Effect of Chlamydia trachomatis infection and subsequent TNFa secretion on apoptosis in the murine genital tract

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Effect of Chlamydia trachomatis Infection and Subsequent Tumor Necrosis Factor Alpha Secretion on Apoptosis in the Murine Genital Tract

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The pathology observed during Chlamydia infection is due initially to localized tissue damage caused by the infection itself, followed by deleterious host inflammatory responses that lead to permanent scarring. We have recently reported that the infection by Chlamydia in vitro results in apoptosis of epithelial cells and macrophages, and that infected monocytes secrete the proinflammatory cytokine interleukin-1β. At the same time, proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α) can also trigger apoptosis of susceptible cells. To study the possible relationship between Chlamydia trachomatis infection and apoptosis in vivo, we used the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling technique to determine whether infection may cause apoptosis in the genital tract of mice and, conversely, whether cytokines produced during the inflammatory response may modulate the level of apoptosis. Our results demonstrate that infected cells in the endocervix at day 2 or 7 after infection are sometimes apoptotic, although there was not a statistically significant change in the number of apoptotic cells in the endocervix. However, large clumps of apoptotic infected cells were observed in the lumen, suggesting that apoptotic cells may be shed from the endocervix. Moreover, there was a large increase in the number of apoptotic cells in the uterine horns and oviducts after 2 or 7 days of infection, which was accompanied by obvious signs of upper tract pathology. Interestingly, depletion of TNF-α led to a decrease in the level of apoptosis in the uterine horns and oviducts of animals infected for 7 days, suggesting that the inflammatory cytokines may exert part of their pathological effect via apoptosis in infected tissues.

Apoptosis is a key phenomenon in the regulation of cell population size and cell life span (18, 52). This process of cell death plays an important role in normal tissue homeostasis and in certain pathological conditions, including cancer. A growing number of studies over the last few years have shown that intracellular microbes can also modulate apoptosis of the host cell, either inhibiting or promoting cell death, and it has been proposed that the persistence and pathogenesis of several pathogenic microbes may be related to their ability to dysregulate apoptosis (2).

Although microbe-induced apoptosis has been extensively characterized for viral infections (2, 6, 47), apoptosis has also been observed during infections in vivo by bacteria or protozoan parasites, such as for Escherichia coli, Yersinia pseudotuberculosis, Trypanosoma cruzi, Shigella flexneri, Bordetella pertussis, Salmonella enterica serovar Typhimurium, and Toxoplasma gondii (13, 21, 26, 27, 34, 40, 59). Apoptosis due to infection by these pathogens may allow the pathogens to exit plasma gondii (13, 21, 26, 27, 34, 40, 59). Apoptosis due to infection by these pathogens may allow the pathogens to exit plasma gondii (13, 21, 26, 27, 34, 40, 59). Apoptosis due to infection by these pathogens may allow the pathogens to exit plasma gondii (13, 21, 26, 27, 34, 40, 59). Apoptosis due to infection by these pathogens may allow the pathogens to exit plasma gondii (13, 21, 26, 27, 34, 40, 59). Apoptosis due to infection by these pathogens may allow the pathogens to exit plasma gondii (13, 21, 26, 27, 34, 40, 59). Apoptosis due to infection by these pathogens may allow the pathogens to exit plasma gondii (13, 21, 26, 27, 34, 40, 59). Apoptosis due to infection by these pathogens may allow the pathogens to exit plasma gondii (13, 21, 26, 27, 34, 40, 59).

In humans, the most common consequence of chlamydial genital infection is salpingitis, which can lead to tubal obstruction and infertility (4). In controlled studies in guinea pigs and mice (3, 9, 38), bacteria are initially detected in the cervical epithelium, but the pathology ascends in most animals to the endometrium and the oviducts within 7 to 9 days after intravaginal inoculation, culminating often in infertility. Most of the damage attributable to Chlamydia is due not to the infection itself but to the inflammation and fibrosis that follow the infection (4).

