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Characterization of Host Cell Death Induced by *Chlamydia trachomatis*▽†

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Chlamydia **are obligate intracellular bacteria that modulate apoptosis of the host cell. Strikingly, chlamydial infection has been reported both to inhibit and to induce apoptosis. Although the ability to inhibit apoptosis has been corroborated by the identification of cellular targets, confirmation of cell death induction has been complicated by a mixture of apoptotic features and atypical cell death during infection, as well as by differences in the experimental techniques used to measure cell death. Here we use a panel of well-established approaches in the study of apoptosis to define the form of cell death induced by *Chlamydia trachomatis* infection. Infected cells displayed apoptotic features such as nuclear condensation and fragmentation, as well as positive TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling) staining. Fragmentation of genomic DNA occurred, but was atypical. Clear evidence against the activation of effector caspases was found. Nuclear changes were measured in fibroblasts lacking one or both of the effectors of mitochondrial apoptosis, Bax and Bak. A slight reduction in nuclear changes was observed in Bax-deficient cells and in Bax/Bak double-deficient cells. Most surprisingly, this reduction was almost complete in Bak-deficient cells. Finally, dying infected cells were efficiently taken up by professional phagocytes, suggesting that *Chlamydia*-induced host-cell death could play a role in the immune response. In conclusion, chlamydial infection can induce cell death. Although *Chlamydia*-induced cell death has certain morphological features of apoptosis, it does not result from activation of the apoptotic pathway.**

Chlamydia are obligate intracellular bacteria that infect mainly epithelial mucosal cells. The various species and strains of *Chlamydia* can cause a wide range of diseases (37). Chlamydiae replicate inside a membrane-bounded vacuole in the cell, the chlamydial inclusion. During the intracellular stages of infection, the bacteria can have dramatic effects on the host cell, as illustrated for instance by a large number of changes in gene expression in infected human cells (16).

Due to their intracellular lifestyle, host cell apoptosis could profoundly affect the ability of chlamydiae to complete their replication cycle. Modulation of the apoptotic pathway has been described for many microbes (15) and could be a relevant component of the chlamydial developmental cycle. Although many studies have addressed this issue, both anti- and pro-apoptotic activities have been described separately. Thus, although some studies have demonstrated apoptosis inhibition by chlamydial infection (7, 9), others have demonstrated the induction of apoptosis by chlamydial infection (29, 32).

This apparent discrepancy has attracted much attention and has been the focus of two recent review articles (1, 26). Some of the differences reported may have resulted from the use of different chlamydial organisms and different host cells. Another factor may be the complexity of apoptosis itself, resulting in a large number of sometimes differing features under different conditions. The various aspects of apoptosis can be detected by a large number of assay systems that may in some circumstances give different answers to the same question. One recent publication suggests an additional possibility, namely, that *C. pneumoniae* induces a form of cell death that has some apoptotic features but is otherwise more consistent with necrosis, for which the term "aponecrosis" has been proposed (4). For all its virtues, it has to be remarked that the latter study used very high infectious doses of *C. pneumoniae*, as well as a host cell (aortic smooth muscle cells) that is neither a typical chlamydial host nor a cell whose apoptotic response is well understood.

In the present study, two laboratories with a long-standing interest in cell death and in chlamydial infection join forces to analyze cell death induction during chlamydial infection. Although there is agreement that *Chlamydia* can block apoptosis (7, 10), there is also irrefutable evidence that the infection can kill the infected host-cell. We therefore used a panel of standard assays used to measure apoptosis in order to compare cell death induction by *Chlamydia* with apoptosis induction by UV light, and we also provide evidence that infection is sufficient for uptake of the dying host cell by antigen-presenting cells.

MATERIALS AND METHODS

Cell lines and chlamydial organisms. Wild-type, Bax-deficient, Bak-deficient, and Bax/Bak-deficient mouse embryonic fibroblasts (MEF) were obtained from David Huang, Melbourne, Australia. The human cervical adenocarcinoma cell line HeLa 229 and the human laryngeal carcinoma cell line Hep2 were obtained from the American Type Culture Collection (ATCC). Cells were maintained in a humidified incubator at 37°C with 5% CO_2 in Dulbecco modified Eagle medium (PAA Laboratories, Pasching, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS). The *C. trachomatis* strain L2 was obtained from ATCC, whereas the mouse pneumonitis (MoPn) strain (*C. muridarum*) was

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obtained from Roger Rank (University of Arkansas for Medical Sciences, Little Rock, AR).

Infection of cells and induction of apoptosis by UV irradiation. Cells were infected with *C. trachomatis* or *C. muridarum* as described previously (8). Host cells (3×10^5 cells/well) were seeded into six-well plates the day before infection. The medium was replaced the next day with Dulbecco modified Eagle medium without FCS (10% FCS was added again after 4 h). *Chlamydia* at the indicated infectious doses was added, and cells were harvested at the indicated time points postinfection for further analysis. The multiplicity of infection (MOI) was determined by intracellular staining for chlamydial inclusions in HeLa cells with a fluorescein isothiocyanate (FITC)-labeled anti-chlamydial lipopolysaccharide (LPS) antibody (Progen, Heidelberg, Germany). The mock-infected cells were subjected to the same procedure in the absence of *Chlamydia*. Apoptosis was induced in uninfected cells by exposure to UV irradiation (1,600 J/m^2) in a transilluminator box (Stratagene, La Jolla, CA), followed by 16 h of incubation at 37°C.

Assay for nuclear apoptosis. MEF, Hep2, or HeLa Cells were inoculated into six-well plates (3×10^5 cells/well) the day prior to infection with chlamydiae or treatment with UV. After the indicated times after treatment, cells were stained with 20 μM Hoechst 33258 (Sigma, Taufkirchen, Germany) for 30 min at 37°C and washed with phosphate-buffered saline (PBS). Nuclear morphological changes (fragmentation and condensation) were determined under a fluorescence microscope. At least 300 nuclei per sample were counted.

TUNEL assay. After the indicated time of infection or treatment, cells were fixed with 4% paraformaldehyde for 25 min or 1 h and washed with PBS. A solution of 0.2% Triton X-100 in PBS was then used to permeabilize the cells. A TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling) kit (Promega [Madison, WI] or Roche Applied Sciences [Indianapolis, IN]) was used according to the instructions provided by the manufacturers. The reaction was revealed with either streptavidin-APC or streptavidin-FITC. Cells were analyzed either by flow cytometry (FACSCalibur; BD, Heidelberg, Germany) or by laser scanning microscopy (Zeiss, Jena, Germany) and wide-field fluorescence microscopy (Leica, Deerfield, IL; see Fig. S1 in the supplemental material).

