Apoptosis-Inducing Factor Mediates Microglial and Neuronal Apoptosis Caused by Pneumococcus

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Streptococcus pneumoniae **is the major cause of bacterial meningitis and it damages the hippocampus by inducing neuronal apoptosis. The blocking of caspases provides only partial protection in experimental meningitis, which suggests that there is an additional apoptotic pathway. A trigger of this pathway is the bacterium itself, as exposure of microglia or neurons to live pneumococci induces rapid apoptosis. In this study, apoptosis was not associated with the activation of caspases-1–10 and was not inhibited by z-VAD-fmk, a broad-spectrum caspase inhibitor. Rather, apoptosis was attributed to damage to mitochondria, which was followed by the release of apoptosis-inducing factor (AIF) from the mitochondria, large-scale DNA fragmentation, and hypodiploidy. Furthermore, intracytoplasmatic microinjection of AIF-specific antiserum markedly impaired pneumococcus-induced apoptosis. These findings indicate that AIF may play a central role in brain cell apoptosis and bacterial pathogenesis.**

Apoptosis is a host cell–deletion mechanism and a trigger of inflammation in bacterial pathogenesis [1, 2], but the precise role and mechanisms of apoptosis during bacterial pathogenesis are poorly understood. Pneumococcus is the major cause of bacterial meningitis and carries a particularly poor prognosis, compared with other meningeal pathogens. Death occurs in up to 30% of cases, and 30%–50% of survivors experience major neurologic sequelae, including seizures, pareses, hearing loss, and learning and cognitive deficits [3–5].

During bacterial meningitis, apoptosis causes permanent loss of neurons in the dentate gyrus of the hippocampus [6, 7]. One of the hallmarks of apoptosis is caspase activation. Caspases are an evolutionarily conserved family of cysteine-dependent, aspartate-directed proteases [8] that have an important role in neuronal programmed cell death. Mice lacking caspase-3, caspase-9, or the caspase-9 activator Apaf-1 display inappropriate survival of neurons during development, which leads to embryonic or perinatal death [9–11]. Furthermore, many disease states of the brain—notably Alzheimer's disease, cerebral ischemia, excitotoxicity, and bacterial infection—are associated, at least in part, with the activation of caspases [7, 12].

The underlying mechanisms of the neuronal apoptosis observed during pneumococcal meningitis are largely unknown. This damage is mediated in part by caspase-3, which is activated in a manner dependent on the host leukocyte inflammatory response in the cerebrospinal fluid. However, blocking the invasion of leukocytes or caspase activation during pneumococcal meningitis protects only half the neurons of the dentate gyrus from undergoing death [7]. Thus, a second and apparently caspase-independent apoptotic pathway also plays an important role in pneumococcal pathogenesis.

Accumulating evidence indicates that caspase activation is not necessary for all forms of apoptosis [13]. For example, the broad-spectrum caspase inhibitor z-VAD-fmk does not prevent chromatin condensation, cell membrane blebbing, and cytoplasmic vacuolation in cells undergoing Bax-induced apoptosis [14]. Furthermore, z-VAD-fmk delays but does not prevent apoptosis induced by DNA damage or overexpression of c-Myc or Bak [15]. Caspases are not activated during promyelocytic leukemia gene product (PML)–induced apoptosis, and caspase inhibitors accelerate PML-induced death [16]. Finally, the blocking of caspase activation only partially improves outcome in several disease states in which cell damage has been attributed to apoptosis [7, 17].

Damage to mitochondria releases proapoptotic factors that can induce either caspase-dependent (e.g., cytochrome *c*) or caspase-independent (e.g., apoptosis-inducing factor [AIF]) apoptosis [18, 19]. Thus, the purpose of this study was to determine the role and mechanisms of brain cell apoptosis during pneumococcal infection.

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Materials and Methods

Brain cell culture. A human microglial cell line was provided by C. A. Colton (Georgetown University, Washington, DC). This cell line was established after transfection of primary embryonic microglial cells [20] with the SV40 large T antigen and grows indefinitely, yet it exhibits many characteristics of primary human microglial cells (positive for CD68/Ki-M7, CD11b, and CD32 and negative for CD68/Ki-M6, CD11c, CD14, CD4, major histocompatibility complex–2, neurofilament, and glial fibrillary acidic protein) [21]. The human cortical neuronal cell (HCN) line HCN-2 was purchased from American Type Culture Collection. HCN-2 cells express a variety of neurotransmitters and are positive for neuronal markers, such as neurofilament or neuron-specific enolase, but they are not positive for nonneuronal markers, such as glial fibrillary acidic protein and S-100 protein.

Cells were grown to 40%–50% confluency. For differentiation, nerve-growth factor (25 ng/mL; Calbiochem), 12-O-tetradecaoylphorbol-13-acetate (20 n*M;* Sigma), and isobutylmethylxanthine (0.5 m*M;* Sigma) were added, as described elsewhere [22], to the cells 1 day before they were challenged with pneumococci. Primary mouse brain cells were isolated and grown from newborn B6/129 mice according to the manufacturer's protocol (Papain Dissociation System; Worthington). Cultures contained ~10% neurons and 90% astrocytes and microglia. Primary rat hippocampal neurons were obtained from fetal rats at embryonic day 18, as described elsewhere [23].

Bacterial cell culture. D39 capsular type 2 pneumococci (Rockefeller University) were grown in casein plus yeast medium [24]. The bacterial inoculum (10^7 cfu/mL) was prepared after growing pneumococci to an optical density of 0.5 (measured at 620 nm).Bacteria were pelleted, were resuspended in cell culture medium, and were incubated with brain cells for 1–12 h. Bacterial multiplication continued slowly under these conditions (1 log every 6 h), with no change in pH of the medium. For some experiments, z-VAD-fmk (Enzyme Systems Products) was added as a broad-spectrum caspase inhibitor.

