Characterization of the interactions of the pneumolysoid, Δ6 PLY, with human neutrophils *in vitro*.

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Running title: Effects of Δ6 PLY on neutrophils

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Abstract

The pneumolysin toxoid, Δ6 PLY, is a prototype pneumococcal protein vaccine candidate. However, its potentially detrimental residual pro-inflammatory interactions with human neutrophils are unknown. In the current study the effects of the toxoid (8-1000 ng/ml) have been compared with those of wild-type pneumolysin (WT/PLY, 8 ng/ml) on neutrophil cytosolic Ca^{2+} fluxes, generation of leukotriene B_4 (LTB_4), and release of matrix metalloproteinase-9 (MMP-9), using spectrofluorimetric, and ELISA procedures (LTB_4 and MMP-9) respectively. Exposure of neutrophils to WT/PLY resulted in influx of Ca^{2+} and significant (P<0.05) release of MMP-9 and generation of LTB_4. However, treatment of the cells with Δ6 PLY at concentrations of up to 1000 ng/ml had only trivial effects on Ca^{2+} influx and no effects on either release of MMP-9 or LTB_4 production. The observed absence of pro-inflammatory interactions of Δ6 PLY with neutrophils is clearly an important property of this pneumococcal protein vaccine candidate.

Key words: Calcium, leukotriene B_4, matrix metalloproteinase-9, neutrophils, pneumolysin.

Introduction

Although they induce capsular polysaccharide-targeted antibody responses which effectively reduce colonization and disease, the restricted number of serotypes and absence of pneumococcal protein antigens represent potential limitations of current pneumococcal conjugate vaccines. Both limitations may be overcome, however, by the development of vaccines based on highly-conserved, broadly serotype-unrestricted, recombinant, surface and sub-surface pneumococcal protein antigens.[1-4] Prominent among the protein antigen vaccine candidates are pneumococcal surface adhesin A (PsaA), and surface proteins A (PspA) and C (PspC, also known as choline-binding protein A), as well as the cholesterol-binding, pore-forming toxin, pneumolysin (PLY). PLY, for the following reasons, is generally considered to be a particularly attractive protein vaccine candidate: i) it is produced by almost all clinical isolates of the pneumococcus; [5] ii) it is generally considered to be the

most potent protein virulence factor of the pneumococcus; [5,6] iii) administration of recombinant PLY into the apical lobe bronchus of rats results in a severe lobar pneumonia with histologic features typical of pneumococcal pneumonia;[7] and iv) the toxin has already been validated as a candidate vaccine antigen.[8,9]

However, because of its cytotoxic effects on eukaryotic cells, the development of PLY-based vaccines necessitates the generation of recombinant variants of the toxin which are attenuated with respect to pore-forming activity in the setting of retention of immunogenicity. One of the most promising of these is Δ6 PLY, generated by site-directed mutagenesis (deletion of alanine 146 and arginine 147 in the pore-forming region of wild-type PLY).[10] Critical criteria for further development include immunological and histological evaluation of protective efficacy and safety in animal models, as well as *in vitro* determination of attenuation of pore-forming activity in haemolytic assays. This latter assessment, although well-accepted, does not, however, detect the sub-lytic activity which underpins the potentially harmful pro-inflammatory activity of the toxin with other cell-types such as neutrophils[11] and epithelial cells.[12] In the current study, we have therefore compared the effects of WT/PLY and Δ6 PLY on several Ca^{2+}-dependent pro-inflammatory activities of human neutrophils *in vitro.*

Materials and Methods

Unless indicated, all chemicals and reagents were obtained from the Sigma Chemical Co. (St Louis, MO, USA).

Wild-type pneumolysin and ∆6 PLY1

Recombinant WT/PLY was expressed in *Escherichia coli* and purified from cell extracts as described previously,[13] while Δ6 PLY, with a double amino acid deletion in the pore-forming region of the toxin (alanine 146 and arginine 147) was generated by site-directed mutagenesis and was also expressed in *E. coli*.[10] The purities of both toxins were confirmed by sodium dodecyl-sulphate-polyacrylamide gel electrophoresis. The stock protein

concentrations of WT/PLY and Δ6 PLY were 493 and 768 μg/ml respectively and were essentially free of contaminating endotoxin (0.103 and 0.073 IU/μg). The respective haemolytic activities (using human erythrocytes, 1% final) were 22700 and 0 haemolytic units/pmol.

