Eur Respir J 2009; 34: 1167–1170
DOI: 10.1183/09031936.00007109

Pneumolysin induces release of matrix metalloproteinase-8 and -9 from human neutrophils

R. Cockeran*, T.J. Mitchell#, C. Feldman¶ and R. Anderson*

ABSTRACT: The research question addressed in the current study was: does the pneumococcal pore-forming toxin, pneumolysin, mobilise matrix metalloproteinase (MMP) -8 and -9 from isolated human blood neutrophils at sublytic concentrations of 5, 10 and 20 ng·mL^{-1}?

MMPs were measured in the supernatants of unstimulated neutrophils and of cells exposed to pneumolysin and the chemoattractant *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (f-MLP; 0.1 μM), individually and in combination, using ELISA procedures, and alterations in cytosolic Ca^{2+} concentrations were monitored using a fura-2 acetoxymethyl ester (fura-2/AM)-based spectrofluorimetric method.

Treatment of neutrophils with pneumolysin alone caused dose-related release of both MMPs, whereas f-MLP caused modest increases; the combination of both activators was, however, most effective. Pneumolysin/f-MLP-activated release of the MMPs from the cells was paralleled by increases in cytosolic Ca^{2+}.

Exposure of human neutrophils to pneumolysin is accompanied by mobilisation of MMPs, which is potentiated by f-MLP. If operative *in vivo*, pneumolysin-mediated release of MMPs from neutrophils and other cell types may contribute to the pathogenesis of severe pneumococcal disease.

KEYWORDS: Calcium, chemoattractants, matrix metalloproteinases, neutrophils, pneumolysin

Pneumolysin, a cholesterol-binding pore-forming protein toxin, is a key virulence factor of the pneumococcus, which promotes dissemination of this microbial pathogen from the lungs to the vascular space *via* its pro-inflammatory and cytotoxic activities [1, 2]. The pro-inflammatory activity of pneumolysin involves several distinct mechanisms. These include activation of immune and inflammatory cells *via* interaction of pneumolysin with Toll-like receptor 4 [3], as well as by induction of Ca^{2+} influx, particularly into phagocytes and epithelial cells, as a consequence of sublytic action of the toxin [4, 5]. In both cases, this results in activation of intracellular signalling cascades, which trigger the synthesis of a range of pro-inflammatory neutrophil- and monocyte/macrophage-activating chemokines/cytokines [6–8].

Although matrix metalloproteinases (MMPs), especially MMP-8 and -9, which are stored in neutrophil granules, have been implicated in lung and brain injury in bacterial pneumonia and meningitis, including pneumococcal meningitis [9–15], the potential of pneumolysin to activate the release of MMPs from these cells has not been investigated. In the present study, the ability of pneumolysin, alone and in combination with the chemoattractant *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (f-MLP), to activate the release of MMP-8 and MMP-9 from isolated human neutrophils was investigated.

MATERIALS AND METHODS

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

Pneumolysin

Recombinant pneumolysin was expressed in *Escherichia coli* and purified from cell extracts as described elsewhere [16]. Protein homogeneity was confirmed by sodium dodecylsulphate-polyacrylamide gel electophoresis. The stock toxin protein concentration was 0.52 mg·mL^{-1}, which corresponds to 6.1×10^5 haemolytic units·mL^{-1}, as determined by one of us (T.J. Mitchell) using sheep erythrocytes, and the stock was essentially free (<2 pg·mL^{-1}) of contaminating bacterial endotoxin. The toxin was diluted in Hank's balanced

AFFILIATIONS
*Medical Research Council, Unit for Inflammation and Immunity, Dept of Immunology, Faculty of Health Sciences, University of Pretoria, Tshwane Academic Division of the National Health Laboratory Service, Pretoria,
¶Division of Pulmonology, Dept of Medicine, Charlotte Maxeke Johannesburg Academic Hospital and Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa, and
#Division of Infection and Immunity, Institute for Biomedical and Life Sciences, University of Glasgow, Glasgow, UK.