Differentiation of live, apoptotic, and necrotic cells. Ethidium bromide (EB; Sigma) and acridine orange (AO; Sigma) are fluorescent-intercalating DNA dyes. AO stains all nuclei green, whereas EB stains nuclei red but is excluded by cells with an intact cell membrane. Double staining with EB and AO allows for differentiation of live, apoptotic, and necrotic cells [25]: live cells have a green, regular-sized nucleus; early apoptotic cells have a green, condensed, shrunken or fragmented nucleus; late apoptotic cells have a red, condensed, shrunken or fragmented nucleus; and necrotic cells have a red and regular-sized or increased nucleus.

After brain cells were incubated with bacteria, 2 μg/mL each of AO and EB in PBS was added to each well. After a 5-min incubation, individual cells in the center of each well were counted. An in situ cell-death detection kit (TUNEL; Boehringer) and a fluorescein isothiocyanate kit (Annexin-V; Coulter), which involves costaining with the membrane-integrity dye propidium iodide, were used as described by the manufacturers. For microinjection experiments with anti-AIF antiserum, microglial cells (200 cells per experiment) were microinjected into the cytoplasm with AIF-specific antiserum or control serum (0.5 pL) marked with Dextran Texas Red and then were incubated with live pneumococci (10^7 cfu/mL) for 4 h before cells were stained for morphologic analyses of apoptosis.

Western blotting and pulsed-field gel electrophoresis. Brain cells were incubated for 3–12 h with pneumococci or for 6 h with the apoptosis inducer staurosporine (0.1 μM; Sigma). Cells were pelleted by centrifugation (153 *g* for 5 min at 4°C), and pellets were lysed on ice in Ripa buffer (50 m*M* Tris, 150 m*M* NaCl, 1% Triton X-100, 0.1% SDS, and 1% sodium deoxycholate) with 10 mg/mL protease inhibitor mixture (Sigma) [26]. The protein extracts were boiled for 5 min, were separated by SDS–polyacrylamide gel electrophoresis, and were transferred to a polyvinylidene fluoride membrane. After the blots were blocked in Tris-buffered saline with 0.1% Tween and 5% nonfat milk, they were incubated with primary anti–human poly(ADP-ribose) polymerase (PARP), cytochrome *c,* caspases-1–10, and actin (1:1000; PharMingen and Santa Cruz Biotechnology) overnight at 4°C. After being rinsed, the blots were incubated with a horseradish peroxidase–conjugated secondary antibody (BioRad) and were developed by use of the ECL kit (Amersham). For cytochrome *c* localization studies, mitochondrial and cytosolic fractions were prepared as described elsewhere [27]. Pulsed-field gel electrophoresis was performed as described elsewhere [28].

Fluorometric analysis of caspase activities. Cells were lysed in 10 m*M* Tris-HCl (pH 7.5), 10 m*M* $NaH_2PO_4/NaHPO_4$ (pH 7.5), 130 m*M* NaCl, 1% Triton X-100, and 10 m*M* sodium pyrophosphate for 5 min on ice (no protease inhibitors). Twenty microliters of lysate was added to 80 μL of reaction buffer (100 m*M* HEPES, 10% sucrose, 10 m*M* dithiothreitol, and 0.5 m*M* EDTA [pH 7.5]) containing a specific fluorogenic caspase substrate (75 μM; Calbiochem). After incubation at 37°C for 60 min, fluorescence was measured by use of a microplate reader (CytoFluor). Standard 7-amino-4-methyl coumarin and 7-4-trifluoromethyl coumarin solutions were used for calculating caspase activity.

Immunocytochemistry and fluorescence staining. Before and after various time points of incubation with pneumococci, brain cell cytospin preparations were made and air-dried. After fixation (4% paraformaldehyde) and permeabilization (0.1% Triton X-100), cytospins were incubated with an anti-AIF antibody, diluted 1:500 in PBS–1% bovine serum albumin [19]. A fluorescent Cy3 antibody (Jackson ImmunoResearch) was used to visualize the binding sites of the primary antibody. A mitochondrion-selective fluorescent dye (MitoTracker; Molecular Probes) was used to assess the integrity of the mitochondrial membrane potential [29]. After the cells were incubated with pneumococci for the indicated intervals, 300 n*M* dye was added for 30 min, and its mitochondrial uptake, which depends on an intact mitochondrial membrane potential, was assessed by fluorescence microscopy. For flow cytometry, cells were detached with trypsin-EDTA and were stained with propidium iodide. For electron microscopy, microglial cells were fixed in 2.5% glutaraldehyde in 100 m*M* cacodylate buffer overnight at 4°C. They then were postfixed in PBS with 1% osmium tetroxide, were stained with 2% uranyl acetate, were dehydrated in gradients of ethanol, and were embedded in resin. Sections were examined by use of a JEOL 1200 EX II transmission electron microscope.

