneous platelet binding induction by this step. Step 3: complexes were resuspended in a volume equal to that of the original whole blood sample and placed into the top chamber of an endothelial cell-lined polycarbonate filter insert. A chemoattractant, *n*-formylmethionylleucylphenylalanine was then added to the lower chamber, and the plate incubated for 2 h. Step 4: the upper and lower layers of the inserts were gently washed, and the insert was processed for electron microscopy. Step 5: leukocytes that had crossed the endothelial cell layer into the lower chamber were collected, stained with fluorochrome-conjugated anti-CD11b (neutrophil) and anti-CD42b (platelet) antibodies, and analysed by dual-colour flow cytometry.

surface of the insert was covered with additional EMBED, and the entire unit polymerized for 48 h at 60 °C. The exposed plastic insert was cut away from the hardened resin with a jeweller's saw, and the blocks were sent to the University of Idaho's core electron microscopy facility. Blocks were cut bisecting the filter along its diameter, and faced using a diamond knife to remove any marks left by the jeweller's saw. The cut faces were given a thin coat of carbon via vacuum evaporation, placed in a specimen vice and viewed with a LEO SUPRA 35VP field emission scanning electron microscope in backscatter electron detection mode. Digital images were captured as TIFF files directly onto the LEO computer.

Inhibition of PLC-induced cellular complex formation by anti-platelet strategies. To investigate the molecular mechanisms

of PLC-induced platelet/neutrophil complex formation, studies used cells that were cytoplasmically, rather than surface, labelled. This system has several advantages over the whole blood system. First, by marking the cell with a cytoplasmic label, it ensures that anti-adhesin strategies will not be hindered by surface-bound fluorochrome-labelled antibodies. Second, aliquots of these samples can be directly taken to the cytospin for visual evaluation. Third, antibodies used to block cell-cell interactions are not taken up by irrelevant cell types, thus less reagent can be used in a more specific manner. The disadvantages of this system are twofold: (1) there is a higher background adherence of platelets to leukocytes compared to the whole blood system using surface labelling (approx. 15 vs 5% in whole blood) – a likely consequence of the more extensive isolation and staining procedures applied to both platelets and neutrophils in the

purified cell system, and (2) there is a lower percentage of PMNL binding platelets (35–45 vs 85–95% in whole blood). This latter observation suggests that soluble or cellular elements of whole blood contribute to complex formation induced by PLC.

For these studies, purified platelets and neutrophils were prepared as follows. Platelets in platelet-rich plasma were cytoplasmically labelled with the green fluorescent dye, BCECF-AM ($2 \mu\text{g ml}^{-1}$; Molecular Probes). After 30 min, platelets were separated from unincorporated dye and plasma proteins by gel filtration as previously described (Bryant *et al.*, 2003). Briefly, platelet-rich plasma (5–6 ml) was loaded onto a column packed with Sepharose 2B that was pre-equilibrated with CIB. Fractions containing BCECF-labelled, fibrinogen-free platelets were pooled and platelets were quantified by flow cytometry using $3.6 \mu\text{m}$ fluorobeads as an internal standard. The platelet concentration was adjusted to 1×10^8 cells ml^{-1} with CIB, and the final volume was supplemented to contain 2.0 mM CaCl_2 and $100 \mu\text{M}$ ZnCl_2 [complete CIB (cCIB)]. This isolation technique consistently yielded high numbers of purified resting platelets as determined by the absence of P-selectin surface staining. Further, these platelets were fully responsive (i.e. expressed high levels of P-selectin) to known activators such as thrombin (data not shown).

Neutrophils were isolated by differential density-gradient centrifugation as previously described (Bryant *et al.*, 1993). Isolated neutrophils were labelled intracellularly with the red fluorescent dye, dihydro-ethidium [$8 \mu\text{g}$ (1×10^7 cells) $^{-1}$; Molecular Probes]. After 30 min, unincorporated dye was removed by a single wash in CIB, and cells were resuspended to a concentration of 2×10^6 ml^{-1} in cCIB.