CORRESPONDENCE
R. Cockeran
Dept of Immunology; P.O. Box 2034
Pretoria 0001
South Africa
E-mail: rcockera@medic.up.ac.za

Received:
Jan 15 2009
Accepted after revision:
March 18 2009

European Respiratory Journal
Print ISSN 0903-1936
Online ISSN 1399-3003

salt solution (HBSS; Highveld Biological (Pty), Johannesburg, South Africa; pH 7.4; indicator-free; 1.25 mM $CaCl_2$) and was used at fixed final concentrations of 5, 10 and 20 ng·mL^{-1}, which have previously been found to sensitise or activate the pro-inflammatory activities of neutrophils *in vivo* [4].

Neutrophils

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

Purified neutrophils were prepared from heparinised (5 units preservative-free heparil·mL^{-1}) venous blood as described previously [4]. The neutrophils, which were routinely of high purity (>90%) and viability (>95%) using a dye (0.1% trypan blue) exclusion procedure, were resuspended at 1×10^7 cells·mL^{-1} in PBS (0.15 M; pH 7.0) and held on ice until used.

Matrix metalloproteinases

Neutrophils were resuspended in HBSS at a density of 2×10^6 cells·mL^{-1} and pre-incubated for 10 min at 37°C. This was followed by addition of pneumolysin at final concentrations of 5, 10 and 20 ng·mL^{-1}, the chemoattractant f-MLP (0.1 μM final concentration) or pneumolysin in combination with f-MLP (the toxin was added to the cells followed 1 min later by f-MLP). The tubes were then incubated for 5 min at 37°C, after which an equal volume of ice-cold HBSS was added to each tube and the tubes placed in an ice bath in order to stop the reactions. The incubation time of 5 min was based on previous experience with f-MLP and pneumolysin, which demonstrated that the neutrophil responses to these agents were complete within this period [4, 17]. The cells were then pelleted by centrifugation and the supernatants decanted and assayed for MMP-8 and -9 using ELISA procedures (Quantikine®; R&D Systems, Minneapolis, MN, USA), and the results expressed as nanograms per millilitre of supernatant.

Spectrofluorimetric measurement of Ca^{2+} fluxes

Fura-2 acetoxymethyl ester (fura-2/AM) was used as the fluorescent Ca^{2+}-sensitive indicator in these experiments [4, 18]. Following loading with fura-2/AM (2 μM; 1×10^7 cells·mL^{-1} for 25 min at 37°C), the cells were pelleted by centrifugation and resuspended in HBSS. The neutrophils (2×10^6 cells·mL^{-1}) were then pre-incubated for 5 min at 37°C, after which the cells were transferred to disposable reaction cuvettes that were maintained at 37°C in a Hitachi 650 10S fluorescence spectrophotometer (Hitachi, Tokyo, Japan), with the excitation and emission wavelengths set at 340 and 500 nm, respectively. After a stable baseline was obtained (1 min), the neutrophils were activated by addition of pneumolysin (5, 10 and 20 ng·mL^{-1} final concentration) and f-MLP (0.1 μM final concentration) individually and in combination (f-MLP added 1 min after pneumolysin). Alterations in fluorescence intensity were then measured over a 5–10-min period. The final volume in each cuvette was 3 mL, containing a total of 6×10^6 neutrophils.

Expression and statistical analysis of results

The results of the MMP experiments are expressed as mean±SEM, and traces are shown for the fura-2/AM experiments. In both cases, n represents the number of different donors used in each series of experiments. Statistical analysis was performed by ANOVA, with subsequent Bonferroni multiple comparisons test.

RESULTS

Matrix metalloproteinases

The effects of exposure of neutrophils to pneumolysin and f-MLP individually and in combination are shown in figure 1. Addition of pneumolysin to the cells resulted in release of MMP-8 (fig. 1a) and -9 (fig. 1b), which was dose-related and achieved significance at 20 ng·mL^{-1} toxin. Exposure of the cells to f-MLP resulted in modest increases in MMP release, whereas the combination of pneumolysin and chemoattractant was more effective than the individual agents alone.