Results

Pneumococci directly induce apoptosis. To assess whether there are direct cytotoxic effects of pneumococci, brain cells

were exposed to living bacteria at a ratio of 1:5, reflecting the concentration in human bacterial meningitis (i.e., 10^7 bacteria/mL) [30]. Exposure of human microglia, neurons, primary mixed mouse brain cells, or primary rat hippocampal neurons to live pneumococci induced rapid morphologic and biochemical signs of apoptosis (figure 1). These included cell shrinkage, the condensation of nuclei (without fragmentation; figure 1*D,* 1*J,* and 1*P*), and the appearance of terminal transferase–mediated dUTP nick end–labeled cells (TUNEL; figure 1*E,* 1*K,* and 1*Q*), and Annexin-V– and propidium iodide–positive cells (figures 1*F,* 1*L,* 1*R,* and 2*C*). These results extend specifically to the neurons of the dentate gyrus that are targeted in vivo, because pneumococci (10^6 cfu/mL for 12 h) also augmented apoptosis of cultured primary rat hippocampal neurons by a mean of 56% ± 5% (vs. 29% ± 5% in control cells; $P = .001$, Mann-Whitney *U* test).

The onset of apoptosis induced by pneumococci was rapid (figure 2) and dependent on bacterial concentration (data not shown). Reduction of bacterial number by 1 log increased the time of onset of apoptosis by 3 h. Human microglia died almost exclusively by apoptosis starting within 3 h of challenge (figure 2*A*). These cells first translocated phosphatidylserine (as shown by green Annexin-V staining) and later underwent cell membrane damage (as shown by red propidium iodide staining; figure 2*C*). Cell death was more protracted in human cortical neurons and involved, to a minor extent, the induction of necrosis (figure 2*B*). Thus, direct exposure of neuronal or glial cells to live pneumococci triggered apoptosis. Exposure of these cells to heat-killed pneumococci or purified pneumococcal cell walls failed to induce apoptosis, with the cells remaining viable for ⩾12 h (data not shown).

Using immunoblotting and caspase activity assays, we evaluated the activation of caspases-1–10 by live pneumococci. The exposure of microglia to staurosporine, a universal inducer of apoptosis, led to the rapid cleavage of the caspase-3 substrate PARP (figure 3*A*), activated all caspases tested (i.e., caspases-1–10; figure 3*B;* data not shown), and caused typical nuclear condensation and fragmentation into micronuclei (figure 3*C*). In contrast, pneumococci failed to activate these caspases in microglia or primary mouse brain cells (figure 3*B*). Dying cells demonstrated a gradual decrease in PARP between 6 and 9 h, but typical cleavage fragments of PARP were not observed, which suggests cleavage to very small fragments. Morphologic analysis showed condensed but not fragmented nuclei (figure 3*A* and 3*C;* data not shown). Finally, although the broad-spectrum caspase inhibitor z-VAD-fmk prevented staurosporine-induced apoptosis, it failed to delay pneumococcus-induced apoptosis of microglial or neuronal cells (figure 3*C;* data not shown). This suggested that activation of caspases-1–10 was not a prominent feature of direct pneumococcus-induced apoptosis.

Pneumococci induce mitochondrial damage and AIF-dependent apoptosis. Apoptosis can be initiated by mitochondrial damage without prominent caspase activation in some models of cell death [31]. We therefore tested whether pneumococci triggered mitochondrial damage, using a mitochondrion-selective fluorescent dye, uptake of which depends on an intact mitochondrial membrane potential [29]. Mitochondrial uptake of this dye was markedly reduced within 45 min of exposure to pneumococci (figure 4*A*). The loss of mitochondrial membrane potential preceded all the morphologic signs of apoptosis induced by pneumococci.

Loss of mitochondrial membrane potential was associated with the release of proapoptotic factors into the cytosol. As determined by immunocytochemistry, mitochondrial localization of AIF decreased within 1.5 h of incubation of microglia or neurons with pneumococci (figure 4*B*). Furthermore, immunoblotting of mitochondrial and cytosolic fractions revealed release of cytochrome *c* from the mitochondrial fraction into the cytosolic fraction (figure 4*C*) after incubation of cells with pneumococci. Of interest, the release of cytochrome *c* did not lead to the activation of caspases in brain cells exposed to pneumococci (figure 3*B*).

We used transmission electron microscopy to determine whether the loss of mitochondrial membrane potential and the release of cytochrome *c* and AIF were associated with ultrastructural changes of mitochondria. Consistent with the loss in mitochondrial membrane potential, the exposure of microglia or neurons to live pneumococci caused rapid and massive swelling of mitochondria (figure 4*D;* data not shown).

The characteristics of microglia undergoing pneumococcus-induced death were reminiscent of many aspects of AIF-induced apoptosis [19], including peripheral condensation of chromatin along the nuclear envelope (without the formation of micronuclei) and the exposure of phosphatidylserine to the outer leaflet of the cell membrane (figures 1 and 2). Because pneumococci induced the rapid release of mitochondrial AIF, we assessed whether AIF function was necessary for pneumococcus-induced apoptosis by microinjecting an AIF-specific and neutralizing antiserum [19] into the cytosol of microglia and then exposing these cells to live pneumococci. Microglia microinjected with anti-AIF antiserum displayed 3–4-fold less apoptosis than did uninjected cells or cells injected with control antiserum (decrease from 34% ± 6% to 11% ± 3% after a 4-h incubation; $P = .03$, Mann-Whitney *U* test) (figure 5*A*). Thus, pneumococcus-induced apoptosis involves the release of AIF from mitochondria and is AIF dependent. Other markers of pneumococcus-induced apoptosis included hypodiploidy, condensation of chromatin (figure 5*B*), and large-scale DNA fragmentation (figure 5*C*) and were similar to the reported effects of AIF [19].