Assay for fragmentation of chromosomal DNA. Three million MEF cells were seeded in 100-mm cell culture dishes the day before infection. On the next day, cells were infected or UV irradiated as described above. After 24 h of infection, cells were harvested by trypsinization, washed with PBS, lysed in detergent-containing buffer (150 mM NaCl, 0.5% sodium dodecyl sulfate) supplemented with 500 μg of proteinase K/ml, and incubated at 37°C overnight. Reactions were extracted with phenol-chloroform-isoamyl alcohol, and DNA was precipitated by addition of 1 volume of isopropanol. Pellets were then washed and dissolved in Tris-EDTA buffer containing RNase A. After 1 h of incubation at 37°C, DNA was run on a 1% agarose gel containing ethidium bromide.

Assay for caspase activity. MEF cells (3×10^5 cells/well in six-well plates) were mock infected or infected with *C. trachomatis*, and an aliquot of uninfected cells was UV irradiated. Cells were harvested by trypsinization, washed with PBS, and lysed by incubation in 40 μl of NP-40 lysis buffer (150 mM NaCl, 1% Ipegal CA-630, 50 mM Tris [pH 8.0]) for 15 min on ice. Cell lysates were cleared by centrifugation for 5 min at 15,000 rpm at 4°C. Triplicates of 10-μl aliquots of the supernatant were added to 90 μl of DEVD assay buffer (50 mM NaCl, 2 mM $MgCl_2$, 40 mM β-glycerophosphate, 5 mM EGTA, 0.1% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, 100 μg of bovine serum albumin/ml, 10 mM HEPES [pH 7.0]) containing 10 μM DEVD-7-amino-4-methyl-coumarin (AMC) fluorimetric substrate. Reactions were incubated for 1 h in 96-well flat-bottom plates at 37°C. Free AMC was measured, and values are presented as arbitrary relative fluorescence units (mean and standard error of the mean for the above-described triplicate reactions).

Detection of active caspase-3 by flow cytometry. After infection or UV irradiation, MEF cells were harvested, fixed in 2% paraformaldehyde for 15 min, permeabilized with 1% saponin, and incubated with anti-active caspase-3 antibody (Pharmingen, Heidelberg, Germany) and FITC-conjugated goat anti-rabbit (Dianova, Hamburg, Germany) as a secondary antibody. Flow cytometry was performed in a FACSCalibur, and at least 10,000 cells per sample were recorded.

Assay for the uptake of MEF by professional phagocytes. PKH26 (red; Sigma)-stained MEF cells (2×10^5/well) were seeded into 12-well plates the day before infection. After the indicated infection or treatment, 4×10^5 PKH67 (green; Sigma)-stained dendritic cells (from the immortalized DC D2SC/1 (25) or RAW264.7 macrophages (from the ATCC) were added to MEF cultures. After 2 h of coincubation at 37°C, all of the cells were collected and fixed. Uptake was then measured by flow cytometry in a FACSCalibur.

RESULTS

Nuclear morphology during cell death induction by *C. trachomatis*. One of the most distinctive features of apoptosis is the condensation and fragmentation of the nuclei of dying cells (recognized as a defining feature of various forms of apoptotic death by Kerr et al. in 1972 (21). We infected MEF at various infectious doses of *C. trachomatis*. The nuclear morphology induced by higher doses was indistinguishable by DNA staining and light microscopy from the morphology during classical apoptosis induced by UV light (Fig. 1A). Earlier reports have suggested that death induction depends in part on the host cell type used (19). We found that the MEF cells we used (E1A transformed) showed a clearly higher susceptibility to cell death induced by chlamydial infection than HEp2 epithelioid cells (Fig. 1B and C). However, higher infectious doses did induce nuclear changes with a relatively high incidence also in HeLa and HEp2 cells (Fig. 1D), reaching levels of up to 30 to 35% at 40 h postinfection (data not shown). Nuclear condensation/fragmentation was a relatively late event. Using an MOI of 5, the first MEF cells with nuclear condensation were detectable at about 18 h. When lower infectious doses were used, higher percentages of cells with condensed nuclei were observed at about 40 to 50 h postinfection, coinciding with the completion of the chlamydial replicative cycle (which takes about 40 h) (27). We also compared the effects of *C. trachomatis* to those caused by infection with the species *C. muridarum*, also known as the mouse pneumonitis (MoPn) strain of *C. trachomatis*; we have reported cell death induction with MoPn previously (19). The nuclear morphology was indistinguishable between the two species (not shown), and nuclear changes occurred at comparable frequencies (Fig. 1E). If cultures were infected at a lower MOI, the same nuclear changes were observed but later in the infection (Fig. 1F); at this infectious dose, protection against experimentally induced apoptosis appears to dominate during most of the developmental cycle (10). Thus, infection with *C. trachomatis* or *C. muridarum* causes morphological nuclear changes typical of apoptosis.

Cells infected with *C. trachomatis* become TUNEL positive. The TUNEL method was introduced over 10 years ago to detect apoptosis (13). The enzyme terminal deoxynucleotidyltransferase is used to add labeled nucleotides to single- or double-strand breaks in nuclei of cells undergoing apoptosis-associated DNA degradation. MEF cells infected with *C. trachomatis* at an MOI of 5 were found to contain nuclei with a positive TUNEL signal, which again looked similar in location to nuclei in cells undergoing UV-induced apoptosis (Fig. 2A). To exclude the possibility that the chlamydial inclusions may have been labeled by the TUNEL technique, cells were costained for chlamydial LPS. There was no overlap between the two stains (Fig. 2B), indicating that it was indeed the nucleus of the infected cell that was labeled by the TUNEL reaction. In order to guarantee that all wells were analyzed, all cells were collected and centrifuged onto slides before staining, which leads to distortions of cell morphology, especially in the inclusions. For comparison, cells were also grown on coverslips (see Fig. S1 in the supplemental material).