Cytosolic Ca^{2+} concentrations

Cytosolic Ca^{2+} concentration results are shown in figure 2. Addition of pneumolysin to neutrophils was followed by a lag phase, the duration of which was inversely related to the concentration of the toxin, followed by a dose-related increase in fura-2 fluorescence intensity, due to influx of Ca^{2+} [4],

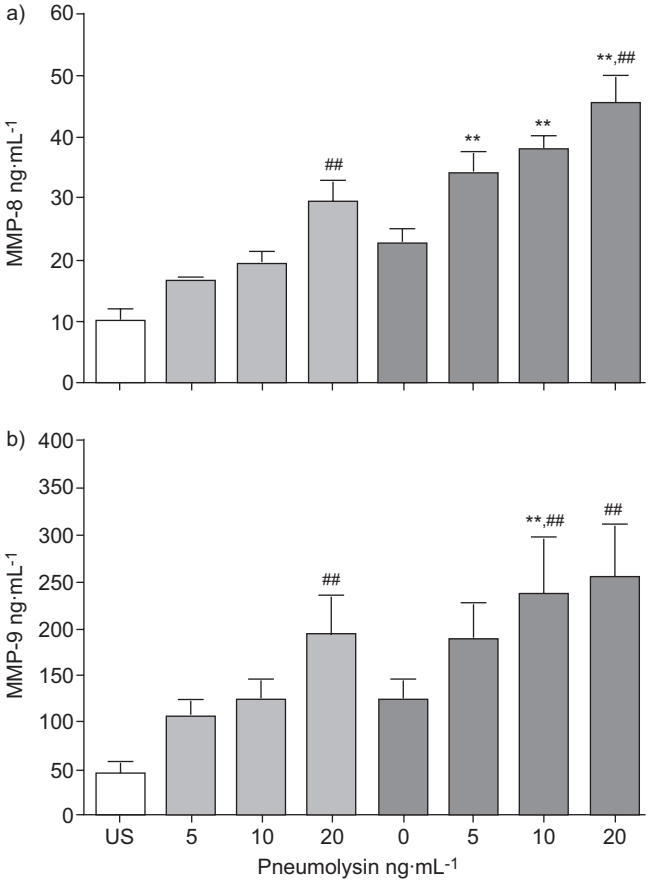

FIGURE 1. Release of: a) matrix metalloproteinase (MMP)-8; and b) MMP-9 from unstimulated neutrophils (US; □) and from cells treated with pneumolysin alone (■) and *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (0.1 μM) alone or in combination with pneumolysin (■). Total cellular (2×10^6 neutrophils) MMP-8 and -9 were 186±33 and 722±142 ng·mL^{-1}, respectively. The results of four different experiments using cells from four different donors are presented as mean±SEM. **: $p<0.01$ *versus* treatment with the individual agents; ##: $p<0.01$ *versus* corresponding treatment without pneumolysin.

reaching sustained peak plateau levels, the magnitudes of which were related to the pneumolysin concentration (fig. 2a). Addition of f-MLP to the cells was accompanied by the typical immediate increase in fura-2 fluorescence intensity compatible with mobilisation of Ca^{2+} from intracellular stores (fig. 2b). This was followed by two successive phases, first a rapid decline in fluorescence intensity due to Ca^{2+} efflux, as well as resequestration of Ca^{2+} into stores, and, secondly, a more gradual decline due to store-operated influx of the cation [17]. When the cells were exposed to the combination of pneumolysin and f-MLP, the magnitude of the abrupt f-MLP-mediated increase in fluorescence intensity remained unchanged. However, the decline in fluorescence intensity was slower and of lesser magnitude than that observed with f-MLP alone, being inversely related to the pneumolysin concentration and compatible with Ca^{2+} flooding of the cytosol (fig. 2b).

DISCUSSION

As is the case with the primary granule protease, elastase, the secondary and tertiary granule MMPs, MMP-8 and -9, are essential for the protective functions of neutrophils. Elastase not only facilitates transendothelial migration of neutrophils [19] but also protects against Gram-negative bacterial pathogens by degrading major outer membrane proteins [20], and the MMPs are necessary for extracellular matrix degradation and neutrophil migration [21, 22]. However, if the release of neutrophil-derived proteolytic enzymes, particularly MMPs, is poorly regulated and excessive, as may occur during hyperacute inflammatory responses, the risk of injury to bystander cells and tissues is likely to be considerable [9–15].

