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Pneumolysin mediates platelet activation *in vitro*

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Short title: Pneumolysin-induces platelet activation

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Abstract

This study has explored the role of the pneumococcal toxin, pneumolysin (Ply), in activating human platelets. Following exposure to Ply [10–80 nanograms (ng)/ml], platelet activation and cytosolic Ca^{2+} concentrations were measured flow cytometrically according to the level of expression of CD62P (P-selectin) and spectrofluorimetrically respectively. Exposure to Ply resulted in marked upregulation of expression of platelet CD62P, achieving statistical significance at concentrations of 40 ng/ml and higher ($p<0.05$), in the setting of increased influx of Ca^{2+}. These potentially pro-thrombotic actions of Ply were attenuated by depletion of Ca^{2+} from the extracellular medium, or by exposure of the cells to a pneumolysoid devoid of pore-forming activity. These findings are consistent with a mechanism of Ply-mediated platelet activation involving sub-lytic pore formation, Ca^{2+} influx, and mobilization of CD62P-expressing α-granules, which, if operative *in vivo,* may contribute to the pathogenesis of associated acute lung and myocardial injury during invasive pneumococcal disease.

Keywords: calcium, CD62P, community-acquired pneumonia, pneumococcus, P-selectin, *Streptococcus pneumoniae*

Introduction

Despite significant advances in diagnosis, care and therapy, bacterial community-acquired pneumonia (CAP), of which *Streptococcus pneumoniae* (also known as the pneumococcus) is the major causative pathogen, continues to carry an unacceptably high mortality rate ranging from 5-50% [1]. Acute lung injury and multi-organ dysfunction syndrome, as well as acute cardiac events mostly occurring early in the course of CAP, have been identified as being significant contributors to mortality [1-7]. In the case of pneumococcal CAP, an increasing body of evidence, derived from both clinical and experimental studies, has implicated the major cytolytic, cholesterol-binding, pore-forming protein toxin, pneumolysin (Ply), in the pathogenesis of associated adverse pulmonary and cardiovascular events [8-13]. Although these harmful activities of Ply have been attributed to direct pulmonary and cardiac toxicity, it is noteworthy that Ply also possesses pro-inflammatory activities [14]. These are evident at lower, sub-lytic concentrations of the toxin and may also contribute to the pathogenesis of acute lung and cardiac injury in CAP.

Importantly, platelets are now recognized to be key players in orchestrating inflammatory responses [15], with platelet activation seemingly associated with acute lung injury in the experimental setting [9] and with myocardial infarction, worsening heart failure or arrhythmias in the clinical setting of invasive pneumococcal disease [16]. To our knowledge, however, only one previous study has addressed the direct involvement of Ply in mediating platelet activation, documenting aggregation of these cells following exposure to very high concentrations of the toxin, but without characterizing the mediators and mechanisms involved [17].

In the current study, we report on the effects of brief exposure of human platelets to Ply, at concentrations representative of severe pneumococcal disease [18], on expression of CD62P (P-selectin), as well as on influx of extracellular Ca^{2+}, an event which precedes and is a prerequisite for platelet activation. In this context, CD62P is widely recognized as being the major platelet-derived mediator of homotypic as well as heterotypic aggregation, the latter involving neutrophils and endothelial cells, activities which are both pro-inflammatory and pro-thrombotic [16,19].

Materials and Methods

Permission to draw blood from healthy, adult human volunteers was granted by the Research Ethics Committee, Faculty of Health Sciences, University of Pretoria.

Recombinant Ply and the pneumolysoid, delta6Ply, attenuated in respect of pore-forming activity, were prepared as described previously [20, 21]. The possible influence of contaminating endotoxin was excluded in both Ply preparations using the Endosafe®-PTS™ system (Charles River Laboratories, Wilmington, MA, USA) which is based on the *Limulus* amebocyte lysate kinetic chromogenic method. Both active Ply and delta6Ply contained <1 endotoxin unit (EU)/microgram of protein after purification (1 EU is the lower limit of detection).

Unless stated otherwise all other chemicals and reagents were purchased from the Sigma Chemical Co., St Louis, MO., USA.

To prepare platelet-rich plasma (PRP), blood (anti-coagulated with 5 units/ml preservative-free heparin) was centrifuged at 1,000 rpm for 10 min at room temperature within 15 min of venepuncture and used immediately. PRP (10µl) in a final volume of 1ml Hanks' balanced salt solution (HBSS, indicator-free, 1.25 mM calcium, pH 7.4) was incubated for 5 min at 37°C after which one of the following was added: i) HBSS (control); ii) recombinant, endotoxin-free Ply (10, 20, 40, 80 ng/ml); iii) Ply (40 ng/ml) in Ca^{2+}-free HBSS; iv) inactive, recombinant delta6Ply (80 ng/ml); v) the conventional platelet activator, adenosine diphosphate (ADP, 100 mM) as a positive control system; or vi) platelet-activating factor (PAF, 400 nM) as an additional positive control system. The platelets were then incubated for a further 5 min period after which flow cytometric analyses were performed to determine CD62P expression.

