

Induction of necrosis and apoptosis of neutrophil granulocytes by *Streptococcus pneumoniae*

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SUMMARY

Apoptosis followed by macrophage phagocytosis is the principal mechanism by which neutrophil granulocytes (PMN) are removed from the site of inflammation. To investigate whether *Streptococcus pneumoniae* causes apoptosis of PMN, we exposed PMN to viable and heat-killed pneumococci and purified pneumococcal cell walls (PCW). The occurrence of PMN cell death was quantified by flow cytometry using annexin V/propidium iodide labelling of the cells. Intracellular histone-associated DNA fragments were quantified by ELISA. The presence of apoptosis was confirmed by *in situ* tailing. Exposure of PMN to viable pneumococci caused necrosis of the cells. The pneumococcal cytotoxin pneumolysin, the bacterial production of hydrogen peroxide, and PCW contributed to necrosis. Heat-killed pneumococci accelerated the process of apoptosis observed in cultivated non-stimulated PMN *in vitro*. These results demonstrated that pneumococci induce PMN cell death. Depending on the intensity of the stimulus, PMN necrosis and apoptosis were observed.

Keywords neutrophil cytology PMN cell death pneumococcal cell walls pneumolysin hydrogen peroxide

INTRODUCTION

Neutrophils (PMN) play a principal role in host defence against bacterial infection. During the onset of inflammation, mononuclear cells and PMN invade the tissue by transmigration through the layer of activated endothelial cells. One major function of PMN consists of phagocytosis of bacteria. After a short life span PMN are removed from the site of inflammation by macrophage engulfment after undergoing apoptosis [1–3]. The programme of apoptosis is constitutive and regulated by microenvironmental signals [4,5]. The process of transmigration itself prolongs the survival time of the granulocytes. In this process cross-linking of β_2 -integrins on the surface of PMN seems to play a major role [5]. Once PMN have reached the site of inflammation, the cells are exposed to a variety of cytokines. Granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), adenosine polyphosphates, IL-2, IL-4, IL-6, IL-8, IL-15, platelet-activating factor (PAF), and the complement component C5a lead to prolonged survival of PMN [6–13]. The interaction of PMN with monocytes and platelets delays apoptotic cell death [14,15]. Glucocorticoids prolong PMN survival [16,17]. Tumour necrosis factor-alpha (TNF- α) and IL-10 are host

mediators inducing PMN apoptosis [18–20]. During anti-bacterial host defence, bacteria and bacterial products act on PMN function and life span. *In vitro* stimulation of PMN with the Gram-negative organisms *Escherichia coli* and *Haemophilus somnus* [21–23] supported the hypothesis that PMN were cleared from the site of inflammation by apoptosis in bacterial infection. PMN underwent apoptosis after contact with these Gram-negative bacteria. However, although stimulation with low-dose *E. coli* resulted in apoptosis, high doses induced necrosis of the PMN [24].

Streptococcus pneumoniae is the most frequent bacterial pathogen causing otitis media, pneumonia and meningitis in humans. Three distinct pneumococcal products are released during bacterial growth which have been shown to damage host cells: the cytotoxin pneumolysin [25], hydrogen peroxide [26,27], and pneumococcal cell wall components [28]. In this study we evaluated the influence of viable and heat-killed pneumococci, and of cytotoxic pneumococcal products on the regulation of PMN survival.

MATERIALS AND METHODS

Preparation of PMN

PMN were isolated from buffy coats derived from healthy human donors using plasma Percoll gradients (Pharmacia Biotech, Freiburg, Germany) and were cultured in Dulbecco's minimal essential medium (DMEM) with 10% fetal calf serum (FCS).