Discussion

Caspase-dependent neuronal apoptosis is induced by the host inflammatory response, in particular by leukocyte invasion into the central nervous system [7], a feature central to all bacterial

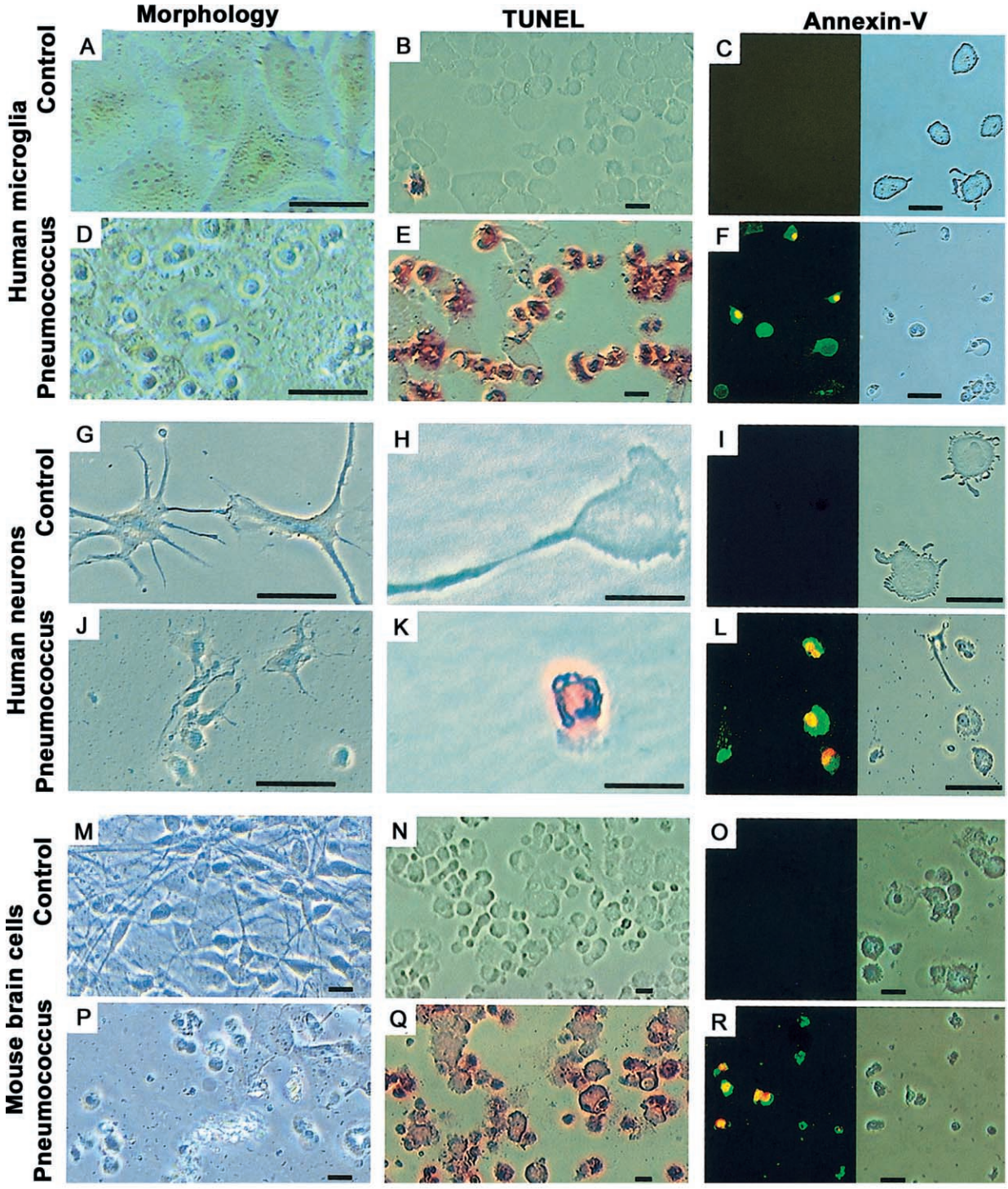

Figure 1. Pneumococci directly induce apoptosis. Human microglial cells *(A–F),* human neurons *(G–L),* and primary mouse brain cells *(M–R)* were incubated with live pneumococci *(D–F, J–L,* and *P–R)* or no stimulus *(A–C, G–I,* and *M–O)* for 6 h. Apoptosis was detected morphologically by shrinkage and condensation of cells and nuclei *(D, J,* and *P)* and biochemically by use of an in situ cell-death detection kit (TUNEL, Boehringer; *E, K,* and *Q*) and a fluorescein isothiocyanate kit (Annexin-V, Coulter), which involves costaining with the membrane-integrity dye propidium iodide *(F, L,* and *R)*. *Bars,* 10 μm.