Atypical fragmentation pattern of genomic DNA. The biochemistry of apoptotic DNA fragmentation is comparatively well understood. The endonuclease caspase-activated DNase

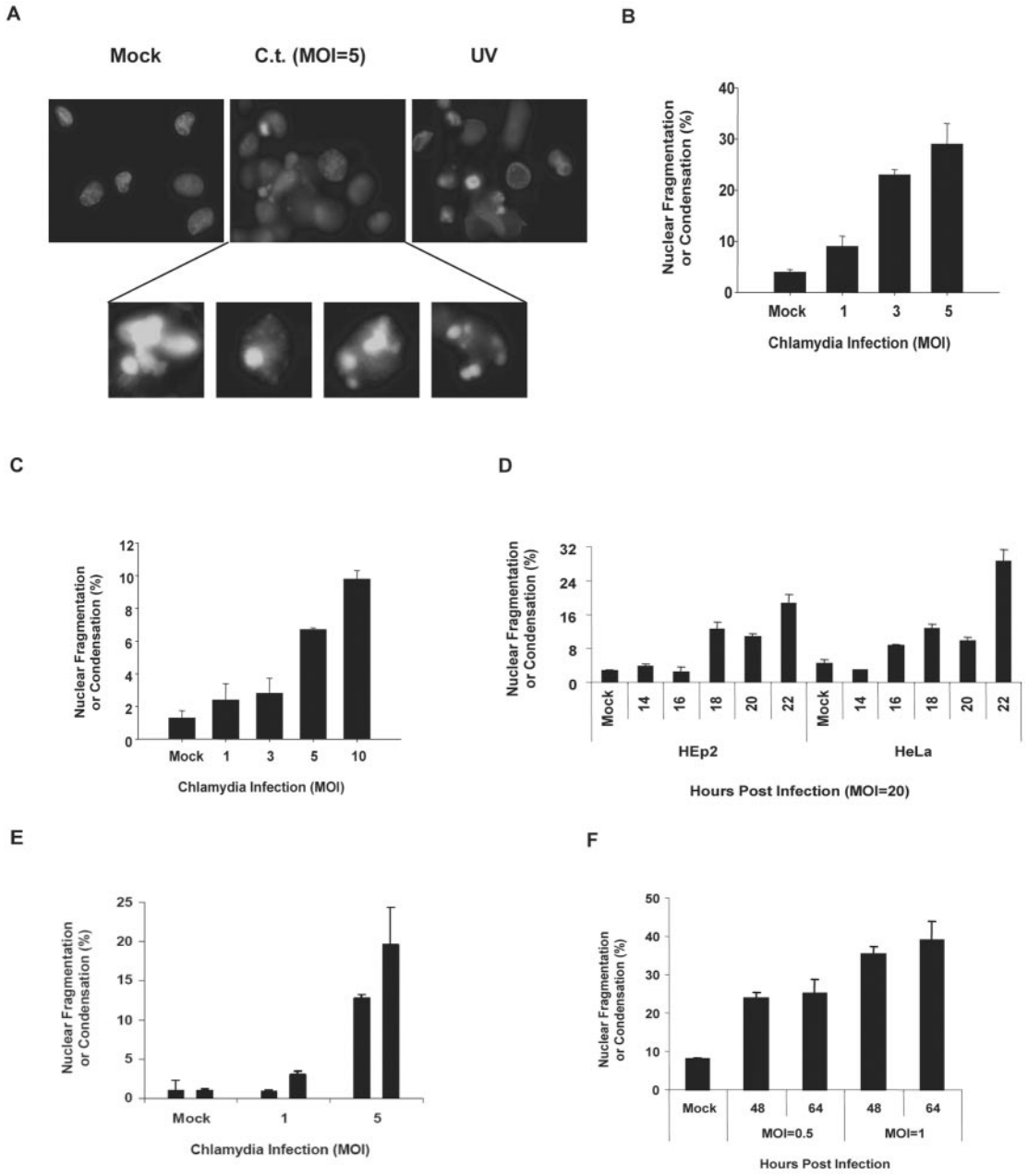

FIG. 1. *C. trachomatis* induces nuclear morphological changes in host cells. (A) MEF cells were infected with *C. trachomatis* at an MOI of 5 for 24 h (under which conditions nearly 100% of cells were infected [Fig. S6 in the supplemental material and data not shown]). Uninfected cells were UV irradiated and incubated for 16 h. All cells floating in the medium and attached to the plates were harvested for Hoechst staining; there was a tendency toward higher numbers of cells with condensed nuclei in the floating population. Cells were analyzed by fluorescence microscopy. Typical examples of nuclear fragmentation or condensation upon infection are shown in enlarged pictures. (B and C) MEF cells (B) or HEp2 cells (C) were either mock infected or infected with *C. trachomatis* at the indicated MOI for 24 h. (D) HEp2 or HeLa cells were infected with *C. trachomatis* at an MOI of 20 for the indicated times. (E) MEF cells were infected with *C. muridarum* at the indicated MOI (the bars refer to two separate experiments). (F) MEF cells were infected with an MOI of 0.5 or 1 and analyzed after 48 or 64 h as indicated. After Hoechst staining, all cells in suspension and attached to the plates were harvested, and at least 300 cells were counted for each experimental condition. The results are given as the percentage of cells with typical fragmented or condensed nuclei in the cell population. The standard deviation was calculated from three separate results counted in one sample. All experiments were performed independently two to five times, with similar results each time.

CAD (5), also called DFF40 in humans [24]), is activated through caspase-dependent inactivation of its inhibitor ICAD/DFF45 (34). CAD then cleaves the genomic DNA at the most easily accessible sites between nucleosomes, yielding fragments of multiples of about 180 bp. Although a positive TUNEL reaction is normally the result of CAD activation, the typical ladder-like pattern of DNA can also be detected by gel electrophoresis. After electrophoretic separations of DNA from UV-irradiated cells, bands were visible that made up the lower rungs of the ladder. At the same time, a smear of higher-