The platelet suspensions (median concentration 3.2 x 10^6/ml, range 2,2 to 4.4 x 10^6/ml) were stained with 5 µl mouse anti-human CD42a-PE-labelled (Beckman Coulter, Miami, USA) and 5 µl mouse anti-human CD62P-FITC-labelled (Becton Dickenson, Franklin Lakes, USA) monoclonal antibodies to detect the total and

activated platelet populations respectively. After 15 minutes of incubation in the dark, the samples were analysed on a Gallios flow cytometer (Beckman Coulter) and the results expressed as the percentage of activated platelets with 50,000 cells interrogated during each measurement.

The Ca^{2+}, cell-permeant, fluorescent probe, fura-2/AM, was used to measure alterations in platelet cytosolic Ca^{2+} following exposure of the cells to Ply. Briefly, platelet-rich plasma, prepared as described above, was incubated with fura-2/AM (4 μM) for 45 min at 37°, and washed in PBS with ethylene glycol tetraacetic acid (EGTA, 3 mM). The platelets were resuspended in HBSS containing 0.1% bovine serum albumin and held at room temperature until used.

The fura-2-loaded cells (1-2 x 10^7/ml) were preincubated for 5 min at 37°C, after which they were transferred to disposable reaction cuvettes, which were maintained at 37°C in a Hitachi 650 10S fluorescence spectrophotometer with excitation and emission wavelengths set at 340 and 500 nm respectively. After a stable baseline was obtained (± 1 min), Ply (40 and 80 ng/ml) in the presence or absence of EGTA (3 mM), the pneumolysoid, delta6Ply, or ADP (100 μM), was added to the platelet suspension and the subsequent alterations in fura-2 fluorescence monitored over a 10 min time period.

Expression and statistical analysis of results

The results of the flow cytometric measurement of CD62P are expressed as the median values with interquartile ranges (10 separate experiments using blood from 5 different donors). Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, San Diego, USA) using a one-way ANOVA with a Bonferroni correction for multiple comparisons. The results of the experiments designed to measure alterations in platelet cytosolic Ca^{2+} are shown as the individual traces from a single representative experiment (6 in the series using cells from 3 separate donors).

Results

As shown in Figure 1, exposure of platelets to Ply caused a dose-related increase in the expression of CD62P, which achieved statistical significance at concentrations of 40 ng/ml and higher of the toxin (P<0.05), reaching levels comparable to those observed with PAF, but less than that with ADP. These effects of Ply were partly attenuated by depletion of Ca^{2+} from the cell-suspending medium, while the pneumolysoid, delta6Ply, was relatively ineffective.

The effects of exposure of platelets to Ply on cytosolic Ca^{2+} concentrations are shown in Figure 2. Treatment of platelets with Ply at concentrations of 40 or 80 ng/ml was accompanied by influx of Ca^{2+}, which following a short lag period, increased from basal levels of 29 (IQR= 23-37) nM to peak concentrations of 84(IQR= 68-100) and 146 (IQR= 120-159) nM, respectively (P <0.05 for comparison of the basal values with the corresponding peak values following addition of Ply). The peak cytosolic Ca^{2+} concentrations did not decline for the full duration that fluorescence intensity was monitored. Inclusion of EGTA in the cell-suspending medium abolished the Ply-mediated influx of Ca^{2+}, while the pneumolysoid, delta6Ply, was ineffective. An abrupt increase in fluorescence intensity which rapidly declined to basal levels was observed in the presence of ADP, indicative of a transient increase in cytosolic Ca^{2+} concentrations (results not shown).

Discussion

These findings demonstrate that exposure of human platelets to Ply at concentrations ranging from 40–80 ng/ml *in vitro* results in significant upregulation of expression of CD62P, a marker of platelet activation. These concentrations of Ply are of probable pathologic relevance being somewhat lower than those measured in the cerebrospinal fluid of patients with confirmed pneumococcal meningitis [18]. The attenuation of Ply-mediated platelet activation observed when the cells were suspended in Ca^{2+}-free medium, or following exposure to the pneumolysoid, delta6Ply, are consistent with the requirement for pore formation and influx of

extracellular Ca^{2+} in upregulation of expression of CD62P. This contention is strongly supported by the finding that exposure of platelets to Ply, but not to delta6Ply, resulted in significant influx of Ca^{2+} which was abolished by depletion of Ca^{2+} from the cell-suspending medium. Unlike the immediate increases in cytosolic Ca^{2+} which were evident following receptor-mediated activation of platelets with ADP, Ply-mediated influx of the cation occurred after a short lag phase, reaching peak responses that remained elevated and did not decline to basal levels in keeping with the pore-forming mechanism of activity of Ply.

