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Apoptosis of Epithelial Cells and Macrophages Due to Infection with the Obligate Intracellular Pathogen *Chlamydia psittaci*¹

David M. Ojcius,² Philippe Souque, Jean-Luc Perfettini, and Alice Dautry-Varsat

We have characterized the cytotoxic activity of the obligate intracellular bacterium *Chlamydia psittaci*, which resides within a membrane-bound vacuole during the 2-day infection cycle. We have established that infected epithelial cells and macrophages die through apoptosis, which is measurable within 1 day of infection and requires productive infection by the bacteria. Inhibition of host cell protein synthesis has no effect on cell death, but blocking bacterial entry or bacterial protein synthesis prevents apoptosis, implying that bacterial growth is required for death of the host cell. Apoptosis was confirmed through the use of electron microscopy, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling, gel agarose electrophoresis of fragmented DNA, and propidium-iodide labeling of host cell nuclei. Although infected cells died preferentially, both infected and uninfected cells became apoptotic, suggesting that the infected cells may secrete proapoptotic factors. Inhibition of either of two proapoptotic enzymes, caspase-1 or caspase-3, did not significantly affect *Chlamydia*-induced apoptosis. These results suggest that, as in the case of apoptosis due to Bax expression or oncogene dysregulation, which initiate the apoptotic program within the cell interior, the *Chlamydia* infection may trigger an apoptotic pathway that is independent of known caspases. As apoptotic cells secrete proinflammatory cytokines, *Chlamydia*-induced apoptosis may contribute to the inflammatory response of the host. *The Journal of Immunology*, 1998, 161: 4220–4226.

Apoptosis (programmed cell death) is a form of cell death distinguishable from necrosis (accidental cell death), which occurs in embryonic development, tissue homeostasis, and immune-cell mediated cytotoxicity and selection of immune cells (1–3). Apoptosis occurs when a normally functioning cell receives any of a variety of death signals, including different cytokines (e.g., TNF- α and the Fas ligand) acting via cell surface receptors (2), but is also associated with a number of pathologic disorders, including AIDS, fulminant hepatitis, cancer, and neurodegenerative disorders (4). The salient features of apoptosis are membrane blebbing, cell shrinkage, chromatin condensation, and DNA fragmentation. At later stages of apoptosis, the doomed cell fragments into membrane-bound vesicles that are rapidly ingested by neighboring cells (1).

Microbe-induced apoptosis was first identified for viral infections and has subsequently been reported in cases of infections with a large number of pathogenic bacteria and parasites (4–7). Most of the latter studies report that infection of macrophages results in apoptosis, although occasionally infection has been found to inhibit apoptosis induced by other triggers (8–10). The ability of pathogens to induce apoptosis may play a role in the initiation of the infection, survival of the pathogens, and escape from the host immune response, as well as promoting inflammatory responses. For some pathogens, trig-

gering of apoptosis may be a host response to reduce or inhibit microbial replication (5, 11–13).

Cytotoxicity due to infection by human and nonhuman strains of the intracellular bacteria *Chlamydia* has been reported for many years (14–21), but the mechanism of cell death has not been investigated. *Chlamydia* species are among the most successful pathogens on earth, being causative agents of conjunctivitis, trachoma, pneumonia, and the most common sexually transmitted bacterial infections (22–24). For all of these *Chlamydia* species, the main pathologic response during the early part of the infection process is due to the acute inflammatory response by the host (25).

Macrophages and monocytes undergoing apoptosis have been found to secrete the proinflammatory cytokine IL-1 β (26). Given the potential role that *Chlamydia*-induced apoptosis may play during onset of the inflammatory response, we investigated apoptosis in macrophages and monocytes, in which infection has been described (27), and epithelial cells, which represent the preferential target cells for *Chlamydia* infection in vivo (25, 28). In epithelial cells, infectious elementary bodies (EB)³ are internalized into vacuoles that avoid fusion with host cell lysosomes. After 6–10 h, the EBs differentiate into noninfectious but metabolically active reticulate bodies (RBs), which proliferate within the expanding vacuole, giving rise to 1000 or more progeny per host cell. The infection cycle ends after ~2 days, when RBs differentiate back to EBs, the bacteria are released through a poorly characterized process, and a new infection cycle begins (28).