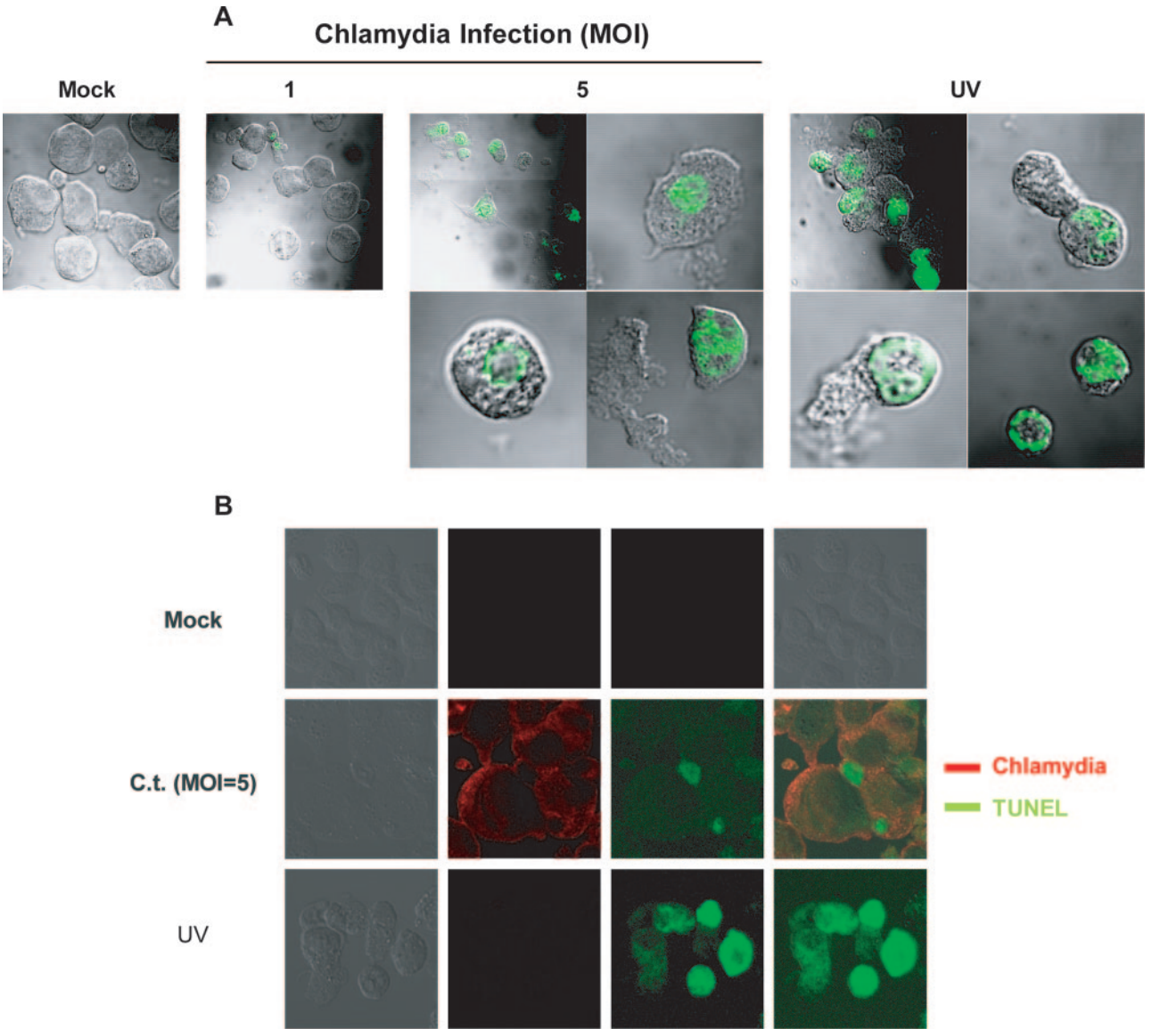

FIG. 2. TUNEL-positive cells during infection of MEF with *C. trachomatis*. (A) MEF cells were either infected with *C. trachomatis* at the indicated MOI for 24 h or treated with UV followed by overnight incubation. All cells in suspension and attached to the plates were harvested and stained by the TUNEL technique. Pictures were then obtained by confocal microscopy. TUNEL-positive cells appear in green. (B) The experiment was performed as described in panel A. Cells were costained by the TUNEL technique (green) and by using an FITC-conjugated antibody detecting chlamydial LPS (red). Images were obtained by confocal microscopy.

molecular-weight DNA appeared, very likely the result of chromosomal DNA that had been cleaved but not completely degraded (Fig. 3). This pattern is very typical of DNA in cells undergoing apoptosis. Surprisingly, the pattern in infected cells looked different. Although some small DNA-fragments were seen, there was no indication of larger fragments (Fig. 3). This indicates that some DNA fragmentation occurs during *Chlamydia*-induced cell death. However, since ca. 25 to 30% of cells show condensed nuclei under these conditions (compared to ca. 40 to 50% after UV irradiation), the pattern must be distinguished from apoptotic nuclear fragmentation. The result suggests that either a small number of infected cells undergo near-complete fragmentation or that only a small part of DNA is subjected to extensive fragmentation. However, it appears unlikely that DNA is cleaved as in apoptosis in all infected cells that show nuclear condensation, as would happen in cells dying by apoptosis (see also the results presented below with TUNEL staining as a measure of apoptosis). Cell populations infected for longer times or at higher infectious doses also failed to show more complete digestion of genomic DNA (see Fig. S2 in the supplemental material), which again suggests that the mechanism of DNA/nuclear destruction during infection is distinct from that seen during apoptosis.

Lack of caspase activation during *Chlamydia*-induced cell death. Caspase activity is an invariable, indispensable feature of normal apoptosis. In practically all known cases of apoptosis, effector caspases (especially caspase-3) are activated and cleave a large number of cellular proteins (11). We assessed

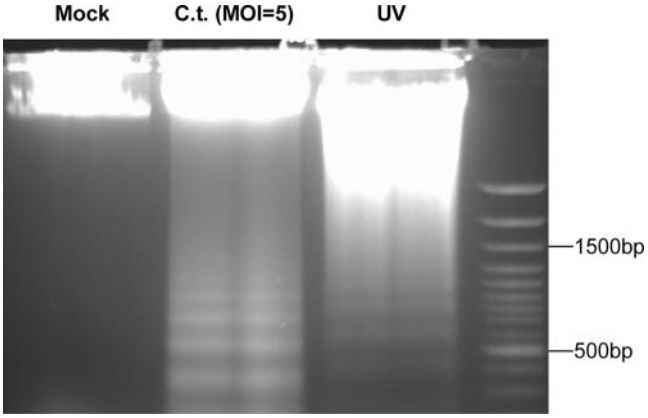

FIG. 3. Pattern of DNA fragmentation during *C. trachomatis* infection and UV-induced apoptosis. MEF cells were either mock infected or infected with *C. trachomatis* at an MOI of 5 for 24 h. Uninfected cells were treated with UV light, as described above. Chromosomal DNA was then extracted and run on a 1% agarose gel containing ethidium bromide. The experiment was done three separate times, with very similar results each time.

the activation and activity of caspase-3 based on two separate assays. Neither enzyme assay for caspase activity in cell extracts (cleavage of a substrate with the recognition sequence DEVD, the preferred site of cleavage of caspase-3 [40]) (Fig. 4A) nor staining for a neo-epitope that is generated during proteolytic activation of caspase-3 during apoptosis (Fig. 4B) yielded any evidence of caspase-3 activation during *C. trachomatis*-induced cell death. In agreement with these results, *Chlamydia*-induced cell death was not blocked by the caspase-inhibitor z-VAD-fmk (see Fig. S3 in the supplemental material). The lack of caspase activity is also relevant to the question of nuclear fragmentation discussed above, since CAD is activated by caspase-dependent cleavage of its inhibitor ICAD. The lack of caspase activity therefore supports the view that the DNA fragmentation seen is not due to apoptosis.