For each test, fluorescently labelled isolated platelets and neutrophils were combined in a total volume of 1 ml (platelet: neutrophil ratio, 50:1), and the solution reconstituted to contain fibrinogen ($100 \mu\text{g ml}^{-1}$). Cells were then preincubated for 10 min with the following agents, either individually or in combination: gpIIb/IIIa inhibitor (eptifibatide, Integrilin, $2 \mu\text{g ml}^{-1}$, a commercially available synthetic heptapeptide that is a high-affinity inhibitor of gpIIb/IIIa; Schering-Plough), a monoclonal anti-CD62P blocking antibody ($8 \mu\text{g ml}^{-1}$, murine IgG₁; Pharmingen) or an isotype-matched control antibody ($8 \mu\text{g ml}^{-1}$, murine IgG₁; Pharmingen). Recombinant PLC [0.25 U (1×10^6 platelets) $^{-1}$] or PBS vehicle was then added for 10 min at 37°C . The reaction was terminated by the addition of an equal volume of 2% paraformaldehyde in PBS and samples immediately analysed by flow cytometry. A portion of each sample ($150 \mu\text{l}$) was fixed to a microscope slide using a cytospin, air-dried and stained first with Hansel stain then with Wright's stain. Images were viewed with a Zeiss Axiophot light microscope and photographed at $\times 100$ magnification. The extent of platelet/platelet aggregation in each specimen was scored over the range of 0 to 4+ by an observer who examined a minimum of three fields per slide in a blinded study.

RESULTS

PLC-induced complex formation in whole blood

PLC-induced platelet/platelet and platelet/neutrophil complex formation in whole blood was analysed by flow cytometry. A typical histogram of unstimulated cells is shown in Fig. 2(a). In this instance, the CD11b-positive neutrophil population was clearly distinguished and was largely devoid of adherent platelets as indicated by the low percentage of dual colour positivity (6%) in this population. Among the platelet-positive neutrophils, each white cell bound only a few platelets as indicated by the low MFI of the platelet signal (i.e. $\text{MFI}_{\text{CD42b}} = 1.9$). A second discrete population was also

Fig. 2. PLC induces large platelet/neutrophil complexes in whole blood. (a) Resting or (b) PLC-induced platelet/neutrophil complexes in whole blood were analysed by dual-colour flow cytometry. In unstimulated blood (a), the percentage of CD11b-positive neutrophils binding platelets is low (6% dual positive, $\text{MFI}_{\text{CD42b}} = 1.9$). A second discrete population, consisting solely of aggregated platelets was also visible (indicated by an arrow). PLC treatment dose-dependently stimulated large numbers of neutrophils to bind large numbers of platelets (1 unit PLC yielded 82% dual positive events with a $\text{MFI}_{\text{CD42b}} = 5.7$) (c) and increased the numbers of platelet/platelet aggregates (b, indicated by an arrow).

visible (Fig. 2a, arrow) and represented 4% of the total events collected. Cytometric analysis showed that this population consisted solely of aggregates of platelets greater than or equal to $10 \mu\text{m}$ in size and that the apparent CD11b positivity of this population was artefact created by the bleed-over of the green (FITC) fluorescence into the red (PE) channel (not shown). No electronic compensation was used to reposition this population. Instead, it was gated and analysed separately, allowing us to study platelet/platelet interactions in the same histogram used to evaluate platelet/neutrophil complexes.

In contrast to unstimulated whole blood, treatment with PLC dose-dependently stimulated large numbers of neutrophils to become platelet (CD42b) positive (Fig. 2b, c). Further, each neutrophil bound multiple platelets as indicated by an increase in $\text{MFI}_{\text{CD42b}}$ to 5.7 (Fig. 2b). In

addition, the separate population of platelet/platelet aggregates became much more prominent (16 % of total events, Fig. 2b, arrow).

Transendothelial cell migration of platelet/leukocyte complexes

The functional capability of neutrophils complexed with platelets was evaluated using a transendothelial cell migration assay. Platelet/neutrophil complexes isolated from PLC-treated whole blood, as shown in Fig. 1, were used for this functional assay. One representative experiment of four is presented in detail here.

PLC stimulated 82 % of neutrophils in whole blood to become platelet (i.e. CD42b) positive compared to 26 % in unstimulated whole blood (Table 1). The increased background complex formation and the higher CD11b surface expression in these experiments were attributable to the added processing of cells (i.e. lysis of red blood cells without subsequent fixation, followed by centrifugation to recover aggregates). Neutrophils from either control or PLC-stimulated samples ($n=6$ per group) were placed on the endothelial cell-covered insert filters and allowed to migrate toward a chemoattractant in the lower chamber. After 2 h, the mean number of transmigrating neutrophils from the untreated whole blood was 16.4 % of those added to the upper chamber (Table 1). Transmigrated neutrophils remained virtually platelet free ($\text{MFI}_{\text{CD42b}} = 1.4$), but had increased size and reduced granularity (not shown). Surface CD11b expression was within 10 % of pre-transmigration levels. In contrast only 6.4 % of neutrophils from PLC-treated samples migrated across the endothelial cell layer (Table 1) – a 61 % reduction in transmigration compared to the control. Interestingly, the transmigrated neutrophils from the PLC-treated samples were virtually platelet-free (CD42b $\text{MFI} = 1.5$) and had a 22.5 % reduction in surface CD11b expression. No large platelet aggregates and only a few individual platelets were detected in this lower chamber (not shown).