Although pneumolysin is considered to be a key player in promoting extrapulmonary dissemination of the pneumococcus and mediating neurological damage in severe pneumococcal infection, *via* both cytotoxic and pro-inflammatory mechanisms [1, 2], the potential of the toxin to induce the release of MMPs from human neutrophils has not, to our knowledge, been addressed. In the present study, we investigated the effects of exposure of isolated human neutrophils to pneumolysin, at concentrations of 5–20 $ng \cdot mL^{-1}$, in the presence and absence of the chemoattractant f-MLP on the release of MMP-8 and -9. Pneumolysin concentrations of up to 9 $\mu g \cdot mL^{-1}$ and 180 $ng \cdot mL^{-1}$ have been detected in pneumococcal culture fluids *in vitro* and in the cerebrospinal fluid of patients with pneumococcal meningitis, respectively [23, 24]. *N*-formylated polypeptide chemoattractants are produced by the pneumococcus [25, 26], and may act in concert with pneumolysin to augment the pro-inflammatory activities of neutrophils, as well as those of monocytes/macrophages [4].

Although exposure of neutrophils to either f-MLP or pneumolysin, in particular, resulted in release of MMP-8 and -9 from the cells, prior treatment with pneumolysin followed by addition of f-MLP resulted in augmentation of MMP release, reaching levels which were significantly higher than those attained with the toxin or chemoattractant individually. Although proportionately similar, the absolute concentrations of MMP-8 released from neutrophils activated with pneumolysin and f-MLP, individually and in combination, were less than those of MMP-9, which appeared to reflect the total intracellular concentrations of these enzymes. The higher intracellular level of MMP-9 observed in the present study may reflect the relative intracellular distribution of MMP-8 and -9, with the former being located exclusively in secondary granules, and the latter in both secondary and tertiary granules [27].

Exocytosis of primary, secondary and tertiary neutrophil granules are Ca^{2+}-dependent events, with the latter two granule types being more readily mobilised than the primary granules, the threshold value for cytosolic Ca^{2+} required for significant release being 200–300 nM [28, 29]. In the present study, this threshold value was attained and exceeded, with the release of both MMPs being closely correlated with pneumolysin/f-MLP-mediated increases in neutrophil cytosolic Ca^{2+}, with the combination of f-MLP and pneumolysin resulting in sustained increases in cytosolic Ca^{2+} levels.

Depending on local concentrations, pneumolysin appears to play a dual role in pneumococcal host defences [2]. At low

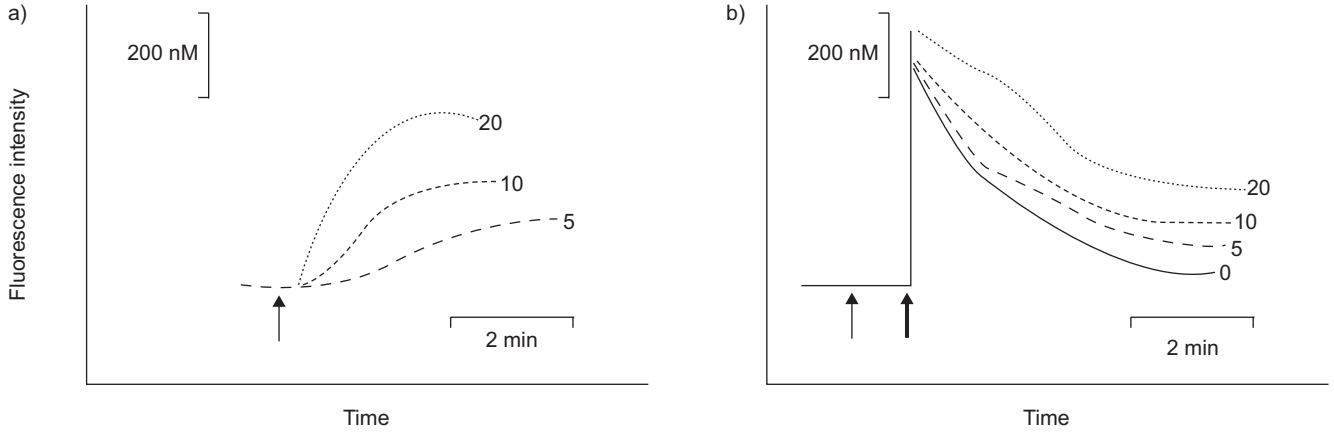

FIGURE 2. Alterations in cytosolic Ca^{2+} concentration (fura-2 fluorescence) in neutrophils treated with: a) pneumolysin; and b) *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (f-MLP; 0.1 μM) alone and in combination with pneumolysin (——: 0 $ng \cdot mL^{-1}$; – – – –: 5 $ng \cdot mL^{-1}$; -------: 10 $ng \cdot mL^{-1}$; ……: 20 $ng \cdot mL^{-1}$). Pneumolysin concentrations (in nanograms per millilitre) are indicated. The thin arrow denotes pneumolysin addition; the thick arrow denotes f-MLP addition. The traces are from a single representative experiment using cells from a single donor (cells from four different donors were used in this series of experiments, all of which produced comparable responses).