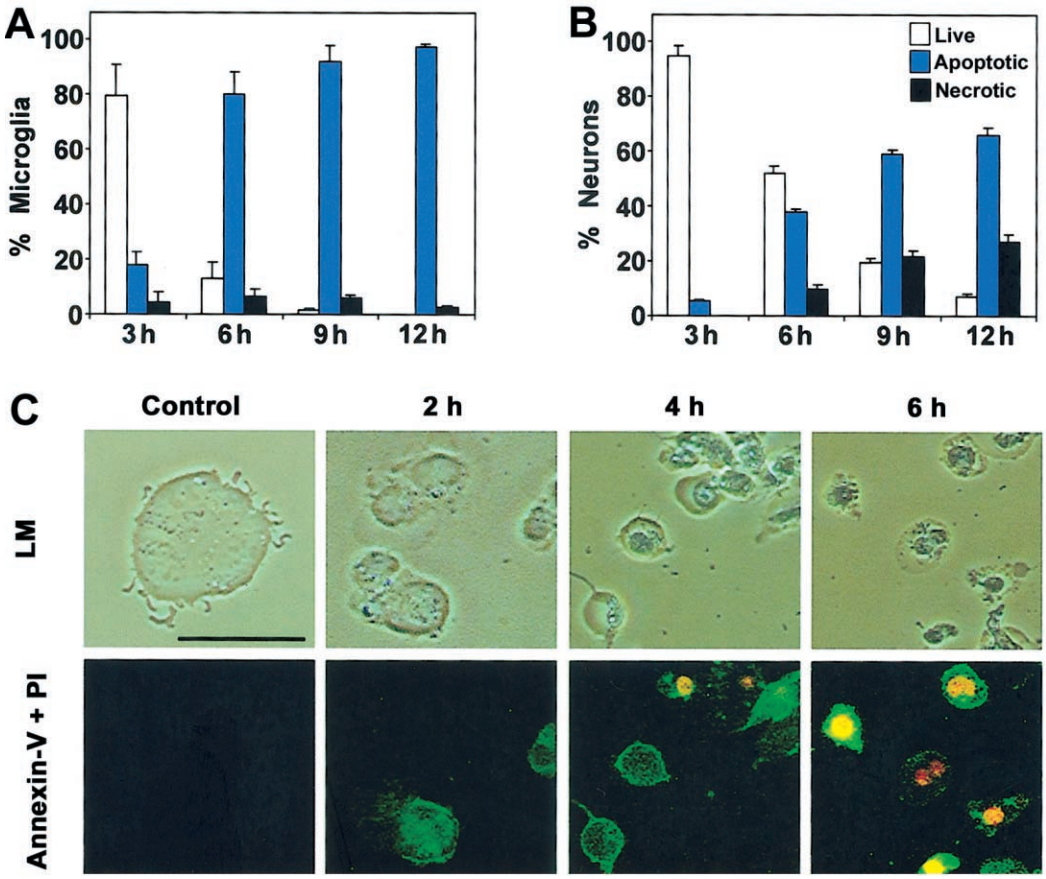

Figure 2. Time course of pneumococcus-induced apoptosis. *A* and *B,* Human neurons and human microglia were exposed to live pneumococci (10^7 cfu/mL). Live, apoptotic, and necrotic cells were differentiated with the ethidium bromide–acridine orange assay. Error bars indicate SD of 3 independent experiments. *C,* Time course of fluorescein isothiocyanate–propidium iodide (Annexin-V + PI; Annexin-V, Coulter) staining of microglia exposed to pneumococci (10^7 cfu/mL). LM, light microscopy. *Bar,* 10 μm.

meningitides. However, this process accounts for only half the neuronal damage to the dentate gyrus during pneumococcal infection. Given the particularly poor outcome of pneumococcal meningitis over and above other forms of central nervous system infection, the existence of additional deleterious processes triggered uniquely by this host-bacterial interaction seemed to be likely. The contribution of direct bacterial effects to brain cell damage and the mechanisms of bacteria-induced brain cell killing are poorly understood.

The present study demonstrates unusual mechanistic features of brain cell apoptosis induced by live pneumococci. Pneumococci induced rapid damage to mitochondria, disrupted mitochondrial membrane potential, caused the release of the proapoptotic mitochondrial factors cytochrome *c* and AIF, and caused large-scale DNA fragmentation and hypodiploidy. However, despite the release of cytochrome *c,* caspases were not broadly activated, and a general caspase inhibitor did not block apoptosis. Pneumococcus-induced apoptosis thus contrasts with that reported for *Salmonella* and *Shigella* species, which induce intensely caspase-dependent host cell apoptosis [32, 33]. This process also is distinct from the caspase-dependent killing of neurons described for group B streptococci [34]. However, apoptosis induced by pneumococcus is AIF dependent, and these findings indicate an important role for AIF in brain cell death and in bacterial pathogenesis.

The novel features of pneumococcal killing of brain cells are as follows. First, although nuclei and chromatin condensed, there was no nuclear fragmentation, and DNA fragments remained large. Caspase-3 activation was not detected, a finding consistent with its role in cleavage of inhibitor of caspase-activated DNase inactivation/DNA fragmentation factor (DFF) 45 and the release of the active DFF40 DNA endonuclease [35, 36]. Second, apoptosis was not associated with a typical degradation of caspase substrates, such as PARP or lamins (figure 3; data not shown). Third, although cytochrome *c* was released, caspases were not activated, indicating a nonfunctional apoptosome. The formation of an active apoptosome, a complex consisting of cytochrome *c,* Apaf-1, dATP, and procaspase-9,