Role of Bax and Bak in *Chlamydia*-induced cell death. A critical step for most forms of apoptosis induction is the release of cytochrome *c* from the mitochondria into the cytosol. This

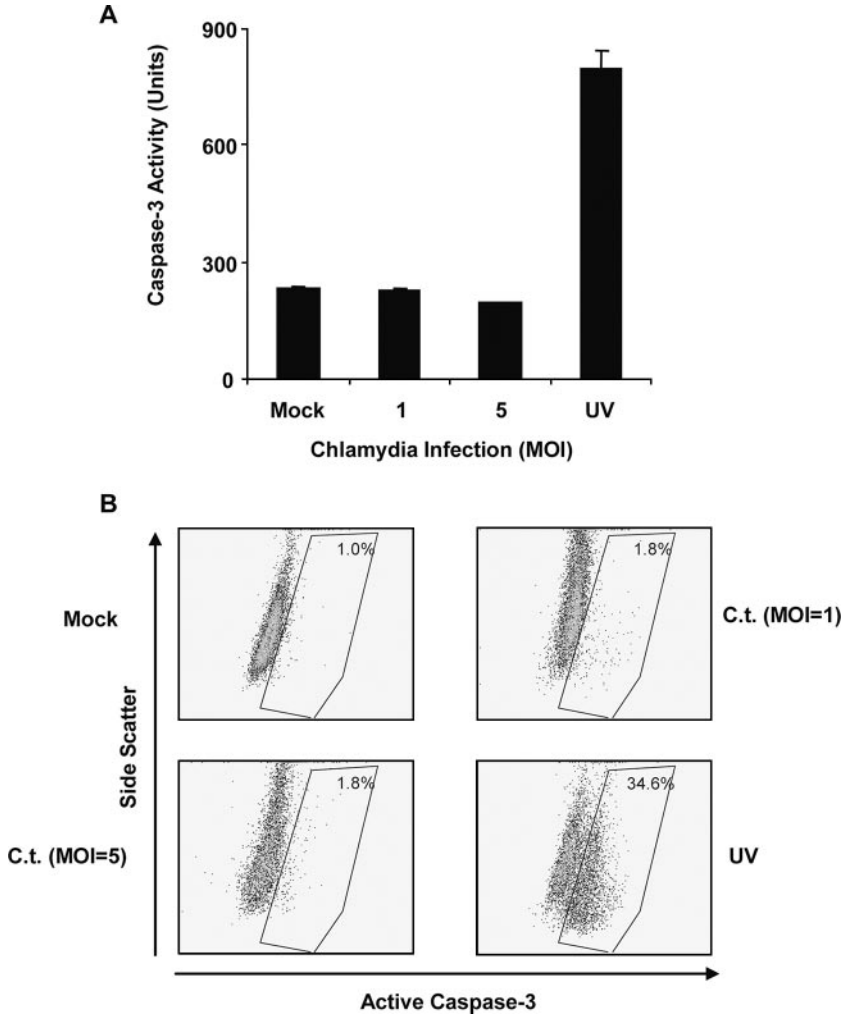

FIG. 4. Lack of detectable caspase-3 activation or activity in MEF cells infected with *C. trachomatis*. MEF cells were either mock infected or infected with *C. trachomatis* at the indicated infectious dose. Uninfected cells were subjected to UV irradiation. (A and B) After 24 h, the caspase activity (A) and activation (B) were assessed. For panel A, cells were harvested and lysed, and the DEVD-cleaving activity was measured in cell extracts. Each bar represents one well of a six-well plate, and the standard deviation refers to separate measurements of one sample. For panel B, cells were harvested and stained with an antibody specific for only the active form of caspase-3, followed by fluorescence-activated cell sorting analysis. The gated cell populations represent cells that express active caspase-3. Experiments were done at least three separate times, with similar results each time.

