

# Pneumococcal pneumolysin and H<sub>2</sub>O<sub>2</sub> mediate brain cell apoptosis during meningitis

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**Pneumococcus is the most common and aggressive cause of bacterial meningitis and induces a novel apoptosis-inducing factor-dependent (AIF-dependent) form of brain cell apoptosis. Loss of production of two pneumococcal toxins, pneumolysin and H<sub>2</sub>O<sub>2</sub>, eliminated mitochondrial damage and apoptosis. Purified pneumolysin or H<sub>2</sub>O<sub>2</sub> induced microglial and neuronal apoptosis in vitro. Both toxins induced increases of intracellular Ca<sup>2+</sup> and triggered the release of AIF from mitochondria. Chelating Ca<sup>2+</sup> effectively blocked AIF release and cell death. In experimental pneumococcal meningitis, pneumolysin colocalized with apoptotic neurons of the hippocampus, and infection with pneumococci unable to produce pneumolysin and H<sub>2</sub>O<sub>2</sub> significantly reduced damage. Two bacterial toxins, pneumolysin and, to a lesser extent, H<sub>2</sub>O<sub>2</sub>, induce apoptosis by translocation of AIF, suggesting new neuroprotective strategies for pneumococcal meningitis.**

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## Introduction

Bacteria-induced apoptosis plays an important role in tissue damage in infectious diseases (1) and may play an essential role in neuronal cell death in meningitis. Survivors of bacterial meningitis suffer from a broad spectrum of neurologic sequelae that arise from neuronal cell damage. Pneumococcus is the most common and most aggressive human meningeal pathogen, causing death in up to 30% of cases and neurologic sequelae in 30–50% of survivors (2–4). Permanent loss of neurons by the induction of apoptosis in the hippocampus (5–8) likely contributes to this poor outcome. One trigger of this damage is the host inflammatory response, as blocking leukocyte invasion into the cerebrospinal fluid (CSF) is partially neuroprotective (7). In addition, pneumococcus directly induces the rapid apoptosis of primary rat hippocampal and cortical neurons, and of human microglial and neuronal cell lines (9). As is true for most disease models including meningitis, the bacterial toxins that trigger the host cell apoptotic response have not been identified.

Apoptosis of brain cells induced by direct exposure to pneumococci is not dependent on caspases but rather involves rapid and massive damage to mitochondria. This results in the release of apoptosis-inducing factor (AIF), and blocking AIF function effectively blocks pneumococcus-induced apoptosis (9). Pneumococcus produces a wide range of potentially toxic factors that could contribute to this apoptosis, including cell wall components and surface-associated and secreted tox-

ins (10–13). Important pathogenicity factors unique to *Streptococcus pneumoniae* include the exotoxins H<sub>2</sub>O<sub>2</sub> and the pore-forming molecule pneumolysin. Pneumococcus is unusual in that it is the only invasive human pathogen that lacks catalase (14), and the release of H<sub>2</sub>O<sub>2</sub> is important in models of pneumococcal pneumonia (15). Pneumolysin is a thiol-activated pore-forming molecule that has potent cytotoxic activity (16), and pneumolysin also plays an important pathogenic role in pneumococcal-induced pneumonia and otitis media (17, 18).

During pneumococcal meningitis, the major site of apoptotic damage is the dentate gyrus of the hippocampus (5, 7). Pneumococci do not penetrate the hippocampus but rather accumulate in high numbers in the CSF of the cerebral ventricles (19). Since the lateral ventricles are in close proximity to the hippocampus, neurons could be directly exposed to secreted bacterial toxins. In particular, the extracellular fluid around brain cells is contiguous with the CSF (20), and the diffusion between the CSF and the cerebral extracellular fluid could deliver soluble, secreted bacterial toxins. The uniquely high morbidity of pneumococcal meningitis versus other meningeal pathogens suggests that pneumococcus-specific toxins induce the apoptotic response. Soluble and secreted factors like pneumolysin and H<sub>2</sub>O<sub>2</sub> fit well to these criteria.

Here we report that two secreted toxins, pneumolysin and H<sub>2</sub>O<sub>2</sub>, mediate pneumococcal-induced apoptosis. The mechanism of this apoptosis involves translocation

of intracellular calcium and AIF. Either pneumolysin or H<sub>2</sub>O<sub>2</sub> is sufficient to induce mitochondrial damage and apoptosis in vitro. Inactivation of these toxins effectively prevents damage to neurons of the dentate gyrus in experimental pneumococcal meningitis.

## Methods

**Brain cell culture.** A human microglial cell line (HMC) (C.A. Colton, Georgetown University, Washington, DC, USA) that expresses most markers common to primary human microglial cells was cultured as previously described (9, 21). Primary rat hippocampal neurons were isolated from fetal rats at embryonic day 18 as described (22). Briefly, hippocampus was dissected, incubated in trypsin-EDTA (Biochrom, Berlin, Germany), dissociated with a Pasteur pipette, and plated in starter medium (neurobasal medium and supplement B27; Invitrogen, Karlsruhe, Germany). The status of cultures was assessed by light microscopy, and viability was tested by trypan blue dye exclusion. TNF- $\alpha$ , IL-1 $\beta$ , and RANTES were measured with ELISA kits (BioSource International, Rattlingen, Germany). Nitrite was detected by Griess reaction. A monoclonal antibody against TNF- $\alpha$  (IP-140; Genzyme GmbH, Konstanz, Germany) was used.

**Bacterial cell culture.** To determine which pneumococcal factors contribute to the induction of host cell apoptosis, we tested a panel of pneumococcal mutants for their ability to induce apoptosis in vitro. Because of their acute susceptibility to pneumococcal-induced apoptosis, human microglial cells were chosen as the most permissive system to screen these mutants. The following pneumococci and isogenic mutants were used: D39 capsular type 2 and R6, its nonencapsulated derivative (Rockefeller University, New York, New York, USA); the pneumolysin-negative mutant *plnA*<sup>-</sup> (provided by D. Briles, University of Alabama, Birmingham, Alabama, USA) (23); the pyruvate oxidase mutant *spxB*<sup>-</sup>, deficient in H<sub>2</sub>O<sub>2</sub> production (12); the *plnA*<sup>-</sup>/*spxB*<sup>-</sup> double mutant; the choline-binding protein A mutant *cbpA*<sup>-</sup>, deficient in adhesion (24); the competence-defective mutant *comA*<sup>-</sup> (25); the permease mutant *psaA*<sup>-</sup> (26), deficient in import of manganese; the IgA1 protease mutant *iga*<sup>-</sup> (27); the mutant *zmpB*<sup>-</sup> (13), deficient in a zinc metalloprotease; and *lytA*<sup>-</sup> (28), which is defective in autolysin.