By electron microscopy, neutrophils in the unstimulated control samples were seen adjacent to the upper endothelial cell layer or within filter pores (Fig. 3a). Most neutrophils in these samples bound none, one or two platelets each. Platelets were generally not aggregated and were round and smooth, suggesting they were in a non-activated state. Neutrophils were rarely seen between the filter and the lower endothelial cell layer, suggesting they had successfully completed their transmigration to the lower chamber. In contrast, large aggregates of platelets and neutrophils were seen adjacent to the upper endothelial cell layer in PLC-treated samples (Fig. 3b). Neutrophils in PLC-treated samples were also seen within pores, but appeared to lack the large platelet aggregates seen on the surface of the insert. When an occasional free neutrophil was seen below the lower endothelial cell layer, it was devoid of adherent platelets (not shown). Platelets themselves formed large aggregates and displayed morphological changes typical of cellular activation, such as irregular shapes and ruffled membranes. In some areas of intense platelet/neutrophil aggregates, destruction of the upper endothelial cell layer was observed (not shown) and likely accounted for the slight reduction in transendothelial electrical resistance in these samples (27.1 vs $34.0 \Omega \text{ cm}^{-2}$ in PLC-treated vs control wells, respectively).

Effects of anti-platelet adhesins on complex formation

The ability of anti-platelet adhesins to block PLC-induced homotypic and heterotypic cellular complexes was also studied by flow cytometry using isolated cells that were cytoplasmically, rather than surface, labelled as described in Methods (Fig. 4). Light microscopy of the unstimulated combination of purified cells demonstrated that platelets were sometimes associated with neutrophils (Fig. 4a, arrow). Addition of PLC stimulated both an increase in the percentage of neutrophils binding platelets and an increase in the number of platelets bound per neutrophil (Fig. 4b), resulting in large aggregates composed of many

Table 1. Transendothelial cell migration of PMNL with/without adherent platelets

Platelet/neutrophil complexes were prepared from whole blood as described in Methods (see Fig. 1). The total number of PMNL and the fluorescent intensity of platelet CD42b and PMNL CD11b expression on PMNL, before and after transmigration through intact endothelial cells, were quantified by flow cytometry. Data shown are from one representative experiment of four and are given as the means of six replicate wells per stimulus $\pm$ SD.

Stimulus	Pre-transmigration				Post-transmigration			
	Total no. of PMNL added to insert ($\times 10^5$)	% of PMNL binding platelets	MFI of the indicated marker on PMNL		Total no. of PMNL transmigrated ($\times 10^5$)	% PMNL transmigrated	MFI of the indicated marker on transmigrated PMNL	
			CD42b	CD11b			CD42b	CD11b
None	4.63 ± 0.35	26.0 ± 5.7	1.57 ± 0.06	105 ± 8.8	0.76 ± 0.13	16.4	1.4 ± 0.03	93.4 ± 0.92
PLC	4.87 ± 0.46	81.5 ± 4.9	4.36 ± 0.06	206 ± 5.1	0.31 ± 0.05	6.4	1.5 ± 0.04	159.6 ± 1.78

Fig. 3. PLC-stimulated aggregate formation reduces neutrophil diapedesis. PLC-induced platelet/neutrophil complexes, prepared from whole blood, were placed on the apical side of an endothelial cell-lined polycarbonate filter insert and a chemoattractant was placed in the chamber below. After 2 h, the inserts were gently washed to removed unbound cells and processed for electron microscopy. (a) Neutrophils from unstimulated whole blood were typically platelet-free and associated either with the apical endothelial cell surface or within insert pores. (b) In contrast, masses of large platelet/neutrophil aggregates were seen in PLC-stimulated whole blood; however, migrating neutrophils within pores and beneath the filter were platelet free. RBCs, red blood cells.

platelets and neutrophils (Fig. 4b, large arrow). Large aggregates of pure platelets were also seen (Fig. 4b, small arrow).