A salient property of apoptotic cells is their detachment from neighboring cells, followed by removal by scavenger phagocytes (1); in vitro, apoptotic cells detach from growth substrate. We have found that many epithelial cells and macrophages are located in the supernatant after infection with *Chlamydia psittaci*, and that the

Unité de Biologie des Interactions Cellulaires, Centre National de la Recherche Scientifique 1960, Institut Pasteur, Paris, France

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² Address correspondence and reprint requests to Dr. David Ojcius, Institut Pasteur, Unité de Biologie des Interactions Cellulaires, 25 rue du Dr. Roux, 75724 Paris Cedex 15, France. E-mail address: ojcius@pasteur.fr

³ Abbreviations used in this paper: EB, elementary body; m.o.i., multiplicity of infection; PI, propidium iodide; RB, reticulate body; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; ICE, IL-1 β -converting enzyme.

cells display characteristic features of apoptosis. Infection-mediated apoptosis has been confirmed with several techniques including electron microscopy, which shows the morphologic changes associated with apoptosis; the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method, which reveals early DNA breaks during apoptosis; gel agarose electrophoresis, showing host cell DNA fragmentation due to infection; and nuclear staining with propidium iodide (PI), which allows for quantitation of apoptosis under different conditions. Results with inactivation of bacteria or inhibition of bacterial adhesion imply that a productive infection is required for apoptosis, which is measurable after a 1-day infection and increases afterward. Since both infected and uninfected cells become apoptotic, secreted factors from infected cells may also trigger apoptosis of uninfected cells. Cells undergoing apoptosis may thus secrete cytokines that contribute to the inflammatory response to *Chlamydia* infection.

Materials and Methods

Cells and materials

The human cervical adenocarcinoma cell line, HeLa 229, the mouse macrophage cell line, J774, and Jurkat cells were from American Type Culture Collection (Manassas, VA). The THP1 cells (29) have been previously described. The cells were maintained at 37°C in an atmosphere of 5% CO₂ in DMEM (Life Technologies, Rockville, MD) (for HeLa) or RPMI 1640 (for J774, Jurkat, and THP1) supplemented with 10% heat-inactivated FBS (Life Technologies) and 2 mM L-glutamine. The *Chlamydia* strain used here, the guinea pig inclusion conjunctivitis serovar of *C. psittaci* (25), was obtained from Dr. Roger Rank (University of Arkansas, Little Rock, AR).

DABCO (1,4-diazabicyclo[2.2.2]octane), chloramphenicol, and heparin were from Sigma (St. Louis, MO), and Mowiol, the IL-1 β -converting enzyme (ICE; caspase-1 inhibitor II (Ac-YVAD-CMK), and the CPP32/apopain (caspase-3) inhibitor II (Z-DEVD-FMK) were from Calbiochem (La Jolla, CA). Texas Red-coupled F(ab')₂ fragment goat anti-mouse Ig were purchased from Molecular Probes (Eugene, OR). The mAb against human Fas (clone CH-11) was from Upstate Biotechnology (Lake Placid, NY), and unconjugated and FITC-labeled anti-*Chlamydia* mAb were from Argene (Varilhes, France). RNase A (DNase free) was from Boehringer Mannheim (Meylan, France).

Preparation of *Chlamydia* and infection of host cells

The chlamydiae were grown in infected HeLa cell monolayer cultures as described (30). For infections, adherent HeLa and J774 cells were typically grown on coverslips or on 75-cm² tissue culture flasks (Costar) until 60 to 70% confluence was obtained, then incubated with chlamydiae in cell culture medium for the indicated times at 37°C in 5% CO₂. THP1 cells were infected in suspension in culture medium (29). Unless indicated otherwise, the *Chlamydia* was used at a multiplicity of infection (m.o.i.) between 1.0 and 2.0.

Confocal microscopy

Samples for confocal microscopy were fixed with paraformaldehyde, incubated with Abs, and mounted as previously described (31, 32). Apoptotic HeLa cells on coverslips were detected by enzymatic labeling of DNA strand breaks with the TUNEL technique (33) using the cell death detection kit from Boehringer Mannheim, following the manufacturer's instructions. For these experiments, infected cells were fixed as described above, and apoptotic cells were detected as green (due to fluorescein-12-dUTP). Cells infected with *Chlamydia* were identified by revealing with unconjugated anti-*Chlamydia* mAb (1:500), followed by incubation with Texas Red-labeled anti-mouse Ig polyclonal Ab.

Fluorescently labeled samples were examined with a Leica confocal microscope (Heidelberg, Germany) attached to a diaphan microscope equipped with a double argon-krypton laser. Serial optical sections were typically recorded at 0.5- μ m intervals with 63 \times and 100 \times lenses.