The *plnA*<sup>-</sup>/*spxB*<sup>-</sup> double mutant was constructed as follows: A 690-bp fragment, generated by PCR using primers that flank *Sau3A* sites in pneumolysin (5'-TAATCCCACTCTTCTTGCGG-3' and 5'-ATAGGAAATCG-GCAAGCCTG-3'), was ligated into the vector pWG5 carrying chloramphenicol resistance (29). The recombinant plasmid was amplified and used to transform *spxB*<sup>-</sup> to create a double knockout mutant (23). Homologous recombination was confirmed by PCR. Pneumococci were grown in C+Y medium (30) without (wild-type) or with (mutants) 1  $\mu$ g/ml erythromycin and, for the double mutant, 2  $\mu$ g/ml chloramphenicol.

Pneumolysin was purified as previously described (31) and used in concentrations of 0.1–10  $\mu$ g/ml. H<sub>2</sub>O<sub>2</sub>

was purchased from Merck KGaA (Darmstadt, Germany) and used in concentrations of 2–2000  $\mu$ M. The concentrations of the toxins used were within physiological levels: 1  $\mu$ g pneumolysin is equivalent to 10<sup>8</sup> CFU pneumococci (32), and the production of up to 410  $\mu$ M H<sub>2</sub>O<sub>2</sub> per gram of pneumococcal protein per minute has been reported (15). *S. pneumoniae* has been shown to produce H<sub>2</sub>O<sub>2</sub> in liquid culture in ranges between 1 and 10 mM (46). Point mutants of pneumolysin were from T.J. Mitchell as described (16).

**Assays for differentiation of live, apoptotic, and necrotic cells.** Ethidium bromide (EB) (Sigma Chemical Co., St. Louis, Missouri, USA) and acridine orange (AO) (Sigma Chemical Co.) are fluorescent intercalating DNA dyes that allow for differentiation of live, apoptotic, and necrotic cells (9, 33). The in situ cell death TUNEL detection kit was purchased from Roche Molecular Biochemicals (Grenzach-Wylen, Germany), and the annexin V-FITC propidium iodide kit was from Beckman Coulter Inc. (Miami, Florida, USA). Both were used as described by the manufacturers. Cell death was also assessed by the release of lactate dehydrogenase (LDH) into the culture medium (34) as determined by a colorimetric assay (Sigma Chemical Co.).

**Immunohistochemistry and fluorescence stainings.** Brain sections were incubated with an anti-pneumolysin specific antibody (Novocastra Laboratories Ltd., Newcastle upon Tyne, United Kingdom), and Vectastain Elite ABC kit (Vector Laboratories Inc., Burlingame, California, USA) was used to visualize primary antibody binding. Following fixation (4% paraformaldehyde) and permeabilization (0.1% Triton X-100), neurons were incubated with an anti-AIF antibody (35) diluted 1:500 for 1 hour (control was with PBS). A fluorescent Cy3 antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania, USA) visualized binding of the primary anti-AIF antibody. Nuclei were stained with HOECHST 33258 dye (Molecular Probes Inc., Eugene, Oregon, USA). Increases of intracellular reactive oxygen species (ROS) were monitored with the ROS-specific dye dihydrorhodamine 123 (DHR123) (36) with a multiwell fluorescence plate reader (PerSeptive Biosystems, Framingham, Massachusetts, USA) according to the manufacturer's instructions and blocked with N-acetyl cysteine (NAC) (10 mM; Sigma Chemical Co.) or catalase (1,250 U/ml; Sigma Chemical Co., C3155, 50,000 units/mg, <0.01 mg Thymol/ml, <0.05 endotoxin units LPS; E-Toxate test, Sigma Chemical Co.). Cells were preincubated with 10  $\mu$ M DHR123 (Molecular Probes Inc.) for 1 hour and then incubated with pneumococci. DHR123 fluoresces when oxidized to rhodamine 123. Increases of intracellular Ca<sup>2+</sup> were monitored by Fluo-4 (37). After incubation with pneumococci, cells were incubated with Fluo-4 (10  $\mu$ M, for 45 minutes) (Molecular Probes Inc.) and intracellular Ca<sup>2+</sup> was determined by fluorescence. BAPTA-AM was used as an intracellular and extracellular Ca<sup>2+</sup> chelator (38). Cells were preincubated with BAPTA-AM (5  $\mu$ M) (Molecular Probes

Inc.) for 1 hour and then incubated with pneumococci in the presence and absence of BAPTA-AM. Fluo-4 was measured with a multiwell fluorescence plate reader (excitation 485 nm, emission 520 nm). For electron microscopy, after pneumococcal challenge, microglial cells were fixed in 2.5% glutaraldehyde in 100 mM cacodylate buffer overnight at 4°C, postfixed, dehydrated, and embedded as described previously (9).

**Rabbit model of pneumococcal meningitis.** Thirty-two 2-kg male New Zealand white rabbits (Myrtle's Rabbitry Inc., Thompson Station, Tennessee, USA) were anesthetized with a mixture of ketamine (35 mg/kg; Fort Dodge Laboratories, Fort Dodge, Iowa, USA) and xylazine (5 mg/kg; Miles Laboratories, Shawnee Mission, Kansas, USA). The bacterial inoculum (200  $\mu$ l, 10<sup>4</sup> CFU, D39 or *plnA*<sup>-</sup> or *plnA*<sup>-</sup>/*spxB*<sup>-</sup>) was introduced into the cisterna magna (39). NAC (300 mg/kg at 0, 8, and 16 hours) was given intravenously and catalase was given intravenously (at 0, 3, 6, 12, 15, 17, 18, and 19 hours) and intracisternally (at 0, 12, and 15 hours) in 12 animals. Animals were anesthetized again at 20 hours and CSF was sampled to confirm bacterial growth. Thereafter, phenobarbital (Abbott Laboratories, Abbott Park, Illinois, USA; 25 mg/kg) was administered to the animals and they were perfused transcardially with 2.5% paraformaldehyde and 0.1% glutaraldehyde (Sigma Chemical Co.) in 0.1 M cacodylate buffer (Ted Pella Inc., Redding, California, USA), pH 7.4, adjusted with sucrose to 300 mOsm. After 10 minutes' perfusion-fixation, brains were removed and postfixed. Neuronal damage was assessed using hematoxylin and eosin (H&E) and TUNEL staining (7). All experiments were performed in compliance with NIH and institutional guidelines.

