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Chlamydia pneumoniae Multiply in Neutrophil Granulocytes and Delay Their Spontaneous Apoptosis

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The obligate intracellular bacterial pathogen Chlamydia pneumoniae (Cp) is responsible for a range of human diseases, including acute respiratory infection. Although experimental intratracheal inoculation with Cp results in a massive recruitment of neutrophil granulocytes (polymorphonuclear neutrophils (PMN)), the role of these cells in the defense against Cp is unclear. In this study the interactions of PMN with Cp were investigated. In vitro coinoculation experiments showed that human granulocytes were able to internalize Chlamydia in an opsonin-independent manner. Importantly, phagocytosed Cp were not killed; the ingested bacteria survived and multiplied within PMN. Although uninfected granulocytes became apoptotic within 10 h, infected PMN survived up to 90 h. Coincubation with Cp significantly decreased the ratio of apoptotic PMN, as detected by morphological analysis, annexin V, and TUNEL staining. The observed antiapoptotic effect was associated with a markedly lower level of procaspase-3 processing and, consequently, reduced caspase-3 activity in infected PMN. LPS was found as a major, but not exclusive, component responsible for the observed antiapoptotic effect. Chlamydia LPS affected PMN apoptosis both by acting directly on the cells and by inducing the autocrine production of the antiapoptotic cytokine IL-8. These data show that, in contrast to other microbial pathogens that drive phagocytes into apoptosis to escape killing, Cp can extend the life span of neutrophil granulocytes, making them suitable host cells for survival and multiplication within the first hours/days after infection.

Chlamydia pneumoniae (Cp)1 has been established as the third species of the genus Chlamydia in 1986 (1). It has emerged as causative agent of ~10% of community-acquired pneumonia cases and 5% of pharyngitis and sinusitis cases (2). As viable chlamydiae have been cultivated from atherosclerotic plaques and cerebrospinal fluid, Cp has also been associated with several nonrespiratory diseases, such as coronary heart disease (3), multiple sclerosis (4), and Alzheimer’s disease (5). Within the bloodstream, monocytes were shown to harbor viable chlamydiae (6), and in vitro studies suggested that infected monocytes transmit the pathogen to endothelial or smooth muscle cells (7). Cp infection of the lung was reported to activate airway epithelial cells and induce the release of IL-8, followed by a rapid transendothelial migration of polymorphonuclear neutrophil granulocytes (polymorphonuclear neutrophils (PMN)) (8). Consequently, in the lung, PMN are among the first leukocytes to encounter Cp.

Although PMN are very important antimicrobial effector cells, recent data suggest that PMN can also serve as a host for intracellular pathogens, as has been shown for the agent of human granulocytic ehrlichiosis (HGE) (9) and Leishmania major (10). PMN were reported to phagocytose Chlamydia elementary bodies, and a small percentage of the internalized bacteria were shown to remain viable up to 10 h after uptake and to retain full virulence (11). The ability of certain intracellular microbes to survive and maintain infectivity in neutrophil granulocytes enables these organisms to establish productive infection. These organisms may use granulocytes as “Trojan horses” for subsequent infection of other cells, such as macrophages (12).

PMN are inherently short-lived cells, with a half-life of only ~6–10 h in the circulation, after which they undergo spontaneous apoptosis (13, 14). The apoptotic process is well regulated and characterized by cell shrinkage, chromatin condensation, internucleosomal DNA fragmentation, membrane blebbing, and, finally, decay into apoptotic bodies (13, 15). Although apoptosis is an intrinsic cell process, it is modulated by environmental signals. For example, the life span of mature neutrophils can be extended in vitro by incubation with either proinflammatory cytokines, including GM-CSF and G-CSF (16), IL-8 (17), IL-1β (18), and glucocorticoids (19), or bacterial products, such as LPS and fMLP (18, 20). Several microbial pathogens have been reported to influence cellular apoptosis (for review, see Ref. 21). Some microbial pathogens, such as Escherichia coli (Ec) (22) and Candida albicans (23), were found to induce apoptosis of PMN. In contrast, inhibition of apoptosis of host cells by intracellular pathogens, such as the agent of HGE (9) or L. major (10), can provide an intracellular niche for the pathogens by extending the life span of their host cells.