Cytofluorometry analysis of apoptosis

Quantitative measurements of apoptosis were performed by cytofluorometry of detergent-permeabilized PI-stained cells as described (34). Cells were first fixed with paraformaldehyde, and bacteria were revealed with FITC-labeled anti-*Chlamydia* mAb (1:500) before incubating with the PI buffer. Cytotoxicity was measured using the standard PI-exclusion assay

with unpermeabilized cells. Unless noted otherwise, both adherent cells and cells in the supernatant were collected for analysis.

The cells were transferred into 12 \times 75 mm Falcon 2052 FACS tubes (Becton Dickinson, San Jose, CA). Data from 10,000 HeLa cells were collected on a FACScan flow cytometer (Becton Dickinson) with an argon laser tuned to 488 nm.

Inhibition of apoptosis and *Chlamydia* adherence by heparin was measured with either HeLa or J774 that had been incubated with bacteria and 120 μ g/ml of heparin (32). The bacteria were incubated in the heparin solutions in PBS for 1 h at 4°C before adding the mixture to an equivalent volume of HeLa or J774 cells in culture medium. After an hour, unbound bacteria were removed by washing once with PBS and replacing the supernatant with culture medium. After an additional 48 h, the percentage of apoptotic cells was determined by cytofluorometry. For UV inactivation experiments, the bacteria were exposed to UV light in a cell culture hood under constant stirring in an ice cold water bath for 60 min. For caspase or chloramphenicol inhibition experiments, 50 μ M of the caspase-1 or caspase-3 inhibitor or 68 μ g/ml chloramphenicol was maintained with the HeLa cells during the duration of the infection.

DNA fragmentation assay

HeLa or J774 cells ($1-3 \times 10^6$) were washed with PBS and centrifuged ($270 \times g$ for 5 min), and the pellet was lysed with 0.6% SDS, 10 mM EDTA, 10 mM Tris, and 20 μ g/ml RNase A, pH 7.5, for 1 h at 37°C in 3 ml. Three hundred microliters of 5 M NaCl was then added, and the preparation was incubated for 1 h on ice and finally centrifuged for 30 min at $13,000 \times g$. The supernatant, containing the DNA, was extracted with phenol-chloroform-isoamyl alcohol (25:24:1), and low-m.w. DNA was precipitated with ethanol. Samples ($\sim 3 \mu$ g DNA per lane) were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining. UV or heparin inhibition experiments were performed as above.

Electron microscopy

HeLa cells infected with chlamydiae for 0 h or 24 h were fixed with 2.5% glutaraldehyde for at least 2 h at room temperature. The fixed cells were then prepared for electron microscopy as previously described (30). Thin sections were poststained with uranyl acetate and lead citrate for examination on a Zeiss electron microscope at an accelerating voltage of 50 kV.

Measurement of IL-1 β secretion

Secretion of IL-1 β from HeLa and THP1 cells was measured using the Genzyme (Cambridge, MA) human IL-1 β ELISA kit following the manufacturer's instructions. Cells were incubated with chlamydiae at an m.o.i. of 1.0 for 1 day. The supernatant was collected, centrifuged in Eppendorf tubes to remove cellular debris, and kept frozen at -80°C until ready for use. Supernatants were diluted in the ELISA kit wash buffer, and the absolute concentrations of IL-1 β were obtained by calibrating the ELISA kit with a known concentration of IL-1 β provided by the manufacturer.

Results

Effect of *Chlamydia* infection on cell death of epithelial cells and macrophages

To characterize the cell death caused by *C. psittaci*, we infected the HeLa cell line (derived from epithelial cells) and measured the percentage of apoptotic cells by cytofluorometry, as described in *Materials and Methods*. Concomitantly, we measured the extent of overall cytotoxicity by the standard PI exclusion method. As apoptotic cells detach from neighboring cells (1), we first determined whether apoptotic cells appearing during infection were located among the adherent cells or the cells in suspension. As almost all apoptotic cells were in suspension (not shown), both adherent cells and cells in suspension were collected for subsequent analysis. Figure 1 shows the dependence of cytotoxicity and apoptosis on the concentration of *Chlamydia* used to infect the cells. After a 48-h infection, almost half of the cells had died when infected with an m.o.i. of 1.0, and most of the cells died after infection with an m.o.i. of 2.0. At all of the bacterial dilutions used, most of the cytotoxicity was due to apoptosis (Fig. 1).