## Results

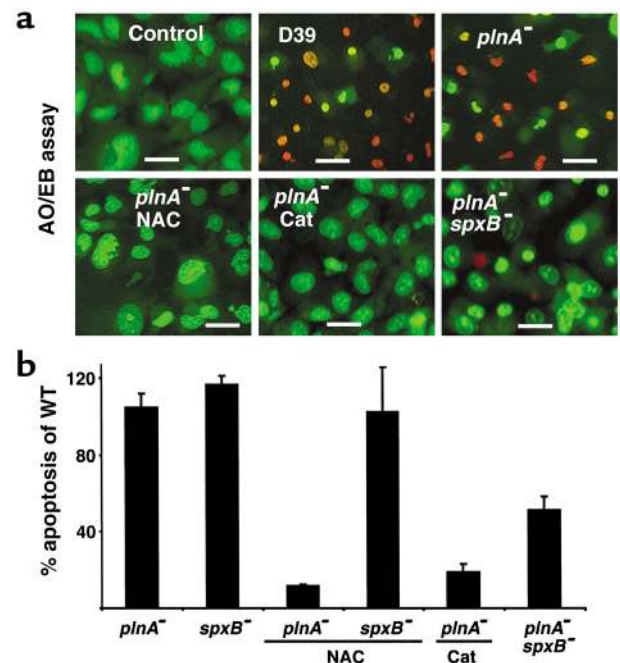
**Identification of possible toxic factors from pneumococci.** To assess whether bacterial toxins were secreted, or were cell wall- or cell surface-associated, we initially evaluated the ability of heat-killed pneumococci or purified pneumococcal cell walls, a potent proinflammatory stimulus in vivo (40), to kill microglial cells. Neither induced apoptosis (data not shown). Further, there was no difference in the proapoptotic activity of encapsulated and unencapsulated pneumococcal strains. Therefore, the pneumococcal toxins that mediate brain cell apoptosis appeared to be factors secreted by the bacterium. In support of this notion, supernatants of bacterial cultures induced rapid apoptosis of human microglial cells even at dilutions of 1:10 (72.4%  $\pm$  6.3% apoptotic cells by 9 hours).

To identify which secreted factor(s) mediates brain cell apoptosis, we assessed the toxicity of pneumococcal mutants defective in production of potential pathogenicity factors. Human microglial cells were exposed to wild-type pneumococcus (D39) or pneumococcal strains bearing the following loss-of-function mutations in the following genes: adherence to host cells (*cbpA*<sup>-</sup>), autolysis (*lytA*<sup>-</sup>), DNA transformation (*comA*<sup>-</sup>),

manganese transport (*psaA*<sup>-</sup>), surface IgA-protease (*igaA*<sup>-</sup>), and zinc metalloprotease (*zmpB*<sup>-</sup>). All of these mutants were as potent as their parental strain in inducing microglial apoptosis (data not shown).

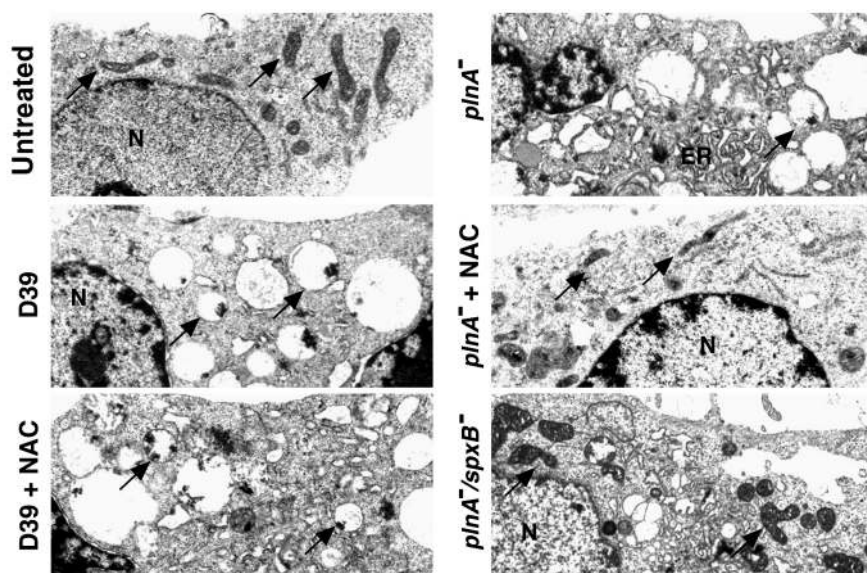
*S. pneumoniae* R6 and pneumolysin did not induce IL-1 $\beta$ , RANTES, or nitric oxide as possible toxic factors in primary rat hippocampal neurons. There was a slight increase of TNF- $\alpha$  by R6 (71.5  $\pm$  62.5 pg/ml) and pneumolysin (12.9  $\pm$  17.2 pg/ml) compared with controls (2.4  $\pm$  4.2 pg/ml). After addition of an anti-TNF antibody, TNF- $\alpha$  was no longer detectable, but no effect on LDH release (181.7  $\pm$  126.5 for R6 vs. 188.7  $\pm$  26.7 for R6 + anti-TNF antibody; 83.2  $\pm$  26.4 for controls vs. 81.7  $\pm$  18.4 for controls + antibody) nor on the number of apoptotic cells (data not shown) was observed.

**Pneumolysin and H<sub>2</sub>O<sub>2</sub> initiate pneumococcal-induced apoptosis.** To assess the role of released H<sub>2</sub>O<sub>2</sub> in apoptosis, we reduced H<sub>2</sub>O<sub>2</sub> concentrations in the pneumo-