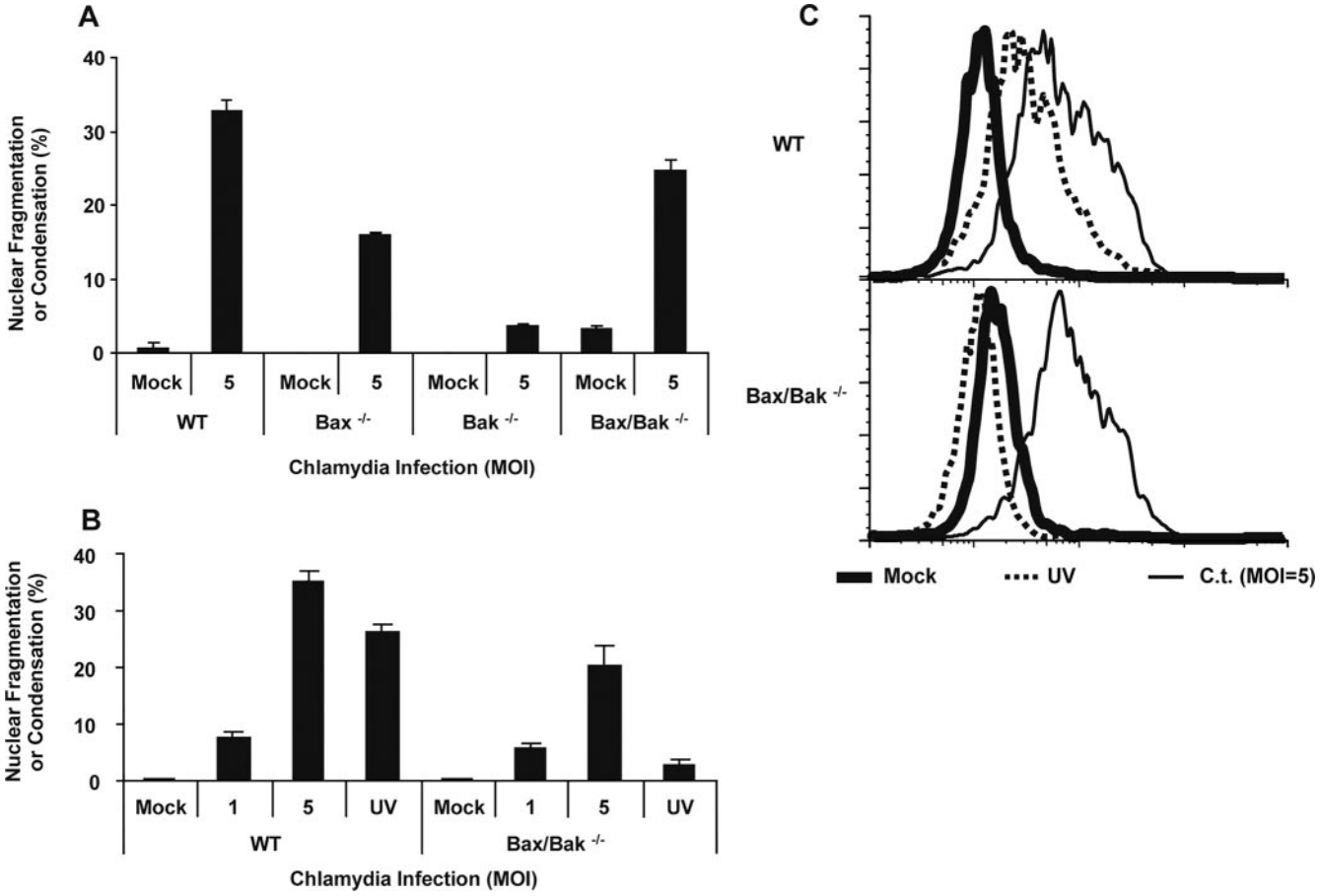

FIG. 5. Effect of combined loss of Bax and Bak on *Chlamydia*-induced cell death. Wild-type, Bax-deficient, Bak-deficient, or Bax/Bak double-deficient MEF cells were infected with *C. trachomatis* as described above. (A) Comparison of the four genotypes at an MOI of 5, analyzed at 24 h. Cells were harvested, and the nuclear morphology was assessed by microscopy as described above. The results are given as a percentage of cells with typical fragmented or condensed nuclei. (B) Comparison of two infectious doses (MOIs of 1 and 5) in wild-type and Bax/Bak double-deficient cells. UV treatment was included as a control. Similar results were obtained in three separate experiments. (C) TUNEL-stained cells were analyzed by flow cytometry. Representative pictures from at least two independent experiments are shown. WT, wild type.

release requires the proapoptotic Bcl-2 family members Bax and/or Bak (3). Analysis of the role played by Bax and Bak is relevant, because we have previously shown that Bax undergoes a structural change during infection that is normally seen during apoptosis (19, 32). Significantly, infected Bax-deficient mice showed a more intense inflammatory response, and *C. muridarum* infection was cleared more rapidly in these mice (31).

MEF cells lacking either Bax, Bak, or both Bax and Bak were infected with *C. trachomatis* and compared for nuclear condensation/fragmentation. There was a clear reduction of nuclear changes in MEF lacking Bax alone (Fig. 5A; we have previously reported similar changes in Bax-deficient cells infected with *C. muridarum* (31). Unexpectedly, cells deficient in Bak alone showed a larger decrease in nuclear changes than in Bax-deficient cells. Surprisingly, cells lacking both Bax and Bak showed nuclear changes in a higher percentage of cells than did Bak-deficient cells (Fig. 5A). A milder phenotype in Bax/Bak double-deficient cells than in Bak-deficient cells has not been described before, and the reason for this behavior during *C. trachomatis* infection is unclear. Therefore, wild-type and Bax/Bak double-deficient MEF cells were infected again, stained for TUNEL, and analyzed quantitatively by flow cytometry. Although *bax/bak*$^{-/-}$ cells were protected against UV-induced apoptosis as measured by the assessment of nuclear morphology (Fig. 5B) and by TUNEL staining (Fig. 5C), there was no difference in TUNEL reactivity between wild-type MEF and *bax/bak*$^{-/-}$ MEF after *C. trachomatis* infection (Fig. 5C).

The function of Bax/Bak during apoptosis is to induce cytochrome *c* release. We therefore also measured the release of cytochrome *c* release during infection. No release of cytochrome *c* was seen under conditions where nuclear changes are present in numerous cells (see Fig. S4 in the supplemental material). The formation of inclusions was similar in the various genotypes (see Fig. S5 in the supplemental material).

Taken together, these data suggest that Bax and Bak have an effect on *Chlamydia*-induced cell death. However, the role played by Bax and Bak is different from their usual role during UV-induced (normal) apoptosis, where Bax/Bak activation is followed by the release of cytochrome *c* and caspase activation.

Infected and dying cells are taken up by professional phagocytes. Cells dying by either apoptosis or necrosis are internalized efficiently by phagocytes. In the case of *Chlamydia*-in-