To exclude the possibility that host cell death may be due to a nonspecific cytotoxic effect of the chlamydiae (16, 17, 19–21), we also evaluated whether UV inactivation of the bacteria, a treatment

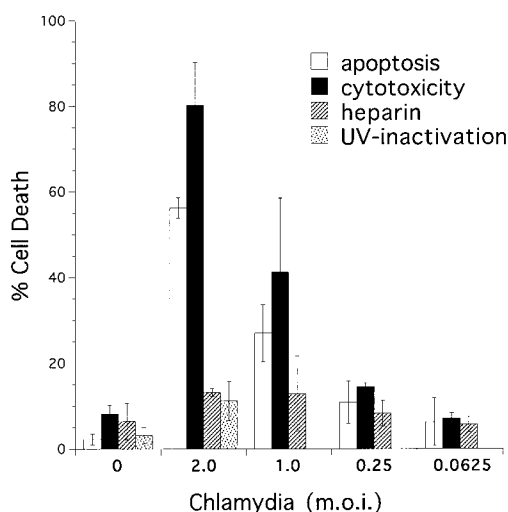


FIGURE 1. Cell death of HeLa cells infected with *C. psittaci*. Percentage of cells dying through apoptosis (white bars) or cytotoxicity (black bars) and effects of heparin pretreatment (hatched bars) or UV inactivation (dotted bars) of chlamydiae on apoptosis of HeLa cells incubated with bacteria for 48 h. Apoptosis and cytotoxicity were measured by cytofluorometry as described in *Materials and Methods*. The data points represent the average and SD from two representative infections.

that prevents gene transcription by the chlamydiae (35, 36), could prevent apoptosis. At the highest bacterial concentration (corresponding to an m.o.i. of 2.0 of viable chlamydiae), using UV-inactivated bacteria induced only background levels of apoptosis (Fig. 1). Similarly, to determine whether the chlamydiae need to invade the host cell, we preincubated viable chlamydiae with 120 μ g/ml heparin, which has previously been shown to inhibit *Chlamydia* adherence and infection of epithelial cells (32, 37, 38). Again, only background levels of cytotoxicity and apoptosis were observed when cells were incubated for 48 h with chlamydiae under these conditions (Fig. 1). Taken together, these results demonstrate that *Chlamydia* induces apoptosis of epithelial cells; the cells undergo apoptosis only if they have been productively infected.

Depending on the *Chlamydia* strain and source of host cells, *Chlamydia* can also infect most macrophages and monocytes tested (27). To determine whether *Chlamydia*-induced apoptosis is specific to epithelial cells, we also studied the effects of the infection on the macrophage cell line J774 and on the monocytic cell line THP1. Incubation with *C. psittaci* resulted in apoptosis of both cell types, with a bacterial concentration dependence similar to that observed for epithelial cells, although the percentage of apoptotic macrophages or monocytes observed was lower than with HeLa cells (shown for macrophages in Figure 2). Likewise, there was no apoptosis when macrophages were incubated with UV-inactivated chlamydiae (not shown), suggesting that an infection may be required for apoptosis.

The kinetics of *Chlamydia*-induced apoptosis were also examined. In epithelial cells, there was very little apoptosis during the first 12 h of infection, but measurable amounts of apoptosis were already discernable after a 24-h infection (Fig. 3). The extent of apoptosis then increased rapidly, attaining approximately half of the cells after a 2-day infection. A similar time course was observed with macrophages, although, consistent with Figure 3, the extent of apoptosis was always lower than with epithelial cells (not shown).

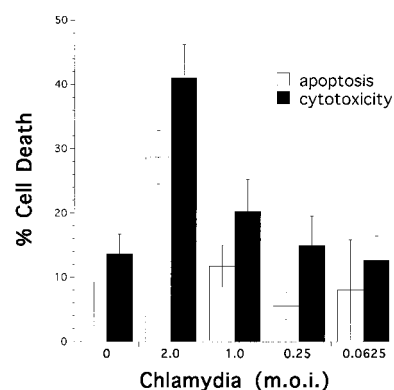


FIGURE 2. Cell death of J774 cells infected with *C. psittaci*. Percentage of macrophages dying through apoptosis (white bars) or cytotoxicity (black bars) after incubation with bacteria for 48 h. The data points represent the average and SD from three separate infections.