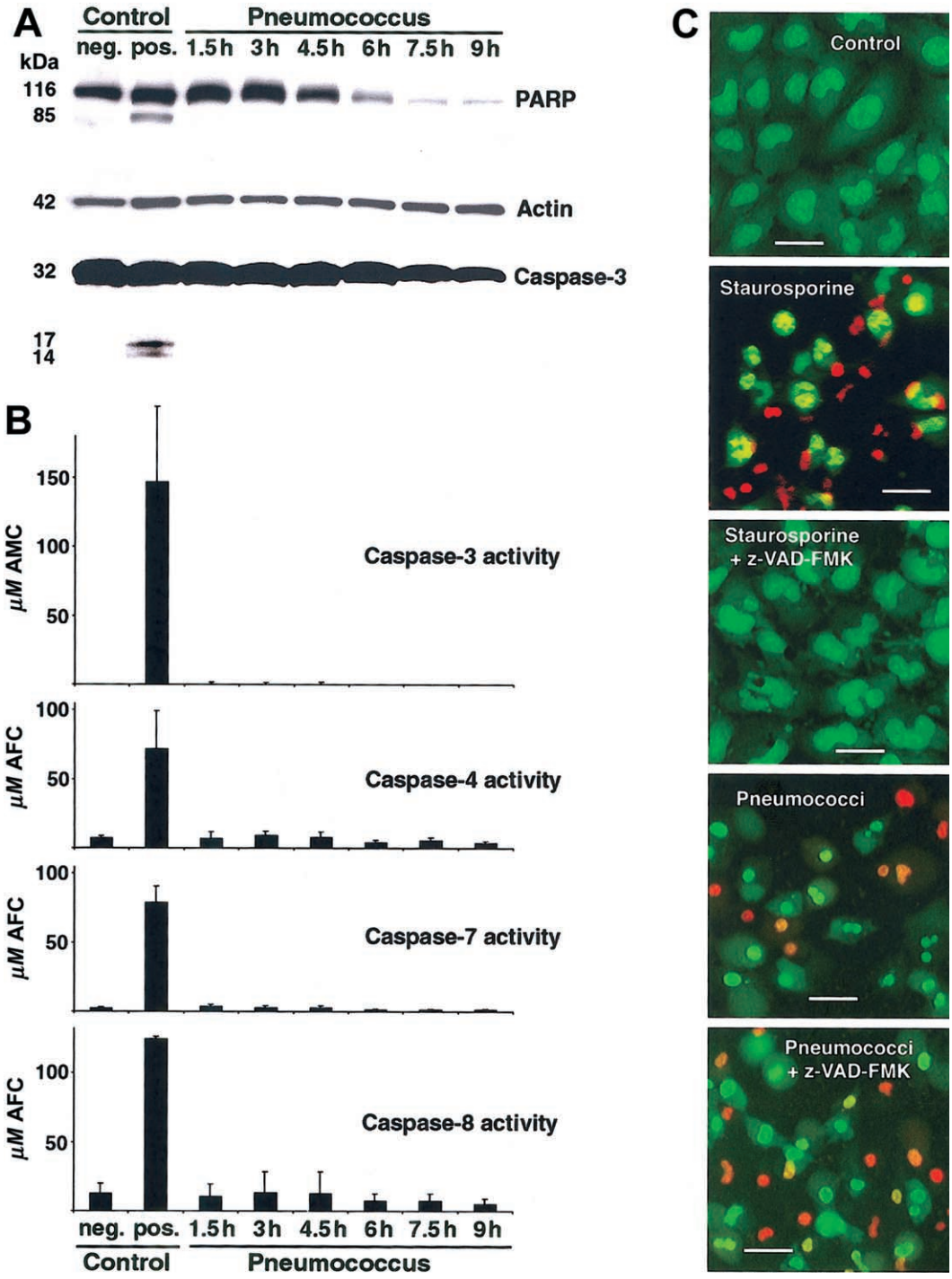

Figure 3. Pneumococcus-induced apoptosis is caspase independent. Microglia were induced to undergo apoptosis by incubation with live D39 wild-type pneumococci or staurosporine (as a positive control). Incubation of microglia with staurosporine (5 h, 0.1 μM) but not with D39 wild-type pneumococci (10^7 cfu/mL, 1.5–9 h) resulted in caspase activation. *A,* Representative results are shown for immunoblotting with caspase-3–specific and primary anti–human poly(ADP-ribose) polymerase (PARP)–specific antibodies. Actin served as a loading control. *B,* Caspases-1–10 are not activated after exposure to pneumococci. Representative results of 4 caspase-activity assays are shown. Similar results were obtained in measurements of activities of caspases-1, -2, -5, -6, and -9 (data not shown). Error bars indicate SD of 1 of 3 experiments done in triplicate. AFC (7-amino-4-trifluoromethyl coumarin) and AMC (7-amino-4-methyl coumarin), standard solutions for determining caspase activity; neg., negative; pos., positive. *C,* The broad-spectrum caspase inhibitor z-VAD-fmk (0.1 $\mu g/\mu L$) prevents nuclear shrinkage and condensation induced by staurosporine (0.1 μM, 6 h) but not by pneumococci (10^7 cfu/mL, 6 h). Nuclei were stained with ethidium bromide (EB) and acridine orange (AO). In this assay, untreated control microglia show normal-sized green AO-stained nuclei and exclude EB. Incubation of microglia with D39 pneumococci (10^7 cfu/mL, 6 h) caused massive shrinkage and condensation of the nuclei, which stained red when cells failed to exclude EB and were thus demonstrated to be late apoptotic cells. Results shown are representative of 2 independent experiments. *Bars,* 10 μm.