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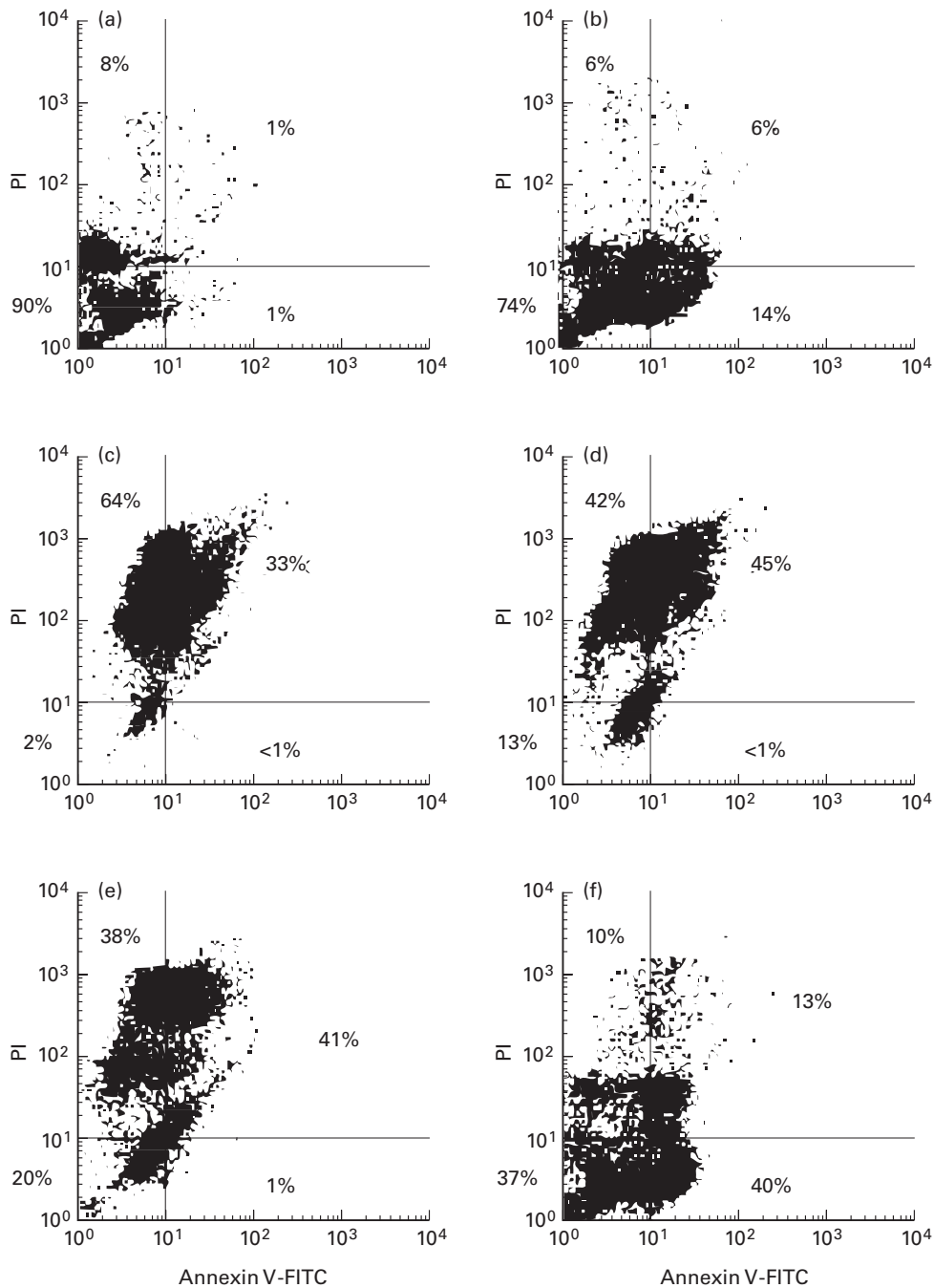


Fig. 1. FACS analysis of PMN after labelling the cells with annexin V-FITC and propidium iodide (PI). Viable cells were negative for annexin V-FITC and PI (lower left quadrant). Annexin V labelling indicated apoptosis (lower right quadrant). Cells which were stained with PI were necrotic (upper quadrants) [32]. Unstimulated freshly isolated PMN (a) and PMN after 19 h *in vitro* culture: unstimulated controls (b), after addition of 10^7 viable pneumococci (D39)/ml (c), 10^7 viable pneumolysin-deficient pneumococci (D39ply::pJDC9)/ml (d), 10^7 viable pneumolysin-deficient pneumococci (D39ply::pJDC9)/ml + catalase (2000 EU/ml) (e), 10^7 heat-killed pneumococci (D39)/ml (f).

Stimulation was performed at 37°C immediately after isolation of the cells. At least three distinct preparations were used for the experiments. Representative results were shown in the Figures.