Neutrophilis

Permission to draw blood from healthy adult human volunteers was granted by the Faculty of Health Sciences Research Ethics Committee of the University of Pretoria, and prior informed consent was obtained from all participants.

Purified neutrophils were prepared from heparinized venous blood as described previously using barrier centrifugation, followed in succession by sedimentation of erythrocytes with 3% gelatin, and removal of residual erythrocytes by hypotonic lysis.[11] The neutrophils, which were routinely of high purity (>90%) and viability (>95%), were resuspended to $1x10^7$ cells/ml in phosphate-buffered saline (PBS, 0.15 M; pH 7.0).

Spectrofluorimetric measurement of Ca^{2+} fluxes

Alterations in neutrophil cytosolic Ca^{2+} following exposure of the cells to WT/PLY (8 ng/ml, fixed, final concentration) and Δ6 PLY (8, 40 and 1000 ng/ml) were measured using the Ca^{2+}-sensitive, fluorescent dye, fura-2/AM as previously described.[11] Briefly, neutrophils (10^7/ml in PBS) were loaded with fura-2/AM (2 μM) for 30 min at 37°C after which they were washed and resuspended in Hanks' Balanced Salt Solution (HBSS, pH 7.4, indicator-free), and preincubated for 5 min at a concentration of $2x10^6$/ml in HBSS. The cells were then transferred to disposable reaction cuvettes that were maintained at 37°C in a thermoregulated Hitachi 650 10S fluorescence spectrophotometer with excitation and emission wavelengths set at 340 and 500 nm respectively. After a stable baseline was obtained, WT/PLY or Δ6 PLY was added to the cells and alterations in fluorescence intensity monitored over a 5-10 min period.

Matrix metalloproteinase-9 and leukotriene B_4

Neutrophils ($2x10^6$/ml in HBSS) were preincubated for 10 min at 37°C followed by the addition of WT/PLY (8 ng/ml) or Δ6 PLY (8, 40 and 1000 ng/ml). After 5 min of incubation at 37°C the reactions were stopped by the addition of an equal volume of ice-cold HBSS and the tubes placed in an ice-bath. The cells were pelleted by centrifugation and the supernatants removed and assayed by ELISA procedures for MMP-9 (Quantikine®, R & D Systems, Minneapolis, MN, USA) and LTB_4 (Correlate-EIA™, Assay Designs Inc, Ann Arbor, MI, USA) and the results expressed as ng/ml and ng/10^7 cells respectively.

Statistical analysis

With the exception of the results of the fura-2/AM fluorescence experiments, which are presented as representative traces, the results of each series of experiments are presented as the mean values ± SEM, where n= the number of different donors used. Levels of statistical significance were determined by comparing the values for systems treated with the two PLY preparations with each other, as well as with the PLY-free control system using Mann-Whitney non-parametric analysis.

Results

Cytosolic Ca^{2+} fluxes

Traces from two typical experiments (n=4 in the series) are shown in Figure 1. Addition of WT/PLY (8 ng/ml) to neutrophils was followed in succession by a short lag phase (~20sec) and a steep increase in fluorescence intensity due to influx of extracellular Ca^{2+},[11] which rapidly reached a sustained plateau phase. Δ6 PLY, at concentrations of 8 and 40 ng/ml, did not promote Ca^{2+} influx, while at 1000 ng/ml the mutant toxin caused a slight influx of Ca^{2+} following a prolonged lag phase of several minutes.

MMP-9 and leukotriene B_4

These results are shown in Figure 2. Addition of WT/PLY (8 ng/ml), but not Δ6 PLY (1000 ng/ml), caused significant release of MMP-9 from neutrophils and a striking increase in the generation of LTB_4.