Morphologic changes and DNA fragmentation in infected cells

To confirm that the cell death measured by cytofluorometry corresponds to apoptosis, we characterized infected HeLa cells by electron microscopy. Both adherent cells and cells in suspension were collected for morphologic characterization (1, 3). While many ostensibly healthy HeLa cells, containing large *Chlamydia* inclusions, were observed after a 1-day infection (Fig. 4A), there were also many condensed cells having distinctive features of apoptosis (Fig. 4, B–D). Thus, many cells displayed cell shrinkage, chromatin condensation, and organelle dilatation (Fig. 4, B and C), as well as more advanced signs of apoptosis, including nuclear segmentation and cellular disintegration (Fig. 4D). Many cells still associated with membrane-bound apoptotic bodies were also observed (Fig. 4D).

Apoptosis was also confirmed by determining whether there was DNA fragmentation (1, 3). The DNA from HeLa cells that had been infected with *C. psittaci* for 48 h showed the typical 196-bp DNA ladder resolved by agarose gel electrophoresis, while uninfected cells under the same conditions had mostly intact DNA (Fig. 5). Entry of bacteria and a productive infection was required for the fragmentation, since UV-inactivated chlamydiae or chlamydiae that had been preincubated with heparin had a much smaller effect (Fig. 5). Consistent with Figure 1, there was a low level of DNA fragmentation in uninfected cells and cells incubated with UV-inactivated bacteria or live bacteria and heparin (Fig. 5).

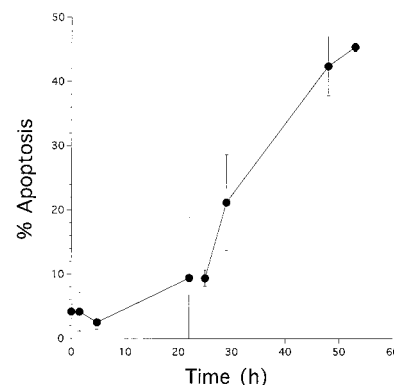


FIGURE 3. Kinetics of *C. psittaci*-induced apoptosis of HeLa cells at an m.o.i. of 1.0. Apoptosis was measured by cytofluorometry as described in *Materials and Methods*. Each data point represents the average and SD from three separate infections.