Pneumococcal strain

An encapsulated virulent pneumococcal strain D39 (NCTC 7466) and its unencapsulated avirulent variant (R6x [29]) were used in

this study. For purification of pneumococcal cell walls (PCW) the unencapsulated strain was used. A pneumolysin-deficient knockout mutant of the strain D39 was generated by insertion duplication mutagenesis of the pneumolysin gene using the plasmid pJDC9 (D39ply::pJDC9) [30]. Mutants were screened on antibiotic selection media (LB-agar, 5% sheep blood, 1 µg/ml erythromycin). Correct insertion was confirmed by polymerase

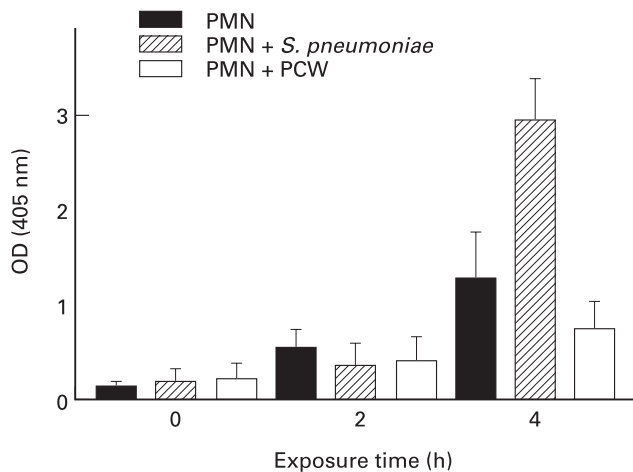


Fig. 2. Apoptosis was determined by ELISA measuring the concentration of cellular histone-associated DNA fragments. The concentrations were determined 0 h, 2 h, and 4 h after the addition of heat-killed pneumococci (equalling 10^7 colony-forming units (CFU)/ml) or pneumococcal cell walls (PCW; $50 \mu\text{g/ml}$) to the *in vitro* culture of PMN. OD, Optical density.

chain reaction (PCR) and sequencing. The deficiency of pneumolysin production was checked by haemolysis assay as described previously [31].

The bacteria were grown in Todd–Hewitt broth to mid-logarithmic phase, harvested by centrifugation, washed in PBS, and heat-killed by incubation at 60°C for 20 min. Bacteria were resuspended in cell culture medium to a final concentration equalling 10^7 colony-forming units (CFU)/ml.

Preparation of PCW

The bacteria were washed in 50 mM Tris buffer pH 7.0 and added to preheated 5% SDS solution and boiled for 15 min. Bacterial cell walls were obtained from the supernatant by centrifugation at $17\,000\text{ g}$, 10 min. The pellets were washed twice in 1 M NaCl and three times in distilled water to remove remaining SDS. The pellet containing larger bacterial cell fragments were broken mechanically by a glass bead stirrer. The supernatant was spun down at 700 g and the cell-free supernatant was spun down again at $38\,000\text{ g}$ for 15 min to obtain bacterial fragments. The pellet was resuspended in 100 mM Tris buffer pH 7.5 containing NaN_3 0.05%, MgSO_4 20 mM, DNase $10 \mu\text{g/ml}$, RNase $50 \mu\text{g/ml}$, and incubated at 37°C for 2 h. Then, trypsin was added to a final concentration of $50 \mu\text{g/ml}$ and CaCl_2 to a concentration of 10 mM. The solution was digested overnight at 37°C . SDS was added to a final concentration of 1% and incubated at 60°C for 15 min. Pneumococcal cell walls were pelleted at $38\,000\text{ g}$ and washed four times with distilled water until SDS had been completely removed. The pellet consisting of PCW was lyophilized and resuspended to a final concentration of 50 mg/ml in distilled water. PCW were used at a final concentration of 50–200 $\mu\text{g/ml}$ for stimulation experiments.

Analysis of PMN by flow cytometry for apoptosis and necrosis

For identification of apoptosis and necrosis of the PMN the binding of annexin V and the uptake of propidium iodide were measured by flow cytometry [32] (FACSCalibur; Becton Dickinson, Heidelberg, Germany) using the apoptosis detection kit TACS annexin V–FITC (R&D Systems, Wiesbaden, Germany)

according to the manufacturer's instructions. Flow cytometric analysis was performed using the Cell-Quest software program (Becton Dickinson). Logarithmic fluorescence intensity of annexin V–FITC was plotted *versus* the fluorescence intensity of propidium iodide (PI) in a dot plot. Data from 10 000 PMN were analysed for each plot. The experiments were performed three times, and results of a representative experiment are shown.