Polymorphonuclear leukocytes are typically observed in the cervix as early as 2 days after infection, and acute inflammation in the uterine horns and oviducts follows within 5 to 7 days after infection (4). A number of inflammatory mediators are present during infection, and these could contribute to tissue damage and fibrosis. Two predominant cytokines usually produced during inflammation are interleukin-1 (IL-1) and tumor necrosis factor (TNF-α), which activate polymorphonuclear leukocytes and contribute to fibrosis due to enhanced production of prostaglandins and collagen and increased expression of integrin, as well as secretion of IL-6, IL-8, and transforming growth factor β (32, 51, 58). TNF-α has in fact been detected in the fallopian tubes of women infected with Chlamydia (48) and in secretions from Chlamydia-infected mice and guinea pigs (7, 8, 54). Results based on studies using mice that display mouse strain-dependent variations in the pathological outcome of Chlamydia genital infection and correspondingly different levels of TNF-α production have suggested that while TNF-α and other inflammatory cytokines may aid in eradication.
ing Chlamydia infection, they may also promote long-term tissue damage (7).

The preferential target tissue of sexually transmitted chlamydial infections in females is the columnar epithelium of the cervix (4, 29), but monocytes and macrophages can also be infected (23) and may aid in disseminating the infection by certain serovars of Chlamydia. As macrophages undergoing apoptosis secrete IL-1 (15), it is conceivable that apoptosis of these cells during Chlamydia infection may contribute to the inflammatory response. Conversely, cytokines such as TNF-α are capable of directly triggering apoptosis of some target cells (12), suggesting that the inflammation following Chlamydia infection may also directly trigger apoptosis.

We have therefore investigated apoptosis in the genital tract of mice using the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) method, which reveals early DNA breaks during apoptosis (42), allowing identification of apoptotic cells that may have evaded detection by conventional histological techniques. The potential effect of the inflammatory response on apoptosis was evaluated by measuring apoptosis in the uterine horns and oviducts in infected mice whose TNF-α levels had been depleted with antibodies.

MATERIALS AND METHODS

Cells and animals. HeLa 293 cells, McCoy cells, and L cells (American Type Culture Collection, Manassas, Va.) were maintained in a humidified incubator at 37°C with 5% CO2 in Dulbecco modified Eagle medium (Life Technologies, Inc., Rockville, Md.) supplemented with 10% heat-inactivated fetal calf serum and 2 mM l-glutamine. The Chlamydia species used here, the mouse pneumonitis strain (MoPn) of C. trachomatis (3, 45), was obtained from Roger King (University of Arkansas) and was grown in L cells and purified by Renografin as previously described for P. piitacci (14). The quantity of bacterial inclusion-forming units (IFU) was determined by incubating the chlamydiae with McCoy cells for 1 day and revealing the presence of bacteria by immunofluorescence with fluorescein isothiocyanate-labeled anti-Chlamydia antibodies (IgG-polysaccharide [LPS]-specific monoclonal antibody clone C6; Argene, Valilhes, France). Four-week-old female C57BL/6 mice were purchased from IFFA CREDO (L’Arbresle, France).

Infections. For infections in vitro, HeLa 293 cells growing at about 70% confluence on tissue culture flasks (Costar) were infected with a multiplicity of infection (MOI) of 6.8 as previously described for P. piitacci (32) and incubated during the indicated times at 37°C.

For infections in vivo, mice first received 2.5 mg of medroxyprogesterone acetate (Sigma Chemical Co., St. Louis, Mo.; or Upjohn, Kalamazoo, Mich.) subcutaneously at 3 and 10 days before infection (7). Infection was initiated by placing 50 μl of SPG buffer (250 mM sucrose–10 mM sodium phosphate–5 mM l-glutamic acid [pH 7.2]), containing 50 μg of gentamicin per ml and 10^2 IFU of C. trachomatis MoPn into the vaginal vault of the mice, corresponding to a 50% infectious dose of 4 × 10^4 (unpublished observations). Uninfected control mice were subcutaneously injected with a saline sample that had been stained with the TUNEL kit without the terminal deoxynucleotidyltransferase enzyme. Under these conditions, the minimal threshold of fluorescence intensity that excludes the false-positive cells was defined. To identify the apoptotic cells in images, the IPLab spectrum program was used to add a green color to the cells with a fluorescence intensity under the threshold level (nonapoptotic cells) and a red color to the cells with a fluorescence intensity higher than the threshold level (apoptotic cells). The relative number of apoptotic cells per image was then counted, and 10 fields were analyzed per tissue.