Pretreatment of the PLC-treated cells with anti-adhesin antibodies dramatically altered these populations. Treatment with the blocking anti-gpIIb/IIIa heptapeptide prevented the formation of large platelet/platelet aggregates (Fig. 4c) and decreased the number of platelets bound per neutrophil. However, this treatment slightly increased the percentage of neutrophils binding platelets. This unexpected finding was explained by light microscopic analysis of the complexes formed. Specifically, by blocking gpIIb/IIIa, large platelet aggregates were not formed; therefore, relatively

more individual platelets were available to bind singly or in small numbers to neutrophils (Fig. 4c, arrowheads), thereby increasing the percentage of dual positive events.

In contrast, incubation with blocking anti-CD62P antibody reduced the percentage of neutrophils binding platelets to baseline levels but did not significantly reduce platelet/platelet aggregates (Fig. 4d, small arrow). Occasionally neutrophils bound these large platelet aggregates likely via a CD11b/fibrinogen/gpIIb/IIIa axis (Spangenberg *et al.*, 1993) such that the MFI_{CD42b} of these few dual-colour events remained elevated. By blocking both adherence molecules, the distribution of cells returned to the untreated background profile (Fig. 4e).

Fig. 4. Anti-platelet adhesins inhibit PLC-induced complex formation. Isolated and cytoplasmically labelled platelets and neutrophils were combined at a platelet:neutrophil ratio of 50:1. Cells were preincubated using blocking strategies against gpIIb/IIIa or P-selectin (CD62P) either alone or in combination, followed by 10 min stimulation with PLC. A portion of each sample was cytospin-fixed to a microscope slide and stained. Photomicrographs are shown in (a–e). (a) Resting unstimulated blood showed few cellular aggregates. (b) PLC-stimulated formation of large platelet/platelet aggregates (small arrows) and platelet/neutrophil aggregates (large arrows). (c) Blockade of gpIIb/IIIa eliminated PLC-induced large platelet aggregate formation; however, neutrophils continued to bind single platelets (arrowheads). (d) Anti-CD62P antibody reduced to baseline levels the percentage of PMNL binding platelets. Yet, of those few PMNL that did bind platelets, the number of platelets/PMNL was increased compared to unstimulated cells (MFI_{control} = 4.85, MFI_{x62P} = 6.0). Anti-CD62P did not significantly reduce the numbers of platelet/platelet aggregates (small arrows). (e) Strategies targeting both CD62P and gpIIb/IIIa restored the normal, unaggregated distribution of cells. The table shows the extent of platelet/platelet co-aggregation scored from the corresponding slides on the left of the figure over the range of 0 to 4+. The percentage of PMNL binding platelets and the relative number of platelets bound per neutrophil (MFI of the platelet signal) were determined by dual-colour flow cytometry. Results are the means $\pm$ SD from two experiments performed in duplicate. * $P < 0.05$ compared to control by *t*-test.

	Toxin	Anti-adhesin strategy	Platelet/platelet complex	PMNL binding one or more platelets (%)	Relative no. of platelets per PMNL (MFI)
(a)	None	None	+/-	17.4 ± 2.2	4.85 ± 0.21
(b)	PLC	Control IgG	++++	30.5 ± 3.8*	5.60 ± 0.42
(c)	PLC	gpIIb/IIIa	+/-	39.5 ± 3.6*	4.25 ± 0.35
(d)	PLC	CD62P	+++	13.4 ± 2.5	6.0 ± 0.01
(e)	PLC	gpIIb/IIIa + CD62P	+/-	12.6 ± 3.1	3.95 ± 0.07

DISCUSSION

C. perfringens type A is the most common organism isolated from patients with trauma-induced gas gangrene (MacLennan, 1962). The local and systemic manifestations of gas gangrene are related to the elaboration of potent extracellular protein

toxins, especially the alpha toxin, a PLC (reviewed by Bryant & Stevens, 1997). Histologically, *C. perfringens* gas gangrene is characterized by extensive destruction of muscle, an absence of leukocytes in the infected tissues and an accumulation of leukocytes within vessels of the affected area. We hypothesized that these pathologies are the consequence of

a dysregulated host response induced by PLC, which leads to vascular thrombosis and injury, subsequent ischaemic necrosis of tissue and impaired PMNL diapedesis.

As evidence that this hypothesis has merit, we have recently demonstrated that intramuscular injection of PLC induced a rapid and irreversible decline in skeletal muscle perfusion due to the formation of occlusive intravascular aggregates of activated platelets, leukocytes and fibrin (Bryant *et al.*, 2000b). Further, we have shown that PLC directly activates gpIIb/IIIa (Bryant *et al.*, 2003, 2000a) by mobilization of calcium stores (Bryant *et al.*, 2003) and that this activation contributes to the formation of both platelet/platelet and large platelet/neutrophil aggregates (Bryant *et al.*, 2000a).