ELISA detection of histone-associated DNA fragments

For measuring intracellular histone-associated DNA fragments of PMN an ELISA was used according to the manufacturer's instructions (Cell Death Detection ELISA^{PLUS}; Roche Molecular Biochemicals, Mannheim, Germany). PMN (10 000) were obtained from the culture and pelleted. The cells were lysed and the supernatant containing the nuclear derived histone-associated DNA fragments was used for ELISA. The optical density (OD)_{405nm} gave the degree of DNA fragmentation.

In situ tailing

Smears of PMN after 24 h *in vitro* culture were used for staining apoptotic nuclei by *in situ* tailing. Cells were fixed by ethanol, rehydrated, and treated for 15 min at 37°C with $50 \mu\text{g/ml}$ proteinase K (Sigma, Deisenhofen, Germany). The preparations were incubated for 1 h at 37°C in a reaction mixture containing $10 \mu\text{l}$ of $5\times$ tailing buffer, $1 \mu\text{l}$ digoxigenin (DIG) DNA labelling mix, $2 \mu\text{l}$ cobalt chloride, 12 U terminal transferase and the necessary amount of distilled water to give a volume of $50 \mu\text{l}$. After washing, the preparations were incubated with 10% FCS for 15 min at room temperature and then washed again. A solution of alkaline phosphatase-labelled anti-DIG antibody in 10% FCS was placed on the sections for 60 min at 37°C . The colour reaction was developed with 4-nitroblue-tetrazolium-chloride/5-bromine-4-chloride-3-indolyl-phosphate (NBT/BCIP). The preparations were counterstained with nuclear fast red–aluminium hydroxide. All reagents were purchased from Roche Molecular Biochemicals.

RESULTS

After preparation of PMN from buffy coats, the cells were cultured *in vitro*. Before adding the bacterial stimuli, fresh isolated PMN were used for annexin V/propidium iodide staining and subsequent FACS analysis to check the viability of the cells. Ninety percent of PMN were viable, only a small population showed signs of necrosis, and few cells were labelled with annexin V (Fig. 1a). After 19 h of *in vitro* culture the PMN were stained for cell viability. In unstimulated control cultures the quantity of annexin V⁺ cells increased (14% *versus* 1% at the time of preparation), indicating that apoptosis had been induced. Only a small population became necrotic, as revealed by propidium iodide staining (12%) (Fig. 1b).

Experiments with encapsulated strain D39 revealed comparable results to those with the unencapsulated strain R6x. Purified cell walls were prepared from the strain R6x. Therefore, the results obtained from stimulation experiments using the virulent strain D39 are shown. Viable pneumococci at 10^7 CFU/ml predominantly induced PMN necrosis, as revealed by propidium iodide uptake (Fig. 1c). To investigate the influence of the major cytotoxic products of the pneumococcus—pneumolysin, hydrogen peroxide, and PCW—the experiments were performed with a pneumolysin-negative pneumococcal mutant (D39ply::pJDC9) in the presence and absence of catalase and with PCW at