RESULTS

Apoptosis of epithelial cells in vitro during infection with the murine strain of C. trachomatis. We have recently reported that the guinea pig inclusion conjunctivitis (GPC) serovar of C. psittaci induces apoptosis of epithelial cells, with apoptosis being measurable after a 1-day infection (33). To determine whether the murine strain of C. trachomatis, MoPn, shares this property, we measured the time course of apoptosis of the epithelial cell line, HeLa, by cytofluorimetry using iodide propidium-stained, detergent-permeabilized cells as described in Materials and Methods. We observed few apoptotic cells during short infection times, but significant levels were detected after a 42-h infection, at which point release of MoPn elementary bodies begins to occur (not shown), and most of the cells were apoptotic after a 2-day infection (Fig. 1A). This time course is reminiscent of the apoptosis previously observed during infection in vitro with C. psittaci (33), although the kinetics were slightly slower for MoPn-induced apoptosis and the MOI required for MoPn (6.6) was higher than that required for GPC (1.0).

To confirm the apoptotic nature of the host cell death, we studied DNA fragmentation of infected cells by DNA electrophoresis on agarose gels. While there was no DNA fragmentation in uninfected HeLa cells, there was a high level of fragmentation in cells that had been infected for 48 h (Fig. 1B). The sizes of the DNA fragments were integral values of 200 bp, which is a characteristic feature of intranucleosomal cleavage due to activated endonucleases during the apoptotic process (57).

To evaluate whether chlamydial growth is required for this type of apoptosis, we infected cells with C. trachomatis for 48 h in the presence or absence of chloramphenicol, an antibiotic that inhibits prokaryotic protein synthesis without altering pro-
Cells were infected at various times with an MOI of 6.6. (A) Time course of C. trachomatis-induced apoptosis, measured by cytofluorimetry. (B) DNA fragmentation of HeLa cells infected on separate days. (C) Effect of chloramphenicol on Chlamydia-induced apoptosis. HeLa cells were infected during 48 h with or without chloramphenicol, and apoptosis was measured by cytofluorimetry. Each experiment was performed with three different preparations of HeLa cells infected on separate days.

Protein synthesis of the eukaryotic host cell (49, 53) is necessary for efficient triggering of apoptosis. In one set of experiments (Fig. 1C), more than half of the apoptosis due to an infection with C. trachomatis MoPn for 48 h was inhibited by incubation with chloramphenicol, and larger inhibitory effects were observed in other experiments, suggesting that chlamydial protein synthesis is necessary for efficient triggering of apoptosis of infected cells. This interpretation is reinforced by the observation that there is no apoptosis if chlamydiae are first inactivated by exposure to UV irradiation (reference 33 and not shown). As we have previously shown that C. psittaci also induces apoptosis in vitro (33), these results suggest that infection may lead to apoptosis regardless of the Chlamydia species or serovar. In addition, infection with C. trachomatis MoPn also induced apoptosis of McCoy cells (not shown), implying that other cell types may also be susceptible to Chlamydia-induced apoptosis.

Apoptosis in the murine genital tract during infection with C. trachomatis. To establish whether C. trachomatis may also induce apoptosis in vivo, we used the murine model of C. trachomatis genital infection (3). After injection of progesterone to synchronize the estrus cycle, female C57BL/6 mice were infected intravaginally with chlamydiae. Tissues for histological analysis were obtained by sacrificing mice after 2 or 7 days of infection. The entire genital tract was excised en bloc, fixed in paraformaldehyde, and processed for histology or TUNEL as described in Materials and Methods. The cervix, uterine horn, and oviduct were then assessed separately for the presence of inflammation and apoptosis.

Two days after infection, the infected cervix had visibly damaged cells, some of which were condensed, and there was a low level of infiltration of inflammatory cells (Fig. 2C). Most of the damaged cells were present in the stratified squamous epithelium of the vagina. The inflammatory response became significantly enhanced after 7 days of infection, with a heavy infiltration of mono- and polymorphonuclear cells in the uterine mucosa (Fig. 2E). As a control, there was no inflammation nor chlamydial staining in uninfected mice (Fig. 2A and data not shown).

We attempted to evaluate whether the chlamydiae are able to directly induce apoptosis in infected cells or indirectly in neighboring cells. Thus, we used the TUNEL method combined with anti-Chlamydia staining to observe simultaneously apoptotic and infected cells. This approach revealed that apoptosis occurred in both infected and uninfected cells (Fig. 2G and H), with the majority of apoptotic cells during infection being localized in the lining cells of the mucosa of the lower uterus and endocervix. We did not find a significant change in the number of apoptotic cells in uninfected mice that were not pretreated with progesterone (not shown). In addition, we observed that infection leads to detachment of apoptotic and infected cells from the epithelium (Fig. 2G). Taken together, these results suggest that the chlamydiae may induce apoptosis directly. However, ostensibly uninfected cells were also apoptotic (Fig. 2H). These may be the same cells as the spontaneously apoptotic cells found in uninfected tissues, but the results also raise the possibility that the chlamydiae may induce apoptosis indirectly, via locally produced cytokines secreted during initial phases of the infection. Finally, although an early feature of apoptosis is detachment of dying cells from neighboring cells, detachment of cells also leads to apoptosis, and we cannot exclude the possibility that infection may first cause detachment, followed by apoptosis.