**Figure 1**

Inactivation of pneumolysin and blocking H<sub>2</sub>O<sub>2</sub> inhibits apoptosis induced by live pneumococci. (a and b) Human microglia incubated with wild-type pneumococci D39 (10<sup>7</sup> CFU/ml, 6 hours) in the absence or presence of NAC (10 mM) underwent massive shrinkage and condensation of their nuclei by AO/EB staining, whereas NAC effectively impaired changes in nuclear morphology and cell membrane damage in cells exposed to *plnA*<sup>-</sup> (10<sup>7</sup> CFU/ml, 6 hours). Bars = 10  $\mu$ m. (b) Quantification of apoptotic microglia after exposure to wild-type (D39) or mutant pneumococci defective in pneumolysin (*plnA*<sup>-</sup>) or H<sub>2</sub>O<sub>2</sub> production (*spxB*<sup>-</sup>) or both (*plnA*<sup>-</sup>/*spxB*<sup>-</sup>). Shown are the results (means  $\pm$  SD) of three independent experiments after 6 hours of incubation. Addition of the antioxidant NAC prevented apoptosis induced by *plnA*<sup>-</sup>, but not that induced by D39 or *spxB*<sup>-</sup> (b). *plnA*<sup>-</sup> in the presence of NAC or catalase (Cat) (1,250 U/ml) and *plnA*<sup>-</sup>/*spxB*<sup>-</sup> were significantly different compared with all others ( $P < 0.05$ ). *plnA*<sup>-</sup> combined with NAC or catalase was more efficient to prevent apoptosis than was the double mutant *plnA*<sup>-</sup>/*spxB*<sup>-</sup> ( $P < 0.05$ ). ANOVA and Student-Newman-Keuls test were used. WT, wild-type.



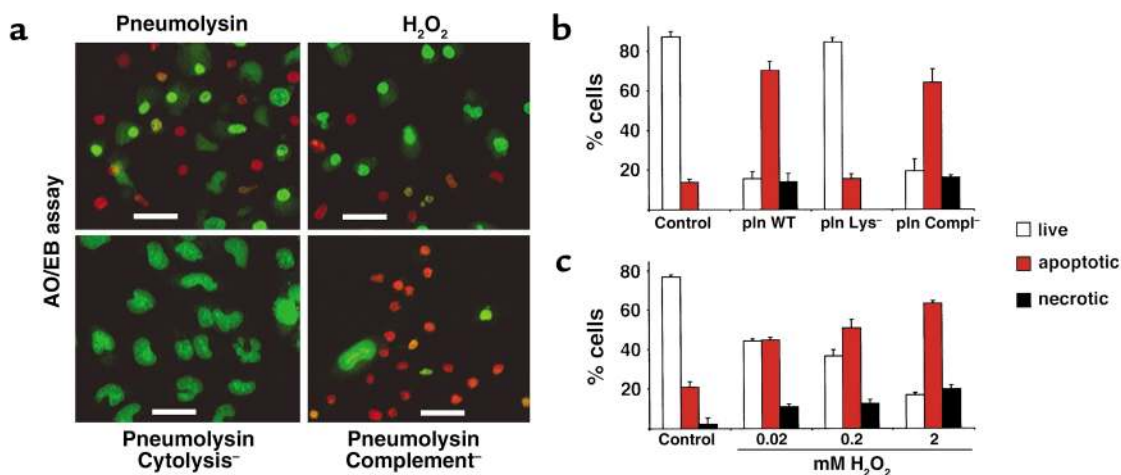
**Figure 2**

Inactivation of pneumolysin and blocking  $H_2O_2$  inhibits mitochondrial damage induced by live pneumococci. Changes of ultrastructure of microglial cells exposed to pneumococci ( $10^7$  CFU/ml) for 3 hours were monitored by transmission electron microscopy. D39 and *plnA*<sup>-</sup> caused massive swelling of the mitochondria (arrows) and endoplasmic reticulum (ER). Treatment of D39-exposed cells with NAC (10 mM) failed to prevent that damage. By contrast, when cells exposed to *plnA*<sup>-</sup> were treated with NAC (10 mM), swelling of mitochondria and the endoplasmic reticulum was markedly attenuated. The double mutant *plnA*<sup>-</sup>/*spxB*<sup>-</sup> also showed attenuated mitochondrial damage.  $\times 7,500$ . N, nucleus.

coccal supernatant by adding the antioxidant NAC or with catalase prior to exposure to microglia. NAC or catalase alone had no effect on microglia cells (data not shown). Apoptosis was verified by typical changes of TUNEL, electron microscopy, and annexin V/propidium iodide and AO/EB staining. We also evaluated the ability of the pneumococcal mutant *spxB*<sup>-</sup>, which produces less than 5% of wild-type levels of  $H_2O_2$  (12), to induce apoptosis. Either pharmacological or genetic inactivation of  $H_2O_2$  failed to prevent microglial apoptosis (Figure 1). Similarly, the pneumolysin-deficient mutant *plnA*<sup>-</sup> (23) was fully active in inducing apoptosis of either microglia or neurons (Figure 1 and data

not shown). Therefore, inactivation of either of these pathogenicity factors alone did not block pneumococcal-induced apoptosis.

We next assessed the possibility that more than one factor secreted by pneumococcus possessed apoptosis-inducing activity. Initially we assessed whether incubation of brain cells with NAC or catalase (Figure 1) blocked apoptosis induced by the *plnA*<sup>-</sup> pneumococcus. Although exposure of microglia to wild-type or *plnA*<sup>-</sup> pneumococci caused early and massive damage to mitochondria (Figure 2) and subsequent apoptosis, treatment of cells exposed to the *plnA*<sup>-</sup> strain with NAC or catalase effectively blocked mito-



**Figure 3**

Pneumolysin and  $H_2O_2$  are each sufficient to induce apoptosis in microglia and neurons. (a) Microglia cells were incubated for 8 hours with purified pneumolysin (0.1  $\mu$ g/ml) or  $H_2O_2$  (0.2 mM) and stained with AO and EB. Either treatment resulted in shrinkage and condensation typical of apoptosis induced by wild-type pneumococci. The apoptosis-inducing activity of pneumolysin correlates with its cytolytic (Lys) but not its complement-activating (Compl) function. Microglial cells were incubated with wild-type or mutant pneumolysin (0.1  $\mu$ g/ml) for 12 hours and stained with AO and EB. A point mutant in the domain required for cytolytic activity of pneumolysin (W433F) failed to induce apoptosis, whereas a point mutant in the domain required for complement activation (D385N) retained full apoptosis-inducing capacity. Bars = 10  $\mu$ m. (b) Quantification of the effects of wild-type or mutant pneumolysin on primary rat hippocampal neurons. Results shown (means + SD) are representative for three independent experiments performed in triplicate. (c) Neurotoxicity (primary rat hippocampal neurons) of various concentrations of  $H_2O_2$ .

chondrial damage and apoptosis (Figures 1 and 2). Based on more than 50 electron micrographs, the percentage of cells with swollen mitochondria was estimated as follows [mean  $\pm$  SD (range);  $n > 4$ ]. Control: 10%  $\pm$  10% (0–26%); D39: 83%  $\pm$  9% (79–95%); D39 + NAC: 78%  $\pm$  12% (70–93%); *plnA*<sup>-</sup>: 86%  $\pm$  17% (62–100%); *plnA*<sup>-</sup> + NAC: 19%  $\pm$  5% (13–27%); *plnA*<sup>-</sup>/*spxB*<sup>-</sup>: 26%  $\pm$  12% (6–43%).