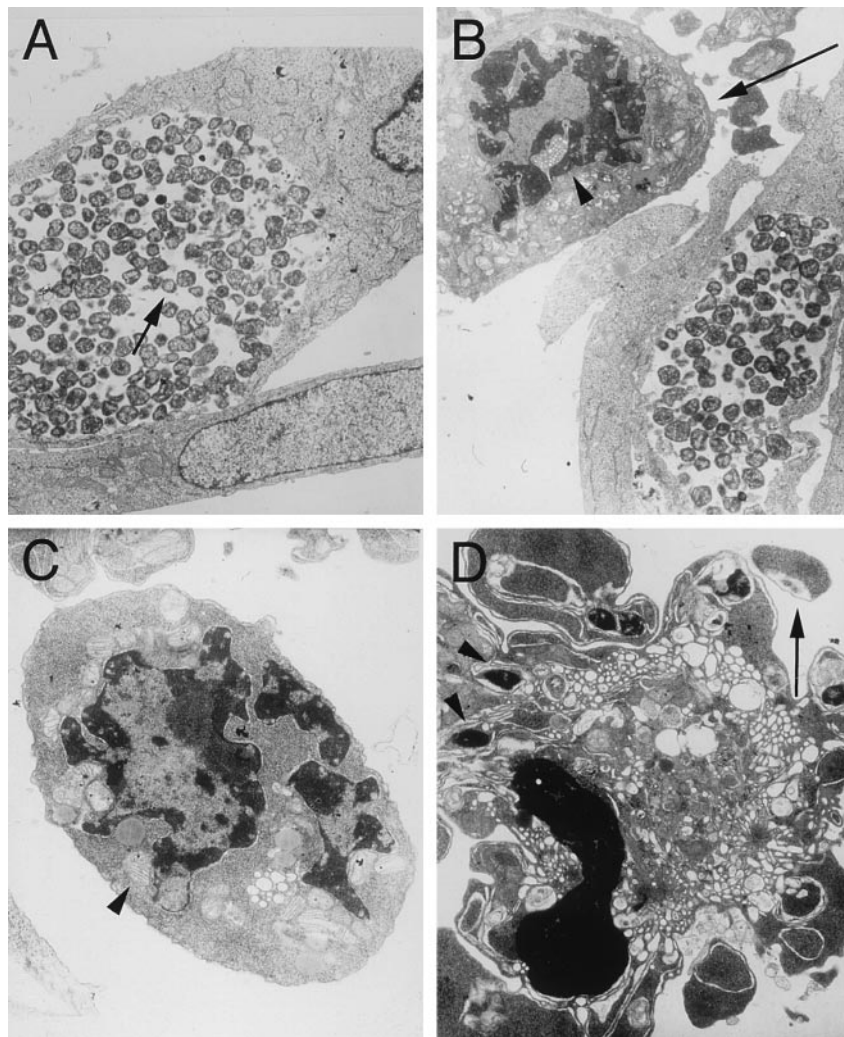


FIGURE 4. Electron micrographs of HeLa cells infected with *C. psittaci* for 24 h. *A*, An infected cell, with inclusion full of chlamydiae at the RB developmental stage, next to an uninfected cell. A typical RB is shown by the arrow. *B*, A viable infected cell next to an apoptotic cell at early stages of condensation (arrow). Arrowhead points to condensed chromatin. *C* and *D*, Apoptotic cells without visible chlamydiae at different stages of apoptosis. Arrowhead in *C* highlights a dilated mitochondrion. Arrowheads in *D* show fragmented nuclei, while the arrow points to a typical apoptotic body.

The relation between *Chlamydia* infection and apoptosis was assessed initially by the TUNEL technique, which identifies enzymatically cells with apoptosis-dependent DNA strand breaks (33). In this experiment, DNA strand breaks were labeled with fluorescein, while chlamydiae were revealed with anti-*Chlamydia* mAb and a second Ab conjugated with Texas Red. Many of the apoptotic cells also contained chlamydiae (Fig. 6*A*), and both infected normal cells and apoptotic cells that were not productively infected were observed (Fig. 6*B*).

The TUNEL results suggest that both infected and uninfected cells can die through apoptosis. This issue was addressed quantitatively with PI-labeled nuclei by cytofluorometry, which allows one to gate specifically on those cells that are apoptotic or infected in the total population of cells. Thus, in two representative experiments, $17.2 \pm 1.7\%$ of the cells were infected with *C. psittaci*, resulting in $33.4 \pm 10.1\%$ of the cells dying from apoptosis. Of the infected cells, however, only 40.4% were apoptotic, while <50% of the apoptotic cells showed any sign of infection. These results indicate that, while infected cells die more often than uninfected cells, both infected and uninfected cells are susceptible to apoptosis.

Effect of inhibitors on apoptosis

Since the eukaryotic cell protein synthesis inhibitor cycloheximide inhibits many, but not all, types of apoptosis (3), we evaluated by cytofluorometry whether cycloheximide had any effect on *Chlamydia*-induced apoptosis. After a 48-h infection, there was no significant effect of cycloheximide on *Chlamydia*-induced apoptosis (Table I). As host cell protein synthesis played no role in infection-mediated apoptosis, we tested whether chloramphenicol, which inhibits protein synthesis by the chlamydiae (39), has any effect. Most of the apoptosis was prevented by this treatment (Table I).

Since cycloheximide can have many effects besides inhibiting apoptosis, we also evaluated the effect of other inhibitors. While many different ligands may trigger apoptosis, the execution of the death program is conducted by highly specific enzymes known as caspases (40). Caspase-3 plays an important role in many types of apoptosis, including Fas-mediated apoptosis (41, 42). Caspase-1 plays a role in the maturation of IL-1 β , although it is not clear whether it is also involved in apoptosis (43). Membrane-permeable irreversible inhibitors of caspase-1 (Ac-YVAD-CMK) and caspase-3 (Z-DEVD-FMK) were tested for their effect on *Chlamydia*-induced apoptosis. While the caspase-3 inhibitor had a small