concentrations, the toxin, possibly acting in concert with bacterially derived chemoattractants, may promote anti-pneumococcal host defences, preventing colonisation of the airways [5]. In severe pneumococcal infection, conversely, excessive production of the toxin may promote hyperacute, damaging inflammatory responses [1].

Notwithstanding the relatively small sample size used in the present study and the requirement for confirmation in animal models of experimental infection, the current findings demonstrate that exposure of human neutrophils to extremely low concentrations of pneumolysin results in the release of MMP-8 and -9, which is potentiated by f-MLP, a mimic of pneumococcal chemoattractants. If operative during severe pneumococcal infection, this mechanism may contribute to the pathology of lung and brain injury, underscoring the potential value of antimicrobial agents, especially macrolides, which target the synthesis of pneumolysin [30, 31].

STATEMENT OF INTEREST

A statement of interest for T.J. Mitchell can be found at www.erj.ersjournals.com/misc/statements.dtl

REFERENCES

1 del Mar García-Suárez M, Flórez N, Astudillo A, *et al.* The role of pneumolysin in mediating lung damage in a lethal pneumococcal pneumonia murine model. *Respir Res* 2007; 8: 3.
2 Marriott HM, Mitchell TJ, Dockrell DH. Pneumolysin: a double-edged sword during the host-pathogen interaction. *Curr Mol Med* 2008; 8: 497–509.
3 Malley R, Henneke P, Morse SC, *et al.* Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. *Proc Natl Acad Sci USA* 2003; 100: 1966–1971.
4 Cockeran R, Theron AJ, Steel HC, *et al.* Proinflammatory interactions of pneumolysin with human neutrophils. *J Infect Dis* 2001; 183: 604–611.
5 Ratner AJ, Hippe KR, Aguilar JL, *et al.* Epithelial cells are sensitive detectors of bacterial pore-forming toxins. *J Biol Chem* 2006; 281: 12994–12998.
6 Cockeran R, Durandt C, Feldman C, *et al.* Pneumolysin activates the synthesis and release of interleukin-8 by human neutrophils *in vivo*. *J Infect Dis* 2002; 186: 562–565.
7 Baba H, Kawamura I, Kohda C, *et al.* Induction of gamma interferon and nitric oxide by truncated pneumolysin that lacks pore-forming activity. *Infect Immun* 2002; 70: 107–113.
8 Koga T, Lim JH, Jono H, *et al.* Tumor suppressor cylindromatosis (CYLD) acts as a negative regulator for *Streptococcus pneumoniae*-induced NFAT signalling. *J Biol Chem* 2008; 283: 12546–12554.
9 Leib SL, Leppert D, Clements J, *et al.* Matrix metalloproteinases contribute to brain damage in experimental pneumococcal meningitis. *Infect Immun* 2000; 68: 615–620.
10 Leppert D, Leib SL, Grygar C, *et al.* Matrix metalloproteinase (MMP)-8 and MMP-9 in cerebrospinal fluid during bacterial meningitis: association with blood-brain barrier damage and neurological sequelae. *Clin Infect Dis* 2000; 31: 80–84.
11 Hartog CM, Wermelt JA, Sommerfeld GO, *et al.* Pulmonary matrix metalloproteinase excess in hospital-acquired pneumonia. *Am J Respir Crit Care Med* 2003; 167: 593–598.
12 Meli DN, Christen S, Leib SL. Matrix metalloproteinase-9 in pneumococcal meningitis: activation *via* an oxidative pathway. *J Infect Dis* 2003; 187: 1411–1415.
13 Kim JH, Suk MH, Yoon DW, *et al.* Inhibition of matrix metalloproteinase-9 prevents neutrophilic inflammation in ventilator-induced lung injury. *Am J Physiol Lung Cell Mol Physiol* 2006; 291: L580–L587.