To confirm that the effects of antioxidants were specific, we generated a pneumococcal mutant defective in both H<sub>2</sub>O<sub>2</sub> and pneumolysin (*spxB*<sup>-</sup>/*plnA*<sup>-</sup>). Although this mutant grew at rates comparable to those of wild-type pneumococci (data not shown), it was significantly impaired in its ability to induce mitochondrial damage and apoptosis of microglial cells or neurons (Figures 1 and 2, and data not shown). Therefore, both pneumolysin and bacteria-derived H<sub>2</sub>O<sub>2</sub> contribute to pneumococcal-induced apoptosis.

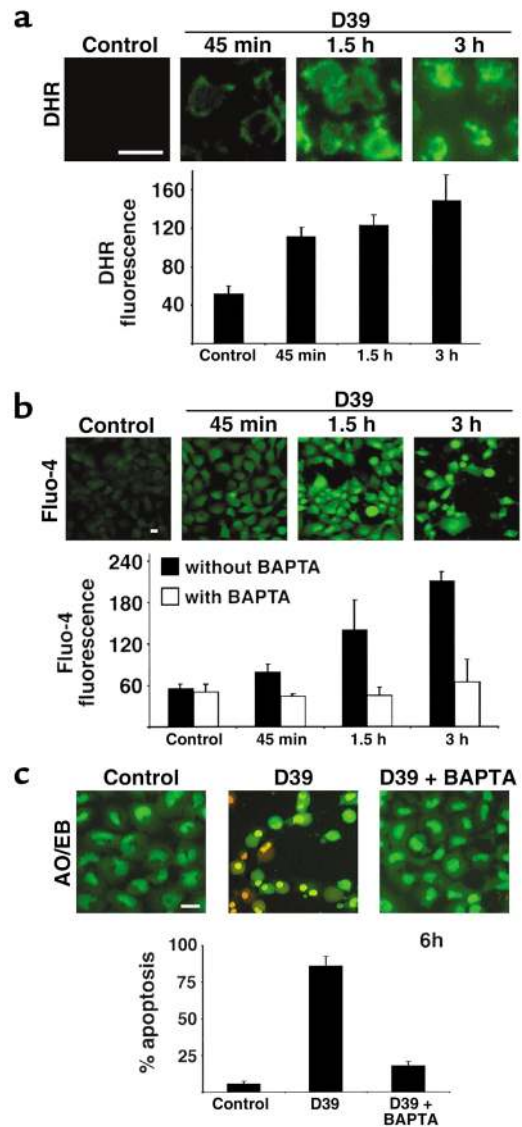
*Purified pneumolysin or H<sub>2</sub>O<sub>2</sub> are sufficient to induce brain cell apoptosis.* To confirm that pneumolysin and H<sub>2</sub>O<sub>2</sub> were each sufficient to induce apoptosis, microglial cells or primary hippocampal neurons were incubated with purified recombinant pneumolysin or H<sub>2</sub>O<sub>2</sub> (Figure 3). Pneumolysin (0.1–10  $\mu$ g/ml) or H<sub>2</sub>O<sub>2</sub> (2–2000  $\mu$ M) induced apoptosis of microglial cells (Figure 3a) as well as primary rat hippocampal neurons (Figure 3, b and c), with kinetics and changes in nuclear morphology indistinguishable from apoptosis induced by live pneumococci (Figure 1), including margination and condensation of chromatin, and cell and nuclear shrinkage. Cell death was confirmed by release of LDH (data not shown).

Pneumolysin activities associated with different domains of the protein include complement activation and cytolysis (16). We incubated microglial cells and primary rat hippocampal neurons with wild-type pneumolysin or with pneumolysin point mutants defective in complement (D385N) or cytolytic (W433F or W433F/C428G) activity (41). Pneumolysin proteins defective in cytolytic activity failed to induce apoptosis, whereas pneumolysin defective in complement activation induced apoptosis to the same extent as did wild-type pneumolysin (Figure 3, a and b). Therefore, the pore-forming activity of pneumolysin was required for its proapoptotic function.

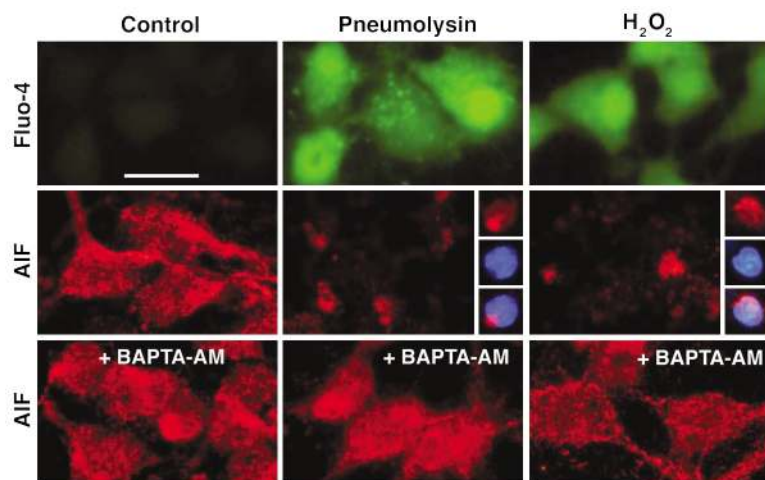
*Pneumococci, pneumolysin, and H<sub>2</sub>O<sub>2</sub> initiate apoptosis by inducing Ca<sup>2+</sup> flux.* Influx of Ca<sup>2+</sup> or the release of Ca<sup>2+</sup> from intracellular sources precedes morphologic changes of brain cell apoptosis induced by several agents (42), and overloading of mitochondria with Ca<sup>2+</sup> induces permeability transition pores (43). We therefore evaluated whether Ca<sup>2+</sup> flux played a role in pneumococcal-induced apoptosis. To assess Ca<sup>2+</sup> levels we used Fluo-4, a dye which fluoresces upon binding to Ca<sup>2+</sup> (44). Within 45 minutes of exposure to pneumococci, there was a profound increase in intracellular Ca<sup>2+</sup> over 3 hours. This effect was blocked after incubation with the extra- and intracellular Ca<sup>2+</sup> chelator BAPTA-AM (Figure 4b). Similarly, the pneumococcal

toxins pneumolysin and H<sub>2</sub>O<sub>2</sub> induced a rapid increase of Ca<sup>2+</sup> concentrations in primary rat hippocampal neurons (Figure 5, top row).