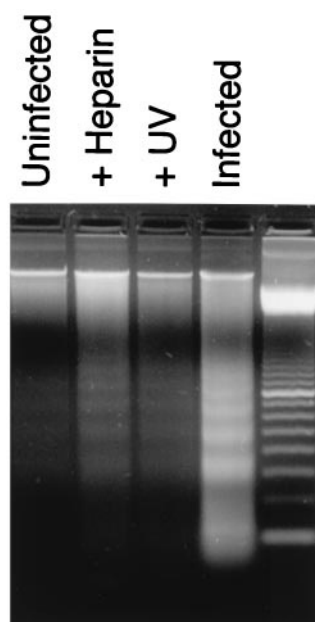


FIGURE 5. Effect of *C. psittaci*-infection on DNA fragmentation. A very low level of spontaneous fragmentation is seen in DNA isolated from uninfected cells, while prominent fragmentation is evident in cells that had been infected for 24 h with *C. psittaci* at an m.o.i. of 2.0. DNA fragmentation could be inhibited by preincubating the chlamydiae with heparin or by previously inactivating the chlamydiae with UV. The DNA ladder was visualized on a 1.5% agarose gel stained with ethidium bromide, and the right lane shows a 100 bp DNA ladder size marker. The experiment was performed with two different preparations of HeLa cells infected on separate days.

but reproducible effect on apoptosis, the caspase-1 inhibitor did not have a significant effect (Table I).

As HeLa cells are not sensitive to Fas-mediated apoptosis in the absence of cycloheximide, the activity of the caspase-3 inhibitor was evaluated by measuring apoptosis of Jurkat cells incubated with anti-Fas Ab. The caspase-3 inhibitor blocked essentially all of the Fas-mediated apoptosis (Table I). The activity of the caspase-1 inhibitor was checked by infecting THP1 cells with *C. psittaci* for 24 h and measuring the secretion of IL-1 β . It has been previously reported that human monocytes incubated with *C. trachomatis* secrete IL-1 β (44), and we have shown that THP1 cells can be infected with *C. psittaci* (29). We therefore measured IL-1 β secre-

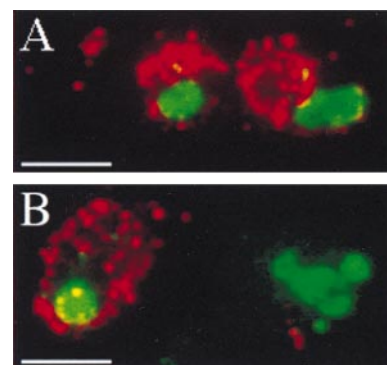


FIGURE 6. Confocal micrographs of HeLa cells double labeled with an anti-*Chlamydia* Ab (red) to identify bacteria and with dUTP (green) to identify DNA strand breaks. Representative images show two infected cells containing apoptotic nuclei (A) and both an infected and uninfected cell undergoing apoptosis (B). Cells were infected with *C. psittaci* at an m.o.i. of 1.0 for 24 h. Scale bars, 10 μ m.

tion from THP1 cells after a 1-day infection in the presence or absence of 50 μ M of the caspase-1 inhibitor. While little if any IL-1 β was secreted from unstimulated THP1 cells, incubation with *C. psittaci* caused a high level of IL-1 β secretion, most of which could be blocked by including the caspase-1 inhibitor in the medium (Table I).

Discussion

We report herein that an infection by *C. psittaci* results in apoptosis of the host cells. As opposed to the immediate cytotoxicity previously reported for infections with a high m.o.i. of a number of *Chlamydia* strains (16, 17, 19–21), we observed a slower rate of cytotoxicity, previously associated with a moderate or low m.o.i. (15, 16, 18, 20, 21). While immediate cytotoxicity does not require a productive infection to take place, the slower cytotoxicity can be inhibited by using UV- or heat-inactivated bacteria or by treatment with chloramphenicol (15, 21). Under the conditions of our experiments, apoptosis is observed only when live bacteria enter the host cell and are allowed to synthesize bacterial protein. Both epithelial cells and macrophages undergo apoptosis after infection. Apoptosis proceeds in the absence of host cell protein synthesis or activation of known eukaryotic proapoptotic enzymes, suggesting that either the chlamydiae may elaborate factors that trigger host cell death or that the heavy load represented by such a large number of

Table I. Effect of caspase inhibitors on apoptosis and IL-1 β secretion

Treatment	Relative Apoptosis Due to <i>Chlamydia</i> Infection ^a	Relative Apoptosis Due to Fas Ligation ^b	<i>Chlamydia</i> -Induced Secretion of IL-1 β (pg/ml) ^c
Control	100	100	359 \pm 62
50 μ M caspase-1 inhibitor	109 \pm 13		149 \pm 10
50 μ M caspase-3 inhibitor	87 \pm 2	11 \pm 9	
50 μ M cycloheximide	90 \pm 25		
68 μ g/ml chloramphenicol	18 \pm 1		

^a HeLa cells were infected with *C. psittaci* at an m.o.i. of 1.0 for 2 days in the presence of the indicated inhibitors. The extent of apoptosis in the absence of inhibitor was defined as 100, and the other values were normalized with respect to the control. Apoptosis was measured by cytofluorometry as described in *Materials and Methods*. The values represent the mean and SD of three experiments.