This study has focused on CD62P because upregulation of expression of this adhesion molecule on platelets is increasingly recognized as being a key mediator of inflammatory events, directing neutrophil trafficking and activation [15]. More recently, CD62P has also been reported to be the primary mediator of heterotypic platelet-neutrophil aggregation [19], a central event in the pathogenesis of arterial thrombogenesis [22]. The mechanism of Ply-mediated platelet activation appears to involve sublytic pore formation and influx of Ca^{2+}, resulting in mobilization of CD62P-expressing α-granules.

Notwithstanding the previously documented direct, cytotoxic activities of Ply [8-13], the results of the current study are consistent with the existence of an additional potential mechanism of toxin-mediated acute adverse pulmonary and cardiac events, *viz.* platelet activation, which remains to be established in the clinical setting Nonetheless, these novel findings raise the important issue of the therapeutic potential of Ply- and platelet-directed therapies in the prevention or attenuation of pneumococcal CAP-related cardiac morbidity and mortality.

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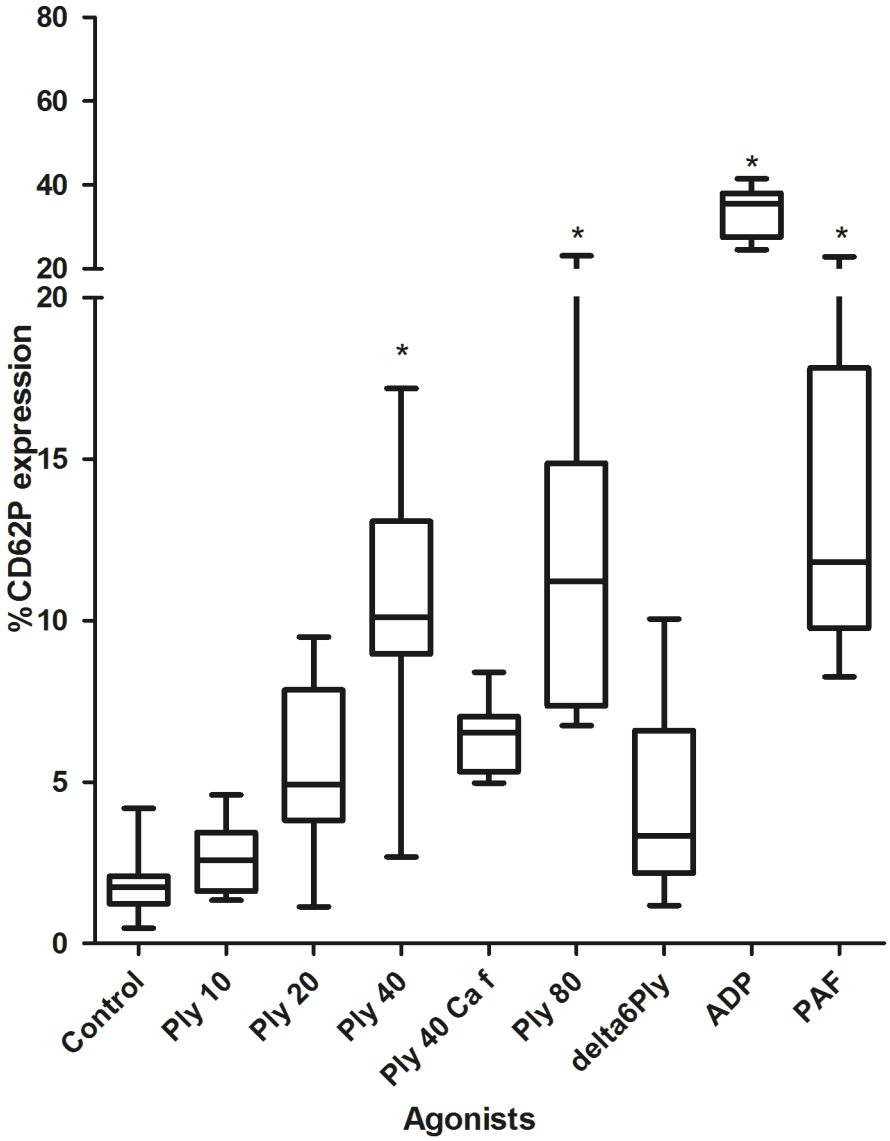
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ADP
PAF
Agonists