Within 45 minutes of exposure of microglial cells to wild-type pneumococcus, we found marked increases of intracellular ROS (Figure 4a), and this was effectively reduced by treatment with NAC (DHR fluorescence units at 0 minutes: 38  $\pm$  13; 90 minutes: 55  $\pm$  11 vs. 123  $\pm$  11 without NAC; 180 minutes: 53  $\pm$  1 vs. 149  $\pm$  26 without NAC) or catalase (0 minutes: 41  $\pm$  13; 90 minutes: 72  $\pm$  7



**Figure 4** Pneumococcus triggers early increases of intracellular ROS and Ca<sup>2+</sup>. Microglial cells were untreated (Control) or incubated with pneumococcus D39 (10<sup>7</sup> CFU/ml) for 45 minutes to 3 hours. ROS (a) and Ca<sup>2+</sup> (b) levels were visualized by fluorescence of the dyes DHR123 (10  $\mu$ M) and Fluo-4 (10  $\mu$ M), respectively, and quantified with a multiwell fluorescence plate reader. Results shown (mean  $\pm$  SD) are representative for three independent experiments performed in triplicate. (b and c) The effects of the Ca<sup>2+</sup> chelator BAPTA-AM (5  $\mu$ M) were investigated with the AO/EB assay after exposure of cells to D39 in the presence and absence of BAPTA-AM. Bars = 10  $\mu$ m.



**Figure 5** Pneumolysin and H<sub>2</sub>O<sub>2</sub> induce increase of intracellular Ca<sup>2+</sup> and release of mitochondrial AIF. Primary rat neurons were exposed to either pneumolysin (0.1 μg/ml) or H<sub>2</sub>O<sub>2</sub> (0.2 mM). Intracellular Ca<sup>2+</sup> was visualized by Fluo-4 (10 μM) after 1.5 hours. Release of mitochondrial AIF, its translocation to the nucleus, and the effects of BAPTA-AM (5 μM) were assessed immunocytochemically after 6 hours' incubation. Insets show colocalization of HOECHST 33258 (blue fluorescence) and AIF (red fluorescence). Bar = 10 μm.

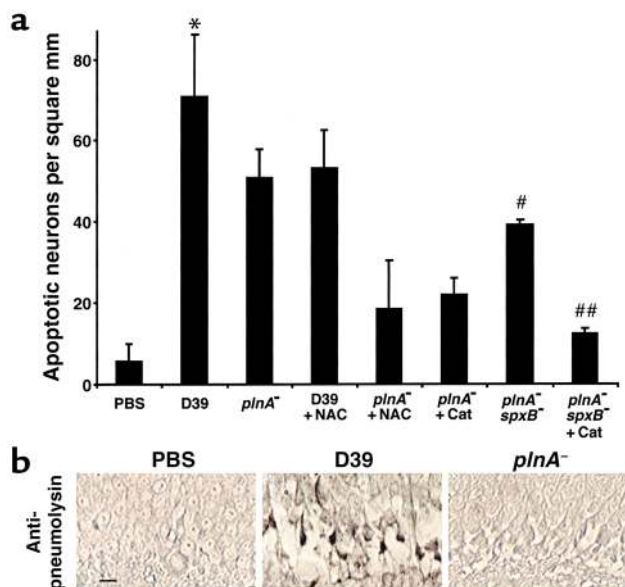
vs.  $126 \pm 9$  without catalase). Exposure to the *spxB*<sup>-</sup> strain caused a delayed and — as expected — an attenuated increase of ROS (0 minutes:  $52 \pm 8$ ; 45 minutes:  $58 \pm 3$ ; 90 minutes:  $62 \pm 12$ ; 180 minutes:  $116 \pm 14$  DHR fluorescence units). This attenuated response was accompanied by a delayed and decreased influx of Ca<sup>2+</sup>. The pneumolysin-defective *plnA*<sup>-</sup> strain was not deficient in inducing rapid increases of intracellular ROS and Ca<sup>2+</sup> (data not shown).

To assess whether this increase in Ca<sup>2+</sup> contributed to the apoptotic response, we pretreated microglia with the extra- and intracellular Ca<sup>2+</sup> chelator BAPTA-AM and then exposed them to pneumococcus. For control, appropriate chelation of calcium by BAPTA-AM was shown by blocking of Ca<sup>2+</sup>-induced increase of Fluo-4 fluorescence. Strikingly, treatment of infected microglia with BAPTA-AM blocked the apoptotic response (Figure 4c), demonstrating that the increases in Ca<sup>2+</sup> were essential for the pneumococcal-induced cell death program. These data therefore suggest that pneumolysin and bacteria-derived H<sub>2</sub>O<sub>2</sub> independently mediate increases in intracellular Ca<sup>2+</sup>, which is essential for apoptosis.

*Pneumolysin and pneumococcal H<sub>2</sub>O<sub>2</sub> induce mitochondrial damage and the release of AIF.* Pneumolysin and bacterial H<sub>2</sub>O<sub>2</sub> recapitulated the changes in Ca<sup>2+</sup> that are associated with pneumococcal-induced apoptosis. To assess whether purified pneumolysin and H<sub>2</sub>O<sub>2</sub> were also capable of inducing mitochondrial damage and the release of AIF, the final steps in pneumococcal-induced brain cell apoptosis (9), primary rat neurons were exposed to the toxins and mitochondrial AIF was monitored by immunocytochemistry. In untreated neurons, AIF colocalized with mitochondria (9). Wild-type pneumococci (9) as well as the supernatant of wild-type pneumococci induced a massive release of mitochondrial AIF into the cytoplasm. In contrast, supernatant of the *plnA*<sup>-</sup>/*spxB*<sup>-</sup> mutant induced only a slight release of AIF (data not shown). H<sub>2</sub>O<sub>2</sub> and pneumolysin induced the release of mitochondrial AIF and translocation to the nucleus (Figure 5, middle row). Pneumolysin defective in complement activation but not pneumolysin defective in pore forming activi-

ty was still able to release AIF (data not shown). Furthermore, BAPTA-AM inhibited the release of AIF (Figure 5, bottom row), suggesting that AIF release caused by pneumolysin or H<sub>2</sub>O<sub>2</sub> was mediated by increases in intracellular Ca<sup>2+</sup>. To address the ability of pneumococcal supernatant-derived pneumolysin and H<sub>2</sub>O<sub>2</sub> to induce the release of AIF, neurons were exposed to culture supernatants from wild-type and double mutant *spxB*<sup>-</sup>/*plnA*<sup>-</sup> bacteria.