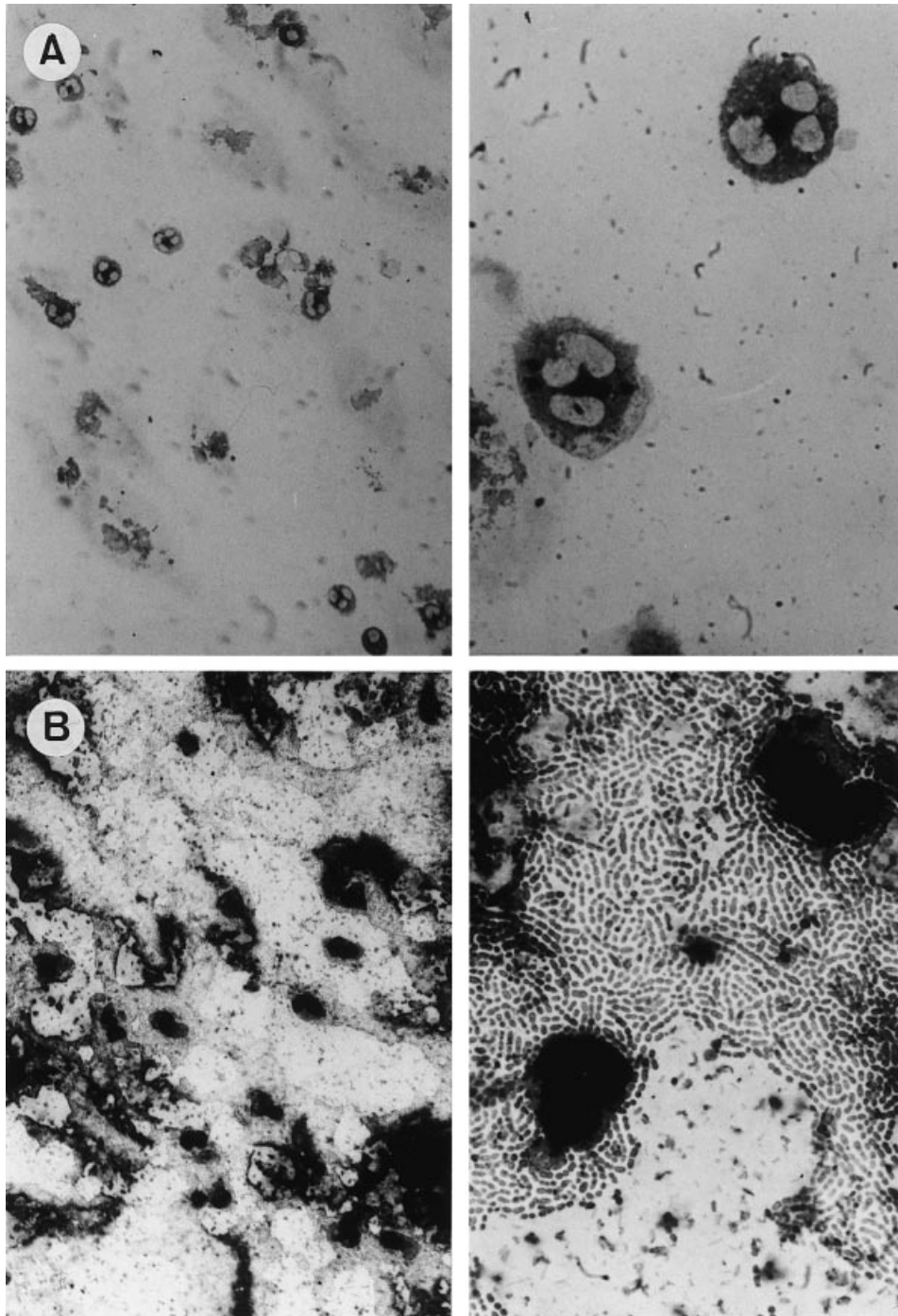


Fig. 3. Detection of apoptotic nuclei (dark staining) of PMN by *in situ* tailing. The tailing reaction was performed 24 h after PMN had been isolated. Controls rarely showed apoptosis (A), whereas in cultures stimulated with heat-killed pneumococci a high rate of PMN apoptosis was observed (B).

concentrations ranging from 50 to 200 $\mu\text{g/ml}$. Viable bacteria of the pneumolysin-negative mutant induced necrosis of PMN (Fig. 1d). However, a slight reduction in cytotoxicity was observed (13% viable cells *versus* 2% in cultures stimulated with the viable D39). The experiment with the pneumolysin-negative mutant was performed in the presence of catalase (2000 EU/ml) to investigate whether the production of hydrogen peroxide by the pneumococcus was the mechanism by which the mutant caused

necrosis of PMN. In this experiment, the rate of viable PMN increased up to 20% (Fig. 1e). These experiments demonstrated that pneumolysin as well as hydrogen peroxide contributed to the necrosis of PMN in the experiments with viable pneumococci. Pneumococci undergo autolysis during growth. During autolysis PCW are released into the surrounding bacteria. Therefore, in another experiment, purified PCW were added to the PMN culture at concentrations up to 200 $\mu\text{g/ml}$. The experiments resulted in

poor reproducibility of the results. Signs of enhanced necrosis were observed, and the rate of apoptotic PMN was comparable to that of unstimulated controls. In some experiments, especially in those with a background of necrotic PMN in the primary preparation, the results did not differ from those of unstimulated controls.

For a second set of experiments, heat-killed pneumococci were used at a concentration equalling 10^7 CFU/ml. Nineteen hours after adding the inactivated bacteria to the PMN culture, 40% of the PMN were labelled with annexin V-FITC and showed no staining with propidium iodide. Therefore, an early phase of apoptosis was observed (Fig. 1f).