The results of the present work also demonstrate a role for P-selectin in PLC-induced platelet/leukocyte complex formation. Thus, as a working model (Fig. 5), we propose that PLC-induced activation of gpIIb/IIIa mediates the formation of large platelet/platelet aggregates that are tethered to the leukocyte principally via toxin-mediated upregulated CD62P.

The pathological consequences of PLC-induced platelet/leukocyte aggregation are two-fold. First, large aggregates irreversibly occlude the vasculature thereby contributing to rapid ischaemic destruction of viable tissue (Bryant *et al.*, 2000a, b). Second, neutrophils, when burdened with large numbers of activated platelets, cannot effectively traverse the

Fig. 5. Working model for PLC-induced platelet/neutrophil complex formation. *In vivo*, absorption of PLC into the microvasculature at the site of infection, causes rapid formation of large platelet/platelet aggregates and platelet/neutrophil complexes (top illustration) that occlude small venules and arterioles leading to ischaemic necrosis of tissue. Formation of such platelet aggregates is mediated by fibrin(ogen) cross-bridging of PLC-activated gpIIb/IIIa molecules on adjacent platelets (indicated by wavy lines). These large aggregates bind neutrophils via PLC-induced platelet P-selectin (CD62P, depicted here as a small dark rectangle on the platelet surface). Immunological blockade of gpIIb/IIIa (second illustration from top) prevents formation of large platelet/platelet aggregates but does not prevent P-selectin-mediated rosetting of single platelets on the neutrophil surface. Blockade of platelet P-selectin (third illustration from top) has no effect on platelet/platelet aggregation, but prevents adherence of these large aggregates to the neutrophil surface. Use of a combined immunotherapeutic modality (bottom illustration) *in vivo* may prevent vascular occlusion and tissue ischaemia, while maintaining neutrophil diapedesis and an effective tissue inflammatory response.

vascular endothelium (this study). This impaired mobility, coupled with PLC-induced upregulation of the endothelial cell adherence molecules, platelet activating factor (Bunting *et al.*, 1997) and intercellular adhesion molecule-1 (ICAM-1, Bryant & Stevens, 1996), and the PMNL adhesion, CD11b/CD18 (this study), irreversibly arrests leukocytes within the vasculature thereby preventing diapedesis and chemotaxis of inflammatory cells into infected tissues. In total, these toxin-induced events thwart the immune response and extend the anaerobic environment – both of which ensure continued clostridial proliferation.

Translating these molecular mechanisms into a clinical application suggests that, as an adjunct to traditional antibiotic therapy, immunotherapeutic agents that prevent platelet aggregation could maintain vessel patency and ensure adequate perfusion of tissues in patients at risk for gas gangrene, just as they do in patients at risk for acute coronary artery events. However, as the present work suggests, targeting a single adherence molecule in gas gangrene may be less than optimal. For instance, blocking gpIIb/IIIa would eliminate large platelet/platelet aggregates and prevent their binding to leukocytes. In the absence of these large complexes, occlusion of vessels would be prevented. Neutrophils, however, would retain the ability to bind multiple activated platelets in a rosette pattern. Such interactions are known to hyperstimulate neutrophil respiratory burst activity (Peters *et al.*, 1999; Ruf *et al.*, 1992) and to facilitate chemical cross-talk between these cells, resulting in direct endothelial cell injury and local vascular dysfunction (Ward *et al.*, 1986), and hyperproduction of thromboxanes (Maugeri *et al.*, 1992), leukotrienes (Maugeri *et al.*, 1994) and cytokines (Neumann *et al.*, 1997). Overproduction of these potent immunomodulators could also contribute to local tissue injury and to the systemic manifestations of gas gangrene. Addition of a strategy targeting platelet P-selectin (CD62P) would prevent these latter complications (Neumann *et al.*, 1997).

In summary, we conclude that rapid tissue destruction in gas gangrene is mediated by the pleiotropic effects of PLC on platelets, leukocytes and endothelial cells, and that a combined immunotherapeutic strategy targeting both gpIIb/IIIa and CD62P may restore the tissue inflammatory response, prevent vascular occlusion, maintain tissue viability, and reduce the need for radical amputation in patients with clostridial gas gangrene. Studies to evaluate these therapeutic approaches in experimental gas gangrene are currently under way in our laboratory.

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