*Pneumolysin and H<sub>2</sub>O<sub>2</sub> contribute to neuronal damage in the rabbit model of pneumococcal meningitis.* To address the role of pneumolysin and H<sub>2</sub>O<sub>2</sub> in hippocampal neuronal apoptosis in experimental pneumococcal meningitis, we used a rabbit model in which bacteria (10<sup>4</sup> CFU per animal) are directly inoculated into the CSF of the animals. Within 24 hours of exposure there is a massive host inflammatory response and damage to neurons of the dentate gyrus of the hippocampus (7). We compared the disease course in rabbits challenged with wild-type (D39) or pneumolysin-negative (*plnA*<sup>-</sup>) pneumococci in the presence or absence of NAC (Figure 6a). CSF was analyzed for bacterial and leukocyte numbers at 26 hours, and the brains of the animals were assessed for hippocampal damage. NAC or catalase treatment did not inhibit bacterial growth in vivo and did not block the influx of leukocytes into the CSF compartment (data not shown). The degree of damage to the dentate gyrus was reduced in animals infected with *plnA*<sup>-</sup> (Figure 6a). However, NAC treatment of *plnA*<sup>-</sup>-infected animals, but not wild-type infected rabbits, drastically reduced neuronal damage (Figure 6a). Catalase treatment also greatly reduced neuronal damage of *plnA*<sup>-</sup>-infected animals (Figure 6a). As NAC or catalase can block both bacteria- and host-derived H<sub>2</sub>O<sub>2</sub>, the double mutant *plnA*<sup>-</sup>/*spxB*<sup>-</sup>, which fails to produce bacterial H<sub>2</sub>O<sub>2</sub>, was tested in vivo. *plnA*<sup>-</sup>/*spxB*<sup>-</sup> induced less neuronal damage than did *plnA*<sup>-</sup> ( $51.1 \pm 6.7$  vs.  $39.5 \pm 0.9$  apoptotic cells per mm<sup>2</sup>;  $P = 0.04$ , *t* test), supporting a partial role for bacterial H<sub>2</sub>O<sub>2</sub>. However, as catalase further decreased neuronal damage in *plnA*<sup>-</sup>/*spxB*<sup>-</sup>-infected versus *plnA*<sup>-</sup>-infected animals ( $22.2 \pm 4.0$  vs.  $12.6 \pm 1.3$  apoptotic cells per mm<sup>2</sup>;  $P = 0.004$ , *t* test), this finding



**Figure 6** Pneumolysin and H<sub>2</sub>O<sub>2</sub> contribute to neuronal damage in the rabbit model of pneumococcal meningitis. (a) Neurotoxicity in the dentate gyrus was quantified by neuronal loss or cell shrinkage assessed in H&E-stained sections. Bars represent means + SD of five brain sections per rabbit. Compared with control animals challenged with PBS, wild-type pneumococci D39 induced significant neurotoxicity (\**P* < 0.05). Damage in D39-challenged animals was also statistically significant compared with all other tested groups except D39 + NAC (*P* < 0.05; ANOVA, Student-Newman-Keuls test). #Significant difference between *plnA*<sup>-</sup>/*spxB*<sup>-</sup> and *plnA*<sup>-</sup>, *P* = 0.04. ##Significant difference between *plnA*<sup>-</sup>/*spxB*<sup>-</sup> with and without catalase, *P* = 0.004; *t* test. (b) Immunohistochemical staining with anti-pneumolysin antibody followed by peroxidase-conjugated secondary antibody and diaminobenzidine (dark color) detected pneumolysin in dentate gyrus neurons of D39- but not *plnA*<sup>-</sup> or PBS-treated animals. Bar = 10 μm.

suggests that host-derived H<sub>2</sub>O<sub>2</sub> also mediates toxic effects in vivo. We have systematically screened other areas of the brain including Purkinje cells in the cerebellum and cortical areas. We could not detect neuronal damage in other areas in our rabbit model of bacterial meningitis after 24 hours.

To test the hypothesis that the neurons of the hippocampus are actually exposed to pneumolysin in vivo, immunohistochemical analyses were performed. Immunostaining with a pneumolysin-specific antibody detected high levels of pneumolysin protein in dying neurons of the dentate gyrus of the hippocampus (Figure 6b). Therefore, released pneumolysin indeed contacts hippocampal neurons, which are susceptible to apoptosis during meningitis.

### Discussion

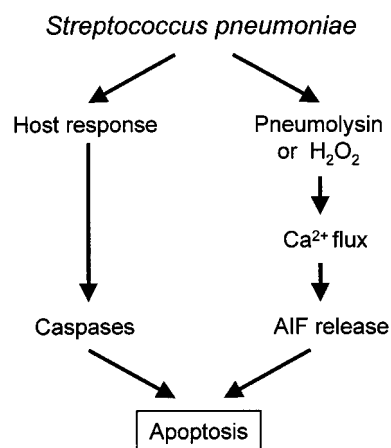
Mechanisms of neuronal damage in pneumococcal meningitis are still largely unknown. Recently, apoptosis has been reported as a major mechanism for damage to the hippocampus (5, 7). We have demonstrated that pneumococci may contribute significantly to neuronal cell death since the bacteria are able to

directly induce apoptosis (9). Here we report that the pneumococcal toxins pneumolysin and H<sub>2</sub>O<sub>2</sub> induce massive and rapid apoptosis. Blocking these proapoptotic factors markedly attenuates mitochondrial damage and apoptosis in vitro and in experimental meningitis, suggesting that strategies targeting these toxins may offer therapeutic benefit.

H<sub>2</sub>O<sub>2</sub> rapidly diffuses through eukaryotic cell membranes to damage intracellular targets (e.g., mitochondria and DNA) (15, 45) and to trigger apoptosis. Pneumococcus is the only meningeal pathogen that lacks catalase and therefore produces excessive amounts of H<sub>2</sub>O<sub>2</sub> (14, 46). Pneumococcal-derived H<sub>2</sub>O<sub>2</sub> appears to be an additional bacterial factor contributing to increases of intracellular ROS and Ca<sup>2+</sup> and release of AIF. Chelating Ca<sup>2+</sup> blocked this AIF release. Increased intracellular Ca<sup>2+</sup> is clearly important for inducing mitochondrial damage and apoptosis, as H<sub>2</sub>O<sub>2</sub> is sufficient to induce apoptosis as well as AIF release and blocking intracellular Ca<sup>2+</sup> prevents mitochondrial damage and cell death.