Discussion

Using human neutrophils as sensitive indicator cells, we have demonstrated that the pneumolysoid, Δ6 PLY, is devoid of detectable pro-inflammatory activity, an essential property of a PLY-based pneumococcal vaccine.[14] The proposed absence of pro-inflammatory activity is based on the observations that following its addition to neutrophils, Δ6 PLY, at concentrations as high as 1000 ng/ml caused only a modest Ca^{2+} influx and failed to activate either the release of MMP-9 or the generation of LTB_4. Both of these are potent effectors of neutrophil-mediated inflammation and tissue damage.[15,16] We cannot, however, exclude the possibility that at longer exposure times, in excess of 10 minutes, significant Ca^{2+} influx and cytotoxicity may occur.

In contrast, treatment of neutrophils with an extremely low, sub-lytic concentration of PLY[11] caused significant release of MMP-9 and generation of LTB_4 as reported previously.[17,18] These pro-inflammatory interactions of the toxin with neutrophils were associated with influx of Ca^{2+},[11] an event which precedes and is a pre-requisite for mobilization of secondary/tertiary granules[19] and eicosanoid production.[20] Although Δ6 PLY at 1000 ng/ml caused modest Ca^{2+} influx following a protracted lag phase, the resultant increment in cytosolic Ca^{2+} did not achieve the necessary threshold for either degranulation or production of LTB_4.

Taken together with retention of immunogenicity and lack of toxicity in a murine model of pulmonary damage,[10] the absence of pro-inflammatory activity described in the current study underscores the promise of Δ6 PLY as a pneumococcal protein vaccine candidate. Δ6 PLY may serve as a vaccine in its own right, as a component of a vaccine containing a cocktail of pneumococcal proteins, or more likely, as a carrier of pneumococcal polysaccharides. In addition to promoting antibody production, Δ6 PLY

should also initiate the Th1/Th17 cell-mediated immune responses which have been reported to contribute to the clearance of pneumococcal colonization and prevention of mucosal disease in experimental models of pneumococcal infection.[1-4]

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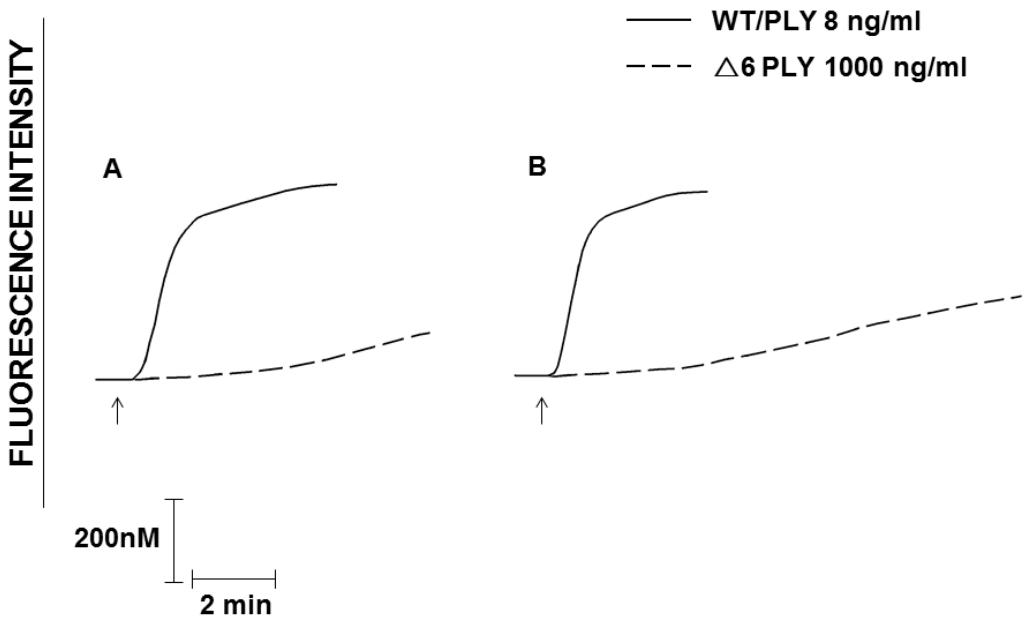

Figure 1. Alterations in cytosolic Ca^{2+} (fura-2 fluorescence) in neutrophils treated with: a) WT/PLY (8 ng/ml); and b) Δ6 PLY (1000 ng/ml) added as indicated by the arrows. The traces shown are from 2 representative experiments (A and B) using cells from 2 different donors (cells from 4 different donors were used in this series of experiments, all of which produced comparable responses).