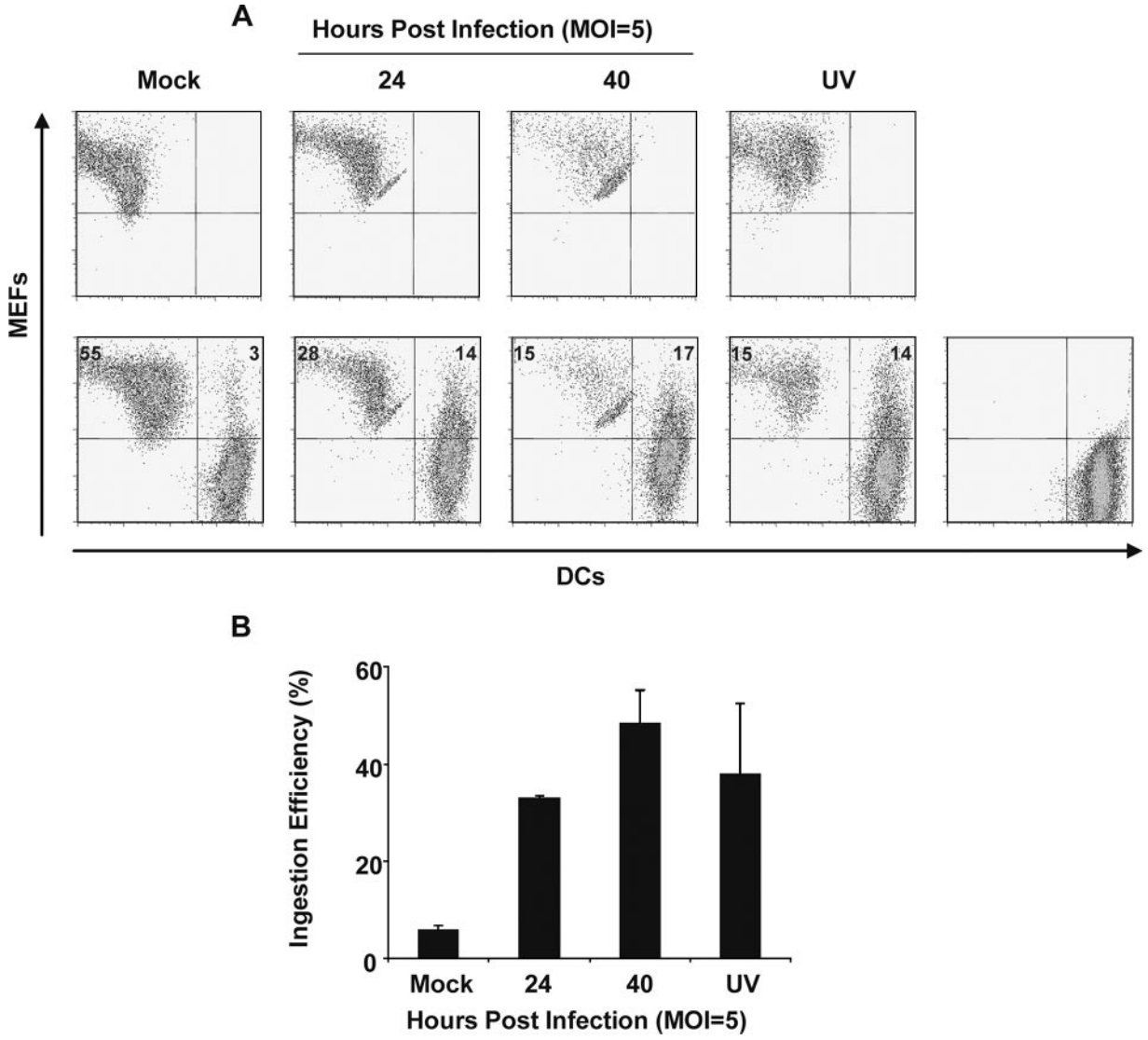

FIG. 6. Professional phagocytes internalize *C. trachomatis*-infected dying cells. (A) MEF were stained red (PKH26) and left either uninfected or infected with *C. trachomatis* at an MOI of 5. Virtually all cells contained inclusions when infected under these conditions (see Fig. S6 in the supplemental material). Samples of uninfected cells were UV irradiated and incubated overnight. At the indicated time points, green (PKH67)-stained DC were added to the MEF cell culture. After 2 h of coincubation, all cells were fixed and analyzed by flow cytometry. Double-positive cells represent DC that have taken up MEF cells (22). The top panel shows MEF cells alone, and the bottom panels show cocultures of MEF cells infected or treated with UV and then coincubated with DC. The far-right bottom panel shows DC that had not been incubated with MEF cells. The decrease in the fluorescence in MEF cells that had not been incubated with DC may be due to membrane turnover and loss of the lipophilic dye, PKH26. (B) Quantitation of the results shown in panel **A**. The efficiency of ingestion was calculated as the percentage of DC containing MEF (upper-right quadrant) divided by the total MEF (upper-left plus upper-right quadrant). The means and standard deviations were calculated from two independent experiments.

fected cells, an uptake by phagocytes may be significant for two reasons. First, it may provide a mechanism for making chlamydial antigens accessible for antigen presentation by major histocompatibility complex molecules. Second, it may contribute to the spread of the infection. To test for uptake, MEF cells were infected with *C. trachomatis* (MOI = 5; virtually all cells are infected at this dose [see Fig. S6 in the supplemental material]) for 24 or 40 h, and the infected cells were then incubated with dendritic cells (DC). Since the cells had been stained with two different fluorescent dyes, the uptake of MEF antigens by DC was revealed by the appearance of double-positive cells. After 2 h of coincubation, the number of double-positive cells was determined by flow cytometry. As shown in Fig. 6, a high percentage of infected cells were taken up by DC. About one-third of the infected cells were internalized by DC after 24 h (comparing the left and right upper quadrants), which is approximately the same number as the cells containing condensed and/or fragmented nuclei after a 24-h infection (Fig. 1). The percentage was slightly higher after 40 h of infection. UV-irradiated cells were used as a positive control. No uptake was seen at 6 h postinfection. Likewise, when heat-killed bacteria were used, MEF cells were not taken up. The

efficiency of the uptake correlated with the infectious dose (see Fig. S7 in the supplemental material). A similar result was seen when RAW264.7 macrophages were used as phagocytes (data not shown). Induction of host cell death by *Chlamydia* therefore appears to be sufficient for the uptake of the dying cell by professional phagocytes. Although we have not further explored this uptake, we have shown previously that MEF cells dying during chlamydial infection expose phosphatidylserine (PS) on their surface (as detectable by annexin V labeling [Perfettini JBC 2003]), and PS can be recognized by phagocytes leading to the uptake of the dead cell (36). PS exposure may therefore be a mechanism for the recognition and uptake of *Chlamydia*-infected cells by phagocytes.

DISCUSSION

In this study we analyzed cell death induced by *C. trachomatis* or *C. muridarum*. We observed nuclear morphological changes that are indistinguishable from apoptosis induced by UV light, as well as positive TUNEL staining in cells dying during chlamydial infection. However, other markers of apoptosis, especially caspase activation or activity, were not seen or were minor. Some DNA fragmentation was observed, but the pattern appeared to be different from the one observed during UV-induced apoptosis. Uptake of infected cells by DC was further seen, hinting at a possible role for *Chlamydia*-induced cell death in stimulating an immune response in vivo.

Cytolytic activity associated with *Chlamydia* infection has been described for more than 30 years (2, 12, 42, 45). Early studies using electron microscopy have investigated the structure of the host cells during infection with *C. psittaci* in vitro and in vivo. Intriguingly, massive changes to organelles were noticed at later stages of infection, such as dilation and vacuolation of the endoplasmic reticulum (ER), distortion of mitochondria, and nuclear condensation (41). Ultrastructural changes were found to be associated with the release of lysosomal enzymes into the cytosol (42), which could be a trigger for changes to organelles. Although all of these changes occur in apoptosis, the observed process and the order of events are not typical. During apoptotic death, an early condensation of the nucleus is normally seen, followed by changes to organelles at later stages (for a review, see reference 14).

Apoptosis is often defined by the morphology of the dying cell, but morphological changes are not the only feature of apoptosis. The features that are tightly linked to apoptosis and that are also observed in *Chlamydia*-induced cell death thus are nuclear morphological changes, positive TUNEL staining, and positive staining for active Bax (the latter one we reported on in references 19 and 32). These results raise a number of obvious questions.