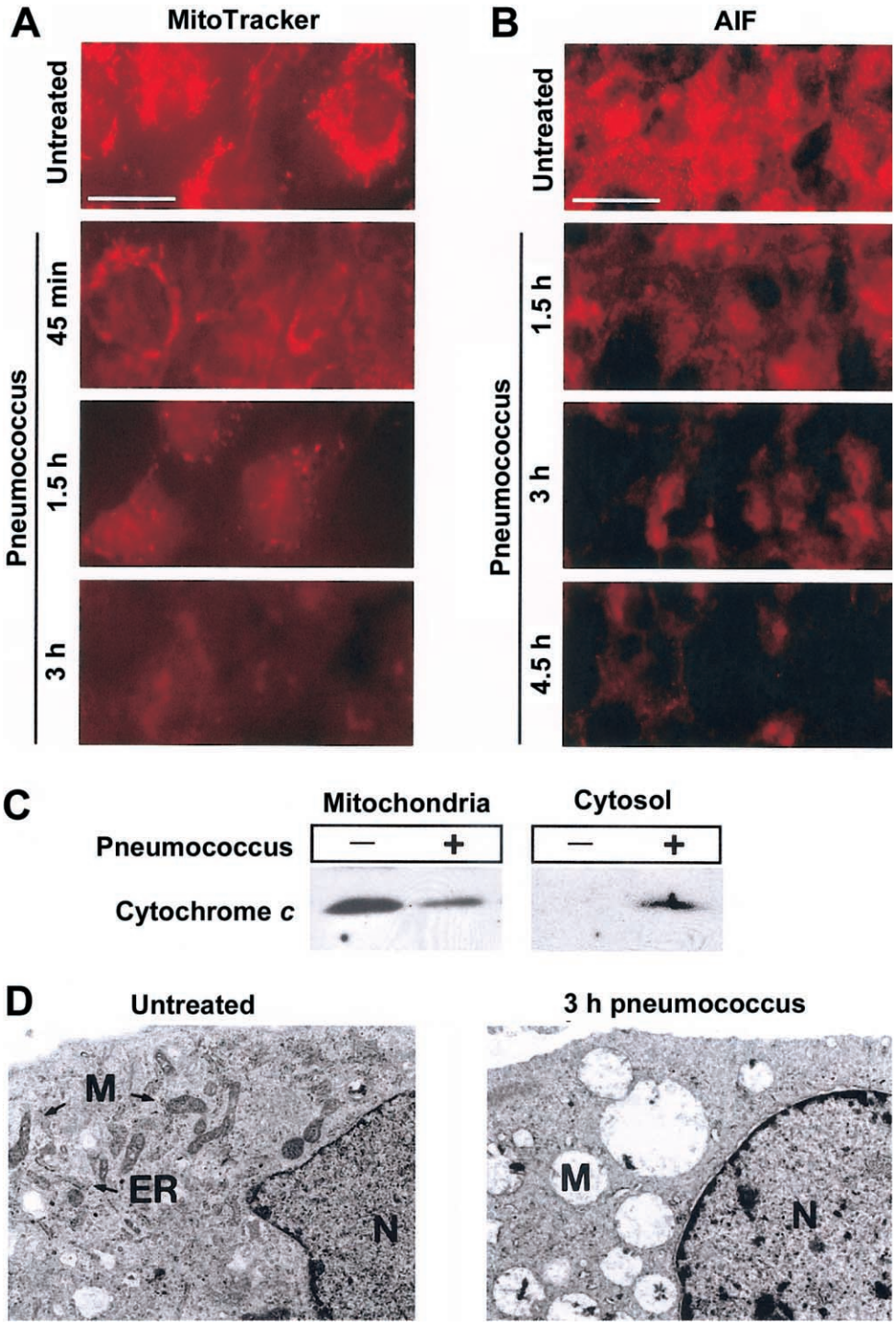

Figure 4. Pneumococci induce rapid damage to mitochondria. Microglial cells were incubated with pneumococci strain D39 (10^7 cfu/mL). Mitochondrial damage was assessed by measuring uptake of a mitochondrion-selective fluorescent dye (MitoTracker, Molecular Probes) that depends on an intact mitochondrial membrane potential *(A)* and by assessing apoptosis-inducing factor (AIF) localization by immunofluorescence *(B)* and cytochrome *c* localization by immunoblotting of mitochondrial and cytosolic fractions *(C)*. Results are representative of 3 independent experiments. *D,* Changes of ultrastructure of microglial cells exposed to pneumococci were monitored by transmission electron microscopy after 3 h of incubation. D39 (10^7 cfu/mL) causes massive swelling of the mitochondria (M). Control microglia show intact ultrastructure of the nucleus (N) and cell organelles (ER). *Bar,* 1 μm.