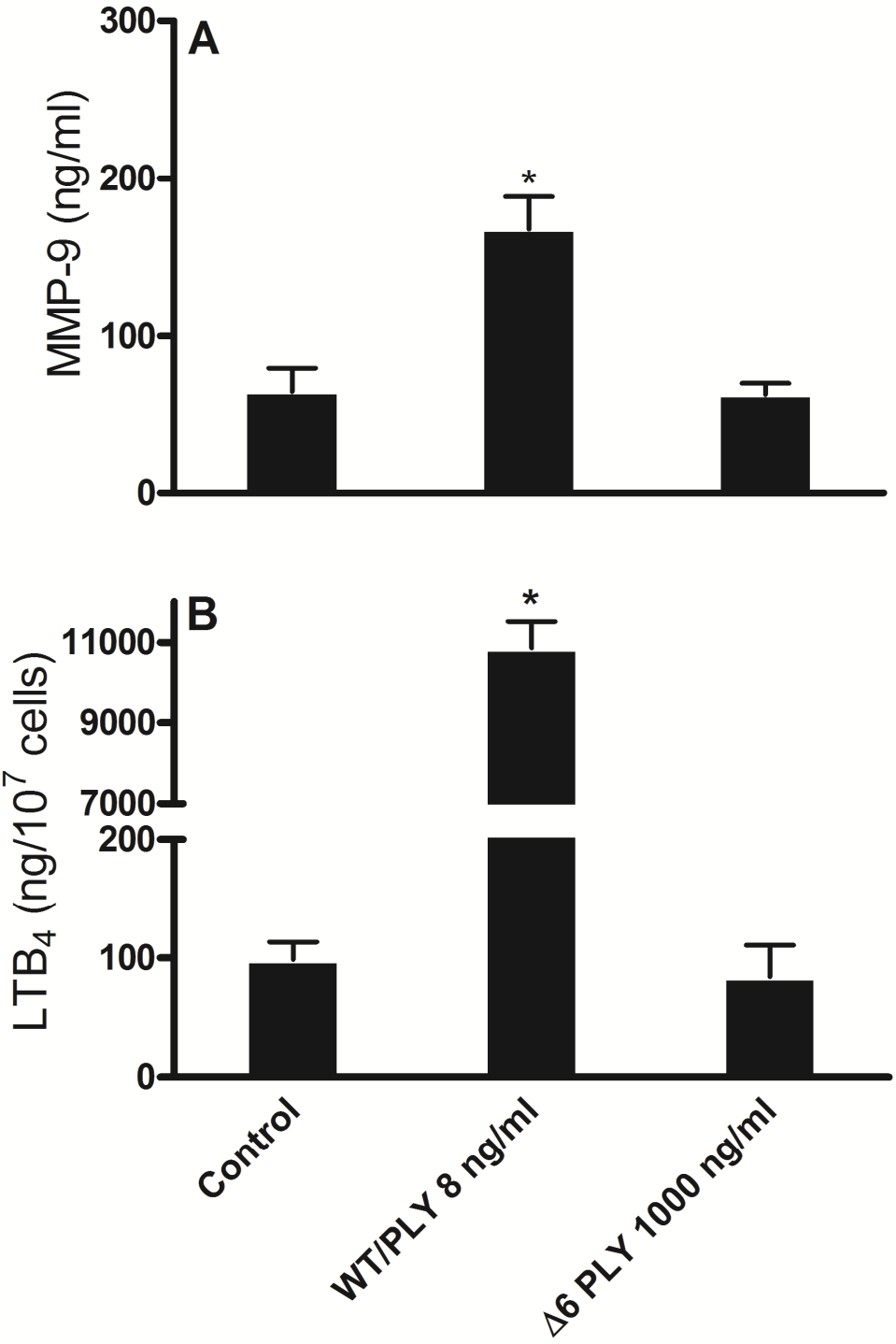

Figure 2. Effects of treatment of neutrophils with WT/PLY (8 ng/ml) and Δ6 PLY (1000 ng/ml) on the release of MMP-9 (A) and production of LTB_4 (B) by neutrophils. The results of 3 different experiments using cells from 3 different donors with 3 replicates for each system are expressed as the mean values ± SEMs. *P <0.05