^b Jurkat cells were incubated with 100 ng/ml anti-Fas Ab (CH-11) for 3 h in the presence or absence of caspase-3 inhibitor. The extent of apoptosis in the absence of inhibitor was defined as 100, and the value in the presence of inhibitor was normalized with respect to the control. Apoptosis was measured by cytofluorometry in two separate experiments.

^c THP1 cells were incubated with *C. psittaci* for 24 h in the presence (control) or absence of caspase-1 inhibitor. The supernatant concentration of IL-1 β was measured in three separate experiments as described in *Materials and Methods*. In the absence of both bacteria and inhibitor, the level of IL-1 β secretion was 4 \pm 49 pg/ml.

proliferating bacteria within the host cell may be sufficient to set off cell death through as yet uncharacterized mechanisms. Consistent with the latter possibility, apoptosis was not observed until after ~1 day of infection (halfway through the infection cycle), when the *Chlamydia* vacuole harbors predominantly bacteria at the metabolically active RB developmental stage. However, both infected and uninfected cells die through apoptosis, suggesting that infected cells may also elaborate soluble factors that induce apoptosis of neighboring uninfected cells.

Apoptosis is mediated by highly specific enzymes known as caspases (40), which have a strong preference for Asp residues in the substrate sequence. Although caspase-1 (or ICE) is specific for the precursors of IL-1 β and IL-18 and is clearly involved in activation of proinflammatory cytokines, it remains to be seen whether caspase-1 is directly involved in executing the apoptotic program (43). Caspase-3 (CPP32, apopain), on the other hand, is clearly involved in promoting cell death (43). We have tested the effects of two inhibitors specific for caspase-1 and caspase-3. The caspase-1 inhibitor had no effect on *Chlamydia*-induced apoptosis, although it inhibited secretion of IL-1 β from monocytes infected with chlamydiae. To our initial surprise, the caspase-3 inhibitor also failed to diminish significantly the extent of apoptosis, implying that *C. psittaci* induces apoptosis through a pathway not dependent on caspase-3. These results are reminiscent of other recent reports showing caspase-independent apoptosis. In cells expressing the proapoptotic protein Bax, a caspase inhibitor failed to inhibit Bax-induced apoptosis (45–47), although it could still inhibit apoptosis triggered by Fas (45), suggesting that known caspases are not involved in Bax-induced apoptosis. Similarly, caspase inhibitors delay but do not prevent apoptosis induced by deregulated oncogenes and DNA damage (47). It has been proposed that, unlike apoptosis initiated by Fas, whose first signal is activation of the early caspase 8, the signal for Bax-triggered apoptosis is integrated within the cell (43). Other enzymes, including nucleases and protein kinases, may also participate in apoptosis (43). As the signal for *Chlamydia*-induced apoptosis would presumably originate at the inclusion, it is thus conceivable that this cell death pathway could resemble the pathway set off by Bax or oncogenes.

Besides the proapoptotic activity described here, it has been reported recently that *Chlamydia* protects infected cells from apoptosis due to other ligands, including TNF- α , Fas Ab, and granzyme B/perforin, and that the protection is evident within 4 h of infection (10). Thus, the antiapoptotic activity may protect infected cells during the initial stages of chlamydial invasion (10). Interestingly, the antiapoptotic activity of *Chlamydia* infection is due to inhibition of caspase 3 activation (10), which is required for apoptosis following the TNF- α and Fas pathways. As the proapoptotic activity of *Chlamydia* is independent of known caspases, it should not be affected by caspase-3 inactivation. Hence, there may be a fine balance struck between the anti- and proapoptotic activities of *Chlamydia* during the course of the infection. While antiapoptotic activity may protect infected cells from lysis by effector cells and cytokines of the host immune system, proapoptotic activity may contribute to the inflammatory response, as macrophages and perhaps other cells undergoing apoptosis may secrete proinflammatory cytokines. The exact contribution from each of these activities to the pathology of the infection will now need to be evaluated, although it is conceivable that the antiapoptotic activity may be operative mainly at the beginning of the infection, with proapoptotic behavior becoming prevalent at later stages.

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