Figure 2

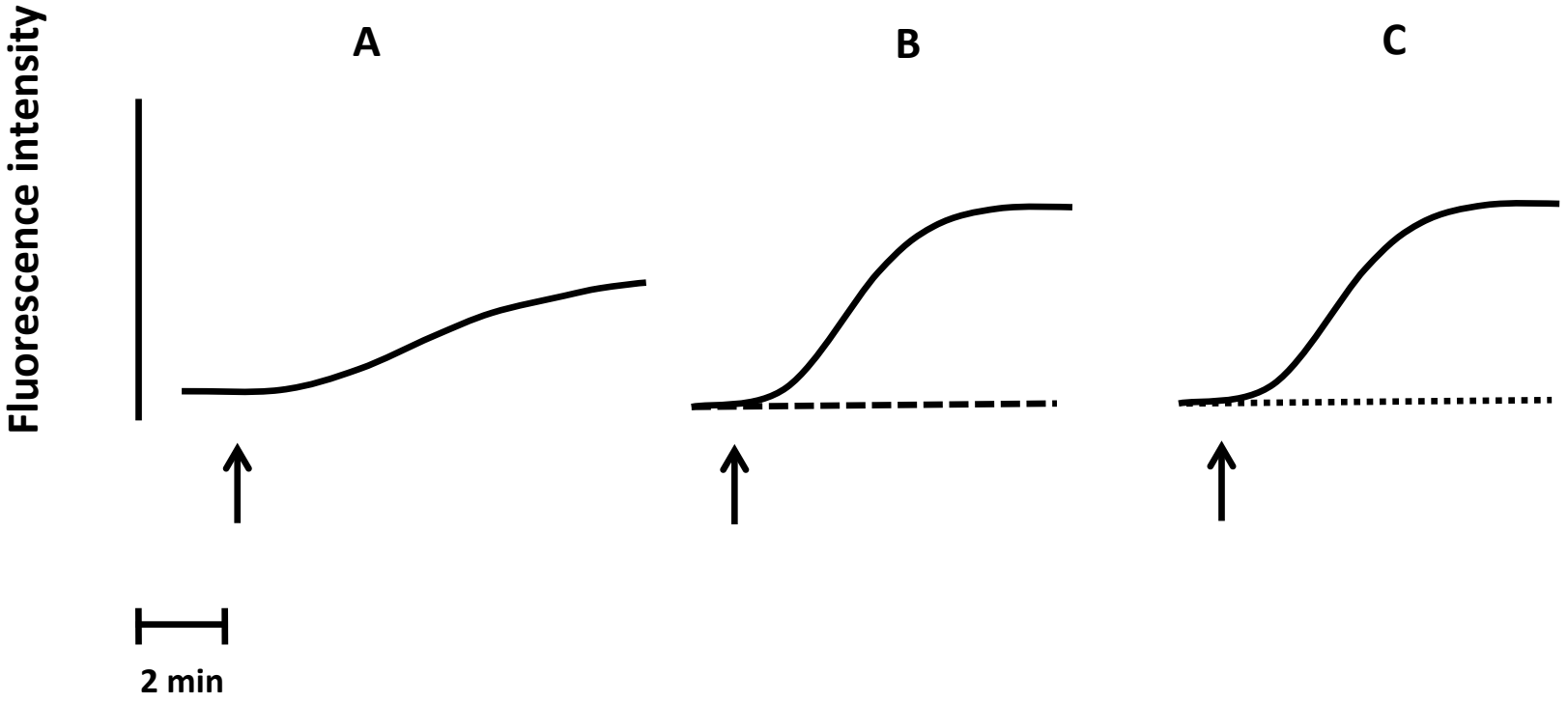

Figure Legends

Figure 1: Box - and – whisker plots showing the effects on platelet activation, measured according to the percentage of cells expressing CD62P, of: i) pneumolysin (Ply) at concentrations of 10, 20, 40, 80 ng/ml; ii) Ply (40 ng/ml) in Ca^{2+}-free medium (Ca f); iii) delta6Ply (80 ng/ml); and iv) ADP (100 μM) and PAF (400 nM) as positive controls. The results of 10 experiments are expressed as the median values with interquartile ranges (25-75%).
*P<0.05

Figure 2: The effects of pneumolysin (——) at a concentration of 40 ng/ml only on the influx of Ca^{2+} into platelets measured according to an increase in fluorescence intensity of intracellular fura-2/AM are shown in A; those of Ply at 80 ng/ml in the absence (——) or presence of EGTA (-- -- --) are shown in B. The effects of active Ply (——) in comparison with those of the pneumolysoid delta6Ply (·····) at a concentration of 80 ng/ml are shown in C. Ply and delta6Ply were added as indicated by the arrow. These traces are from a single representative experiment (6 in the series).