The number of apoptotic cells during infection was quantified as described in Materials and Methods. After a 2- or 7-day infection, there was no significant change in the number of apoptotic cells in the endocervix and lower uterus of infected mice compared to uninfected mice (Fig. 2B and D and data not shown). Hence, it is possible that infection by C. trachomatis in vivo does not lead directly to apoptosis. Alternatively, as suggested by Fig. 2G, it is conceivable that the dying lining epithelial cells may be quickly shed into the lumen of the uterus, as these cells were not observed in the lumen of uninfected mice. However, it was not possible to quantify the apoptosis in the lumen due to the large number of apoptotic cells found in clumps, and they were therefore not taken into account when determining the level of apoptosis in the lower genital tract.

In the uterine horns and oviducts, we also observed an inflammatory response that was often accompanied by an accumulation of nucleated cells in the lumen (Fig. 3A and C). At these infection times, we found lining epithelial cells with large cytoplasmic Chlamydia inclusions in the uterine horns and oviducts, as well as infected cells in the submucosa of the uterine horns and the underlying stroma (Fig. 3B and D).

In contrast to the endocervix, it was easier to demonstrate a difference in the level of apoptosis in the uterine horns and oviducts due to the low physiological level of apoptosis in the absence of infection. The number of apoptotic cells in the uterine horns and oviducts increased significantly in mice infected for 2 or 7 days compared to uninfected controls (Fig. 3E and F; Fig. 4 [see below]). As in the case of the endocervix, however, both infected and uninfected cells were apoptotic (not shown).

Role of TNF-α in modulating apoptosis in the uterine horns and oviducts. In C57BL/6 mice, TNF-α has been detected in genital tract secretions of animals during the first week of infection with C. trachomatis MoPn (7, 8). TNF-α is also known to trigger apoptosis of a diverse range of cells in vitro (12). We therefore depleted TNF-α levels in vivo by injecting mice with a polyclonal anti-mouse TNF-α before and during infection with C. trachomatis, as described in Materials and Methods.
TNF-α levels in endocervical secretions from mice injected with anti-TNF-α antibodies, determined by enzyme-linked immunosorbent assay (ELISA), were significantly lower than in controls \((P < 0.001\) by two-way analysis of variance). The Tukey test revealed significant decreases on days 4 through 7 (not shown). Thus, TNF-α levels on day 5 were 1,070 ± 470 pg/ml in controls and 270 ± 22 pg/ml in the antibody-treated group. On day 7, TNF-α levels were 780 ± 450 pg/ml in controls and 100 ± 60 pg/ml in the antibody-treated group (Fig. 5).

Treatment with anti-TNF-α antibodies had no effect on levels of gamma interferon and IL-1β (not shown).

The number of apoptotic cells was quantified with the
TUNEL technique and image analysis software as described in Materials and Methods. As seen in Fig. 4, there was a significant decrease in the number of apoptotic cells in mice infected for 7 days whose TNF-α levels were diminished by administration of anti-TNF-α antibodies, suggesting that TNF-α produced during Chlamydia infection contributes to apoptosis in the upper genital tract.

**DISCUSSION**

We have previously reported that epithelial cells and macrophages die through apoptosis during infection with C. psittaci in vitro (33). To determine whether apoptosis may be induced by infection in vivo, we used the TUNEL technique to quantify the number of apoptotic cells in the genital tract of female mice during infection with C. trachomatis, having verified first that C. trachomatis infection leads to apoptosis in vitro with similar characteristics as observed during C. psittaci-induced apoptosis.