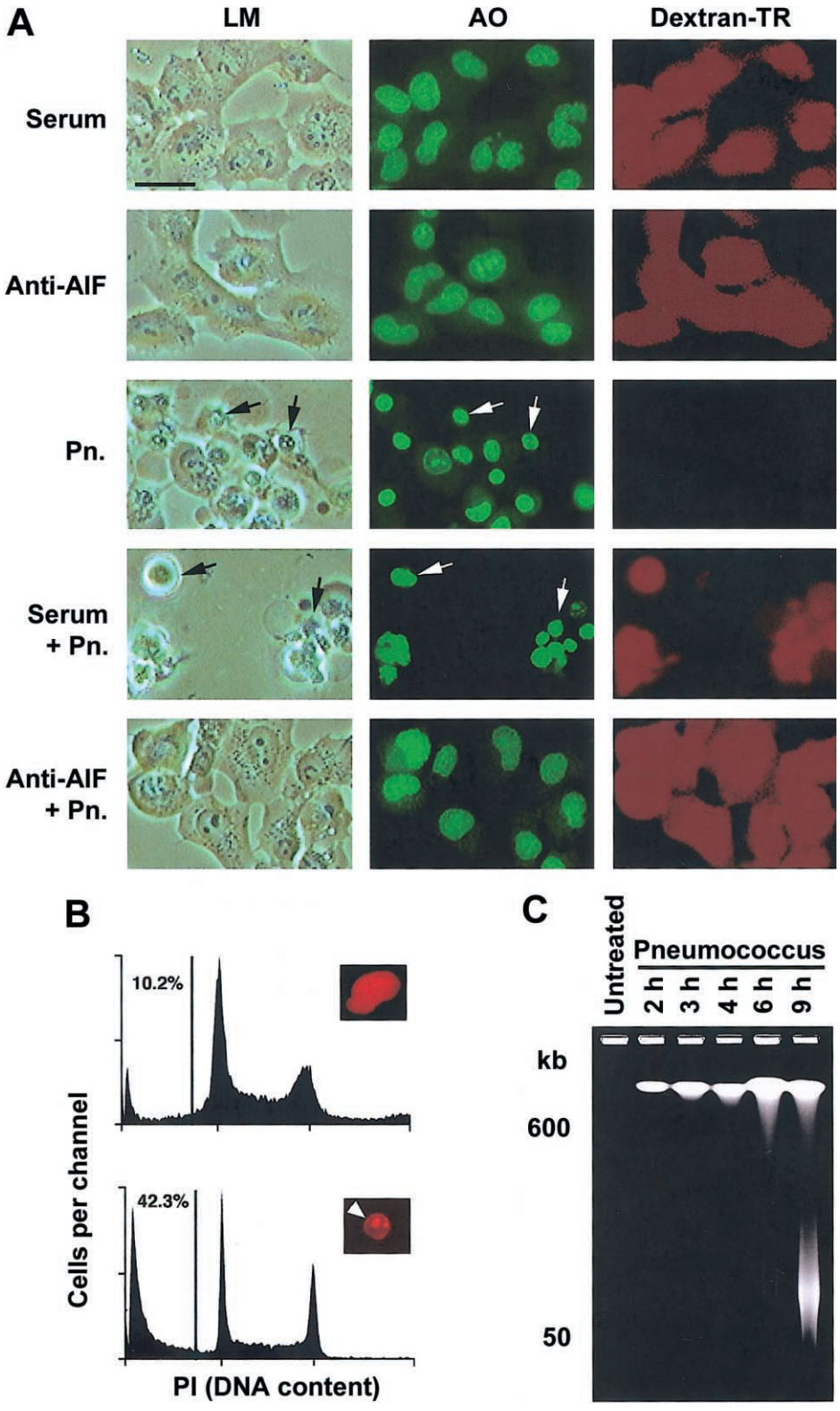

Figure 5. Pneumococcus-induced apoptosis is apoptosis-inducing factor (AIF) dependent. *A,* Human microglia cells were microinjected into the cytoplasm with Dextran Texas Red (TR) dye and AIF-specific antiserum or control serum and then were incubated with pneumococci (Pn.; 10^7 cfu/mL) for 4 h. Results are representative of 2 independent experiments. AO, acridine orange; LM, light microscopy. Arrows indicate nuclear condensation and shrinkage, indicating apoptotic cells. *Bar,* 10 μm. *B,* Flow cytometry results show hypodiploidy of microglia incubated with pneumococci (10^7 cfu/mL) for 3 h. Inset shows morphology of propidium iodide (PI)–stained nuclei. *C,* Pulsed-field gel electrophoresis of genomic DNA prepared from microglia exposed to pneumococci (10^7 cfu/mL).

is required for activation of the caspase-9–to–caspase-3 cascade [37], and presumably one of the other components of the complex is inactivated by pneumococcus. However, host cell death induced by the bacterium involved massive mitochondrial swelling and damage of both the mitochondrial membrane potential and ultrastructure. This resulted in the release of AIF from mitochondria and initiated AIF-dependent cell death, large-scale DNA fragmentation, and hypodiploidy.

AIF is a soluble protein of unknown function localized in the intermembrane space of mitochondria in healthy cells [19]. However, after the induction of permeability transition pores in the outer mitochondrial membrane, AIF is released into the cytoplasm and induces apoptosis. The precise mechanism by which this occurs has not been resolved; however, it is known that AIF causes nuclear shrinkage and condensation and large-scale DNA fragmentation. Furthermore, AIF activity is caspase independent and is not inhibitable with z-VAD-fmk [19]. Thus, AIF is thought to be an important executioner of apoptosis in scenarios where the role of caspases is not prominent, and, as shown here, this is the case in pneumococcus-induced apoptosis.

Overall, the temporal sequence of events in pneumococcus-induced apoptosis appears to be as follows: 45 min after the destruction of the mitochondrial membrane potential, mitochondria release AIF and cytochrome *c,* and AIF release is associated the cleavage of genomic DNA into large fragments of ~600 kb. At this juncture, phosphatidylserine also starts to flip from the inner to the outer leaflet of the cell membrane. Ultimately, the cells become markedly hypodiploid, the nuclei shrink and become TUNEL positive, and the DNA degrades further to fragments of ~50 kb. All in all, this process is rapid, and most brain cells exposed to pneumococcus are dead in 9 h.

During meningitis, bacteria multiply in the subarachnoid space and do not invade brain parenchyma until the end stage of disease. Thus, bacteria do not directly contact cells of the dentate gyrus of the hippocampus. The fact that apoptosis was induced by living pneumococci and not heat-killed bacteria or cell walls is consistent with a secreted product of active bacterial metabolism causing damage to neurons at a distance from the bacteria itself. Preliminary results suggest that bacterial supernatants contain more than one toxic element, including pneumolysin (J. S. Braun, J. L. Cleveland, T. Mitchell, J. R. Weber, and E. I. Tuomanen, unpublished data). That the apoptotic process induced by pneumococci was characterized by condensed but not fragmented nuclei is also consistent with described in vivo morphology [7].

These findings also have important implications for the design of an effective treatment regimen for bacterial meningitis. First, one must consider blocking the host inflammatory response, as inhibitors of caspases or leukocytosis provide partial protection to the hippocampus [7]. Second, adjuvant therapy must be developed that inhibits the direct pneumococcus-induced apoptotic pathway (e.g., by using agents that will prevent mitochondrial damage in the host cell).

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