Despite the proapoptotic effects of H<sub>2</sub>O<sub>2</sub>, it is clearly not the only trigger of pneumococcal-induced apoptosis. A mutant defective in H<sub>2</sub>O<sub>2</sub> production is similar to wild-type pneumococci in its ability to induce death, and blocking ROS with antioxidants does not prevent death induced by wild-type pneumococci. However, this strategy effectively disables the apoptotic potential of the pneumolysin-deficient strain *plnA*<sup>-</sup>, suggesting that the other toxin that contributes to the response is pneumolysin. In support of this notion, the H<sub>2</sub>O<sub>2</sub>/pneumolysin-deficient double mutant has little apoptotic potential.

Pneumolysin is a multifunctional protein toxin that induces cytolysis, complement activation, and cytokine and nitric oxide production (16, 31, 47). Here we have



**Figure 7** Schematic model of pneumococcal-induced apoptosis of neurons. Neuronal apoptosis is triggered in part by the host inflammation and is mediated in part by caspase activation. Pneumolysin and H<sub>2</sub>O<sub>2</sub> are direct triggers of *S. pneumoniae*, inducing apoptosis by increasing intracellular Ca<sup>2+</sup>, damaging mitochondria, and causing the release and translocation of mitochondrial AIF.

shown that pneumolysin is also a potent neurotoxin that triggers apoptosis in primary hippocampal neurons by inducing mitochondrial damage and the release of the proapoptotic factor AIF. Pneumolysin-induced increases of intracellular  $\text{Ca}^{2+}$  and AIF release could be blocked by chelating  $\text{Ca}^{2+}$ , indicating a central role for  $\text{Ca}^{2+}$  in the pathophysiological effects of both pneumococcal toxins. This is consistent with the finding that  $\text{Ca}^{2+}$  is one known factor initiating the release of AIF from mitochondria (35). Specific domains of pneumolysin independently cause proinflammatory and pore-forming activities (16). Following binding to cholesterol, pneumolysin monomers insert into the lipid bilayer, oligomerize, and create pores 35–45 nm in diameter (48). The pore-forming activity of pneumolysin was required for inducing AIF release and apoptosis in vitro since the cytolysis- but not the complement-deficient mutant of pneumolysin protein was unable to induce AIF release and death. Pneumolysin is related to other bacterial hemolysins that have also been noted to induce apoptosis, including listeriolysin of *Listeria monocytogenes* (49),  $\alpha$  toxin of *Staphylococcus aureus* (50), and *Escherichia coli* hemolysin (51). It will therefore be interesting to determine whether this hemolysin family shares a common mechanism in inducing mitochondrial damage and AIF-dependent apoptosis.

A central issue for the in vivo relevance of pneumococcal-induced cell death is the ability of the toxins to invade the site of damage. Pneumolysin was found in the hippocampus colocalized to damaged neurons. The extracellular fluid around brain cells is contiguous with the CSF (20), and pneumolysin and other soluble, secreted bacterial toxins may diffuse into the parenchyma. Meningitis caused by the  $\text{H}_2\text{O}_2$ /pneumolysin-deficient double mutant reduced hippocampal apoptosis by 50%, clearly demonstrating the in vivo role of these pneumococcal toxins.

Since both NAC and catalase scavenge host and bacterial  $\text{H}_2\text{O}_2$ , one way to determine whether the source of the  $\text{H}_2\text{O}_2$  is the host or the bacteria was to test the *plnA*<sup>-</sup>/*spxB*<sup>-</sup> mutant. Removing the ability of the bacteria to make  $\text{H}_2\text{O}_2$  decreased apoptosis compared with *plnA*<sup>-</sup> alone. However, since this effect was not as strong as the benefit of NAC or catalase, the bacteria can be said to contribute to damage by producing  $\text{H}_2\text{O}_2$ , but the host also represents a major source of damaging  $\text{H}_2\text{O}_2$ .

Overall the combined data (refs. 7, 9, and this report) suggest that hippocampal apoptosis in *S. pneumoniae*-induced meningitis is a two-pronged problem (Figure 7). On the one hand, there is caspase-dependent apoptosis of neurons in the dentate gyrus that is due to the host inflammatory response (Figure 7). Thus, strategies to inhibit the host inflammatory response by inhibiting migration, adhesion, or function of leukocytes, or the production of reactive oxygen intermediates, matrix metalloproteinases (52), and/or caspases, are needed to decrease apoptosis and subsequent morbidity. The relevance of the complex host response has been demonstrated by the fact that in a model of

experimental meningitis using group B streptococcus, a pathogen that does not produce the candidate factors of pneumococcus caused a comparable apoptotic injury in the dentate gyrus (53).

Hippocampal neurons have been shown to be susceptible to various noxious stimuli, e.g., ischemia, inflammation, or irradiation. Unlike other neurons in the brain, hippocampal dentate gyrus neurons have the ability to undergo proliferation and neurogenesis (54). In the specific case of bacterial meningitis, the proximity to the ventricle with high bacterial concentrations and diffusible factors like pneumolysin has to be considered. Both factors may contribute to this specific vulnerability.

On the other hand, the direct exposure of these neurons to pneumococcal toxins induces AIF-dependent apoptosis (9). As shown here, the toxins released by pneumococcus include pneumolysin and  $\text{H}_2\text{O}_2$ , and to eliminate neuronal damage during meningitis these also need to be targeted, for example by treating with antioxidants and neutralizing pneumolysin. Pneumolysin and, to a lesser extent, pneumococcal-derived  $\text{H}_2\text{O}_2$  initiate the AIF death cascade by increasing intracellular  $\text{Ca}^{2+}$ , and agents that can block the release of or sequester  $\text{Ca}^{2+}$  may also be of therapeutic benefit, as blocking  $\text{Ca}^{2+}$  dramatically impairs the pneumococcal toxin-induced apoptotic response. The multiplicity of mechanisms by which pneumococci kill CNS cells may explain the exceptionally devastating course of this infection. Using pneumococci as a bioprobe also allows the study of the diversity of apoptotic mechanisms in the CNS.

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