In uninfected cells, nuclear condensation is a consistent feature of apoptosis. However, the precise molecular basis for the condensation is not known. Normally, nuclear condensation occurs together with DNA fragmentation through CAD. In a cell-free system, inhibition of CAD also blocks nuclear condensation (44), although the same approach in intact cells (expression of an uncleavable form of the inhibitor of CAD, ICAD) prevents DNA fragmentation but not nuclear condensation (35). One report suggested that apoptotic condensation may be due to the activity of a specific factor termed acinus (33). However, recent work shows that acinus is part of the spliceosome (38), and knockdown of the acinus failed to prevent apoptotic DNA condensation (18). The mediators of nuclear condensation during apoptosis thus remain to be identified. However, nuclear condensation can also be found under certain conditions in vitro. In isolated nuclei, incubation with $CaCl_2$, $MgCl_2$, and $ZnCl_2$ induced nuclear condensation in the absence of DNA fragmentation (39). It is therefore clear that the apoptotic pathway does not need to be activated in order to obtain the nuclear condensation observed during chlamydial infection.

Some DNA degradation was detectable during chlamydial infection. However, the pattern was atypical for apoptosis. DNase activation during apoptosis usually leads to digestion events that first generate large DNA fragments of 50 to 200 kb (see, for instance, reference 35) and then smaller fragments, causing a ladder-like appearance. A large part of the chromosomal DNA is normally attacked, which causes the accumulation of larger (>5-kb) fragments, observed as a smear on an agarose gel (see Fig. 3, UV treatment). In contrast, DNA fragmentation during infection caused the appearance of some small fragments (the lower ladder rungs) but no bulk degradation of the remaining DNA. This suggests that only a small part of DNA (either a small part in each cell or a larger part in a few cells) is degraded specifically. As mentioned above, CAD activation requires caspase-mediated ICAD-inactivation, but there was no caspase activity in infected cells. Taken together, the results therefore suggest that the DNA fragmentation seen during infection was not the result of CAD activation. However, DNA fragmentation can also be caused by other DNases. In isolated nuclei, micrococcal DNase causes DNA fragmentation (39). Indeed, it had been suggested in the past that DNase I is the enzyme responsible for apoptotic DNA cleavage (30). As mentioned above, the release of lysosomal contents during infection has been observed by ultrastructural cytochemical analysis (42); a lysosomal DNase such as, for instance, DNase II (28) might thus play a role. It is therefore easily conceivable that a nuclease distinct from CAD is activated in the course of infection, whose activity is perhaps limited to a small volume of the nucleus and which generates a limited number of DNA fragments. This might also explain the positive TUNEL reaction that we and others (4) have observed. Since TUNEL also detects single-strand breaks, this may also be a feature of cell death induced by chlamydial infection; single-strand breaks would not be seen by normal agarose gel electrophoresis.

What may be the mechanism and importance of the Bax activation during chlamydial infection? Bax is one of two proteins (Bax and Bak) that can achieve permeabilization of the mitochondrial outer membrane, apparently without the requirement for other protein partners (23). Bax is normally activated by the activation of proapoptotic BH3-only proteins, although the precise mechanism of this activation remains elusive. This raises an apparent paradox, since the ability of chlamydial infection to protect the cell against apoptosis is mostly the result of *Chlamydia*-associated degradation of BH3-only proteins (10, 46). However, there is evidence that Bak can be activated by alternative mechanisms. Bak has been shown to be sequestered to the antiapoptotic proteins Bcl-X_L and Mcl-1, and disruption of this interaction could activate Bak (43). Al-

though the disruption occurs through BH3-only proteins in apoptosis, this may also be achieved by other means during infection. Although it is unclear whether Bax may be regulated in the same way, it is a possibility that should be considered. It should also be noted that, in previous reports, we had only looked at an N-terminal conformational change that is typical of Bax activation (19, 32). Full Bax activation also requires its oligomerization and insertion into the outer mitochondrial membrane (6), which we did not investigate. A recent report shows even a lack of correlation between Bax N-terminal change and Bax activation during T-cell apoptosis (47). It is therefore conceivable that Bax is only partially activated and does not release cytochrome *c*. Finally, there is evidence from a cell-free system that cytochrome *c* in infected cells has a much-reduced capacity to activate caspases (9). If Bax therefore did cause cytochrome *c* release, apoptosis might be blocked at that step.

The high level of nuclear changes in MEF cells lacking Bax, Bak, or both Bax and Bak was very surprising and is difficult to explain. We described earlier that the loss of Bax caused a moderate decrease in the number of cells with nuclear changes during infection with *C. muridarum* (31). We now confirm this result for *C. trachomatis*-infected cells and find in addition that the loss of Bak very strongly inhibits the appearance of nuclear changes. However, the combined loss of Bax and Bak resulted in an increase in the extent of nuclear apoptosis during infection, becoming similar to the level of apoptosis seen in wild-type cells. This paradoxical result suggests that the combined loss of Bax and Bak may be compensated for by other molecular mechanisms that are relevant for *Chlamydia*-induced cell death, albeit not for apoptosis. We can also not exclude the possibility that the cells had acquired secondary mutations due to the loss of Bax/Bak. However, such mutations would very likely be in genes that are unrelated to the apoptotic pathway (as UV-induced apoptosis was blocked in $bax^{-/-}/bak^{-/-}$ cells). In any case, these data are consistent with the view that—whatever the function of Bax/Bak may be—they are not playing the role that they normally have during apoptosis, i.e., cause apoptosis through cytochrome *c* release and subsequent caspase activation. Recent reports suggest that mitochondrial Bax also affects mitochondrial fission (20). It is tempting to speculate that Bax could influence *Chlamydia*-induced cell death at a level unrelated to apoptosis, for instance by affecting mitochondrial metabolism, which might in turn affect morphological changes. Moreover, it has recently been suggested that Bax and Bak are important for the unfolded protein response in the ER (17). ER changes are among the earliest morphological alterations observed in cells infected with *C. psittaci* (41); it is therefore conceivable that the effects of a Bax/Bak double deficiency on *Chlamydia*-induced cell death may instead be related to the ER-dependent stress response.

Cell death during chlamydial infection thus has features that are reminiscent of apoptosis, but there is strong evidence against the participation of the apoptotic pathway. What term should we use to describe this form of cell death? In the absence of essential factors such as caspase activation, it is probably not appropriate to call it apoptosis. Even the term aponecrosis, which has been proposed for cell death induced by high doses of *C. pneumoniae* in smooth muscle cells (4), does not, in our opinion, describe *Chlamydia*-induced cell death in a sufficiently precise and meaningful way. Traditionally, all forms of cell death that do not occur as a consequence of the activation of the apoptotic pathway have been classified as necrotic, although there have also been attempts to distinguish between different forms of nonapoptotic death (for a review, see reference 14). While waiting for the mechanistic basis for *Chlamydia*-induced cell death to be identified, and despite our earlier suggestions (29, 32), we propose that "*Chlamydia*-induced cell death" be used in the interim to describe this type of host cell death.

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