14 Nguyen X, O'Barr TJ, Anderson AJ. Polymorphonuclear leukocytes promote neurotoxicity through release of matrix metalloproteinases, reactive oxygen species, and TNF-α. *J Neurochem* 2007; 102: 900–912.
15 Schaaf B, Liebau C, Kurowski V, *et al.* Hospital acquired pneumonia with high-risk bacteria is associated with increased pulmonary matrix metalloproteinase activity. *BMC Pulm Med* 2008; 8: 12.
16 Saunders FK, Mitchell TJ, Walker JA, *et al.* Pneumolysin, the thiol-activated toxin of *Streptococcus pneumoniae*, does not require a thiol group for *in vitro* activity. *Infect Immun* 1989; 57: 2547–2552.
17 Anderson R, Goolam Mahomed A. Calcium efflux and influx in f-met-leu-phe (fMLP)-activated human neutrophils are chronologically distinct events. *Clin Exp Immunol* 1997; 110: 132–138.
18 Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 1985; 260: 3440–3450.
19 Kaynar AM, Houghton AM, Lum EH, *et al.* Neutrophil elastase is needed for neutrophil emigration into lungs in ventilator-induced lung injury. *Am J Respir Cell Mol Biol* 2008; 39: 53–60.
20 Hirche TO, Benabid R, Deslee G, *et al.* Neutrophil elastase mediates innate host protection against *Pseudomonas aeruginosa*. *J Immunol* 2008; 181: 4945–4954.
21 D'Haese A, Wuyts A, Dillen C, *et al. In vivo* neutrophil recruitment by granulocyte chemotactic protein-2 is assisted by gelatinase B/MMP-9 in the mouse. *J Interferon Cytokine Res* 2000; 20: 667–674.
22 Renckens R, Roelofs JJ, Florquin S, *et al.* Matrix metalloproteinase-9 deficiency impairs host defence against abdominal sepsis. *J Immunol* 2006; 176: 3735–3741.
23 Feldman C, Mitchell TJ, Andrew PW, *et al.* The effect of *Streptococcus pneumoniae* pneumolysin on human respiratory epithelium *in vitro*. *Microb Pathog* 1990; 9: 275–284.
24 Spreer A, Kerstan H, Böttcher T, *et al.* Reduced release of pneumolysin by *Streptococcus pneumoniae in vitro* and *in vivo* after treatment with nonbacteriolytic antibiotics in comparison to ceftriaxone. *Antimicrob Agents Chemother* 2003; 47: 2649–2654.
25 Gao J-L, Lee EJ, Murphy PM. Impaired antibacterial host defence in mice lacking the *N*-formylpeptide receptor. *J Exp Med* 1999; 189: 657–662.
26 Partida-Sanchez S, Cockayne DA, Monard S, *et al.* Cyclic ADP-ribose production by CD38 regulates intracellular calcium release, extracellular calcium influx and chemotaxis in neutrophils and is required for bacterial clearance *in vivo*. *Nat Med* 2001; 7: 1209–1216.
27 Borregaard N, Lollike K, Kjeldsen L, *et al.* Human neutrophil granules and secretory vesicles. *Eur J Haematol* 1993; 51: 187–198.
28 Lew PD, Monod A, Waldvogel FA, *et al.* Quantitative analysis of the cytosolic free calcium dependency of exocytosis from three subcellular compartments in intact human neutrophils. *J Cell Biol* 1986; 102: 2197–2204.
29 Pivot-Pajot C, Varoqueaux F, de Saint Basile G, *et al.* Munc13-4 regulates granule secretion in human neutrophils. *J Immunol* 2008; 180: 6786–6797.
30 Fukuda Y, Yanagihara K, Higashiyama Y, *et al.* Effects of macrolides against pneumolysin of macrolide-resistant *Streptococcus pneumoniae*. *Eur Respir J* 2006; 27: 1020–1025.
31 Anderson R, Steel HC, Cockeran R, *et al.* Comparison of the effects of macrolides, amoxicillin, ceftriaxone, doxycycline, tobramycin and fluoroquinolones, on the production of pneumolysin by *Streptococcus pneumoniae in vitro*. *J Antimicrob Chemother* 2007; 60: 1155–1158.