We observed a large increase in the number of apoptotic cells in the uterine horns and oviducts of mice infected with C. trachomatis, and the apoptotic cells were present in both the epithelium and submucosa. However, the results were ambiguous in the case of the lower genital tract, which already had a large number of spontaneously apoptotic cells in uninfected mice. Infected cells that were also apoptotic were in fact observed in the endocervix of infected mice, but we did not observe a quantitative difference in the levels of apoptosis between infected and uninfected mice. Clusters of apoptotic infected cells were found in the lumen of the vagina after a 2-day infection, and it is thus possible that the apoptotic cells may be shed from the surface epithelial cells, as has been
depleted mice were used in the analysis. Infected mice at day 7, and eight TNF-α
Methods. Two uninfected mice for each group, three infected mice at day 2, ten
the number of apoptotic cells was determined as described in Materials and
Samples were stained with the TUNEL technique as shown in Fig. 3E and F, and
inhibited in mice that had been pretreated with antibodies against TNF-α.
increase after a 7-day infection. The increase after a 7-day infection was partially
number of apoptotic cells after a 2-day infection with
observed in the oviducts of uninfected mice. There was a large increase in the
depletion. A low level of spontaneous apoptosis is
a cytokine IL-1β
of uninfected cells. Several cytokines are in fact secreted by
proposed that chlamydiae may induce apoptosis directly
apoptotic cells were also observed (33). We had therefore
infection, but that locally produced cytokines secreted
during initial phases of the infection may also cause apoptosis
uninfected cells. Several cytokines are in fact secreted by
cells infected by Chlamydia both in vitro and in vivo. Infection of
an epithelial cell line causes release of the proinflammatory
cytokine IL-1α (39), and incubation of dendritic cells and
monocytes with chlamydiae in vitro results in secretion of
TNF-α and IL-1β, respectively (31, 33). In vivo, an acute
inflammatory response is the dominant host response in the early
stages of the infection and is observed in the endometrium and
oviducts within a few days of infection (4). In mice, TNF-α has
been detected in the genital tract secretions of animals inoculated with C. trachomatis
MoPn. High levels were measured within 2 days after intravaginal inoculation, peaked at 6 days,
and decreased after a week (7). It had been proposed that
TNF-α may aid the host to eradicate the chlamydiae, thus
preventing infection of the oviduct (7). Hence, TNF-α, in
conjunction with gamma interferon, transforming growth factor β,
and IL-1 (25, 37, 55, 56), may be a factor responsible for the
inflammatory response and consequent fibrosis.
While TNF-α is a pleiotropic cytokine that is cytotoxic
tumor cells, it also has effects on untransformed cells (1, 24, 35,
41), and it has been proposed that TNF-α may serve as the
local signal contributing to the processes of shedding and
bleeding in humans during menstruation (46). Favoring the
likelihood that TNF-α could also trigger apoptosis during
Chlamydia infection, we have observed a large increase in the
number of apoptotic cells in the uterine horns and oviducts,
which was partially inhibited by depletion with antibodies
against TNF-α. In addition, the number of apoptotic cells in
the upper tract of TNF-α-depleted mice that had been infected
for 7 days is close to the number observed in untreated mice
that had been infected for 2 days, further reinforcing the pos-
sibility that the increased level of apoptosis in untreated mice
infected for 7 days may be due to proinflammatory cytokines,
since the inflammatory response in oviducts of C57 mice
infected with C. trachomatis MoPn does not begin until at least
3 days of infection (7).
It has previously been reported that Chlamydia infections in
vitro induce apoptosis toward the end of the infection cycle
(reference 33 and this work) and protect infected cells against
apoptosis due to external ligands (11). During infections in
vivo, most of the cells dying as a result of the inflammatory
response in the upper genital tract at day 7 do not appear to be
infected and thus should not be protected. Alternatively, in-
fected cells in vivo may be protected only partially, compared
to the results previously reported with cell lines in vitro.
The observation that TNF-α can modulate apoptosis during
infection with C. trachomatis is reminiscent of the role played
by this cytokine during infections with Mycobacterium tubercu-
Chlamydia-induced apoptosis in vivo

The northwestern branch of the Chlamydia species is weak inducers of TNF-α secretion from isolated whole blood cells (16), it is possible that a small population of effector cells could be responsible for the high levels of TNF-α observed during Chlamydia infection in vivo (7, 8).

In conclusion, we propose that Chlamydia-induced release of IL-1 and TNF-α, possibly acting in concert with other inflammatory cytokines, could lead to apoptosis of directly infected and/or neighboring cells. Finally, as macrophages and monocytes undergoing apoptosis also release IL-1 (15), the cells secreting the proinflammatory cytokines, as well as the cells undergoing Chlamydia-induced apoptosis in vivo, need to be identified in order to evaluate their contribution to the pathology of Chlamydia infections.

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