The induction of PMN apoptosis by heat-killed pneumococci was confirmed by two additional methods. After 4 h, the concentration of histone-associated DNA fragments (HA-DNA) in the cellular fraction was measured. The HA-DNA in the cellular fraction should be elevated if apoptosis has induced. Heat-inactivated pneumococci used at a concentration equalling 10^7 CFU/ml led to a significant increase of HA-DNA compared with controls. PCW (50 μ g/ml) decelerated the increase, indicating a lower quantity of apoptotic cells (Fig. 2). The induction of PMN apoptosis by heat-killed pneumococci was visualized by *in situ* tailing. Twenty-four hours after pneumococci had been added to the culture medium almost all PMN were stained positively by *in situ* tailing, whereas only few PMN of control or PCW-stimulated cultures showed nuclei marked by this method (Fig. 3).

DISCUSSION

This study demonstrates that *S. pneumoniae* is capable of inducing cell death in PMN. Viable pneumococci undergo autolysis during growth and treatment with anti-bacterial agents which primarily affect cell wall synthesis [33,34]. During bacterial lysis not only cell wall components are released into the surrounding but also the cytotoxin pneumolysin [35]. Hydrogen peroxide has been identified as a product of pneumococcal metabolism. Hydrogen peroxide and pneumolysin are toxic to eukaryotic cells [26,27]. After heat inactivation pneumococci do not produce hydrogen peroxide and the bacteria do not undergo further autolysis. Therefore, neither pneumolysin nor cell wall components are released. The bacteria act on eukaryotic cells by the components present at the cell surface.

In this study, PMN were exposed to metabolically active pneumococci, pneumolysin-negative pneumococcal mutants, the latter in the presence of hydrogen peroxide-neutralizing activity of catalase, heat-killed pneumococci, and PCW. The results of the study demonstrate differences in the cytotoxic capacity of the stimuli. Whereas viable wild-type pneumococci cause necrosis of most of the PMN, knocking out the pneumolysin gene and neutralization of hydrogen peroxide attenuate the cytotoxic potential of pneumococci. Heat-killed pneumococci caused PMN apoptosis instead of necrosis. In the experiments with heat-killed pneumococci, neither pneumolysin nor hydrogen peroxide, nor the release of PCW could play a significant role in the interaction with PMN. Therefore, heat-killed pneumococci are a less potent stimulus with reduced cytotoxic potential. Probably, the switch between apoptosis and necrosis primarily depends on the intensity of the stimulus. Similarly, in stimulation experiments of PMN with *E. coli* low concentrations of viable bacteria caused apoptosis, whereas high concentrations induced necrosis [24].

In vivo, PMN are exposed to both toxic products of the bacterial metabolism and bacterial components. Which of the tested bacterial stimuli displayed best the situation *in vivo* remains speculative. *In vivo*, apoptosis has been identified as the major form of death by which leucocytes terminate inflammation. Up to 40% of apoptotic PMN have been reported in the cerebrospinal fluid of patients suffering from bacterial meningitis [2]. In an animal model of pneumococcal meningitis the rate of apoptotic PMN peaked 8 h after the initiation of antibiotic treatment with β -lactam antibiotics [2]. These experiments suggest that killed pneumococci and the release of pneumococcal products trigger the induction of PMN apoptosis *in vivo*.

In this study, we could not clarify the role of PCW in necrosis and apoptosis induction *in vitro*. According to present knowledge about the pneumococcus, the remaining cytotoxic potential of the pneumolysin-negative pneumococcus in the presence of catalase should be due to PCW released during autolysis of the cells. Therefore, we performed stimulation experiments with purified PCW. However, these experiments did not give clear results. By flow cytometry, necrosis and apoptosis were observed. The rate of apoptosis was comparable to the rate of apoptosis induced during *in vitro* culture without any further stimulus, a well-described phenomenon [36]. The additional necrosis could be interpreted as being induced by PCW. In stimulation experiments with PCW, reduced concentrations of cellular histone-associated DNA fragments were measured, revealing either reduced spontaneously occurring apoptosis or induction of necrosis. The latter in conjunction with the results described above appears the most likely interpretation.

In conclusion, pneumococcal components including pneumolysin, hydrogen peroxide, and cell wall components initiate inflammation and oppress host defence. The induction of necrosis of PMN by viable pneumococci supports the spreading of bacterial infection. The induction of PMN apoptosis later in the course of disease, induced by metabolically inactive bacteria and probably followed by ingestion of total bacteria, is teleologically beneficial for resolving inflammation.

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