In the present study we investigated the interactions of neutrophil granulocytes with Cp. We monitored the uptake of Cp by

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3 Abbreviations used in this paper: Cp, Chlamydia pneumoniae; Ec, Escherichia coli; HGE, human granulocytic ehrlichiosis; IFU, inclusion-forming unit; PI, propidium iodide; PMN, polymorphonuclear neutrophil, PS, phosphatidylserine; rhIl-8, recombinant human IL-8; Sr, Salmonella enterica.
PMN and demonstrated that intracellular chlamydiae survive and multiply within PMN. Coincubation with Cp resulted in a delay of neutrophil apoptosis up to 3 days, which was associated with a decrease in caspase-3 activity and was mediated by both Cp LPS and the autocrine production of IL-8 by PMN. Up to 42 h after Cp–PMN coincubation, Cp LPS has the strongest antiapoptotic effect, whereas Cp-induced IL-8 mediates apoptosis inhibition at longer time intervals.

Materials and Methods

Bacterial strain, propagation, and purification

The Cp strain CV-6, used in this study, was isolated from a coronary artery plaque and continuously propagated on HeP-2 cells as described previously (24). Briefly, confluent sheets of HeP-2 cells grown in 24-well plates in Eagle’s MEM (Life Technologies, Gaithersburg, MD) were infected with Cp by centrifugation at 3,000 × g for 45 min. After incubation at 37°C in an atmosphere containing 5% CO2 for 72 h, the infected monolayers were mechanically disrupted. Cell debris was removed by centrifugation at 1,000 × g for 10 min, and chlamydiae were concentrated by ultracentrifugation of the supernatant by 24,000 × g for 1 h. The Chlamydia pellet was resuspended in 0.55 ml of PBS, 0.05 ml of it was used to inoculate new monolayers to determine the inclusion-forming units (IFUs) by staining with a Chlamydia LPS-specific, FITC-coupled mAb (clone RR402, IgG3; DAKO, Hamburg, Germany) as described previously (24). Mock-infected cell lysates were prepared in the same way and used for inoculation of controls. Chlamydiae with known IFU number were suspended in 10% FBS (Seromed-Biochrom, Berlin, Germany) as described previously (24). Mock-infected monolayers to determine the inclusion-forming units (IFUs) by staining with a Chlamydia LPS-specific, FITC-coupled mAb (clone RR402, IgG3; DAKO, Hamburg, Germany) as described previously (24). Mock-infected cell lysates were prepared in the same way and used for inoculation of controls. Chlamydiae with known IFU number were suspended in 10% FBS (Seromed-Biochrom, Berlin, Germany) as described previously (24). Mock-infected monolayers to determine the inclusion-forming units (IFUs) by staining with a Chlamydia LPS-specific, FITC-coupled mAb (clone RR402, IgG3; DAKO, Hamburg, Germany) as described previously (24). Mock-infected cell lysates were prepared in the same way and used for inoculation of controls. Chlamydiae with known IFU number were suspended in 10% FBS (Seromed-Biochrom, Berlin, Germany) as described previously (24). Mock-infected monolayers to determine the inclusion-forming units (IFUs) by staining with a Chlamydia LPS-specific, FITC-coupled mAb (clone RR402, IgG3; DAKO, Hamburg, Germany) as described previously (24). Mock-infected cell lysates were prepared in the same way and used for inoculation of controls. Chlamydiae with known IFU number were suspended in 10% FBS (Seromed-Biochrom, Berlin, Germany) as described previously (24). Mock-infected monolayers to determine the inclusion-forming units (IFUs) by staining with a Chlamydia LPS-specific, FITC-coupled mAb (clone RR402, IgG3; DAKO, Hamburg, Germany) as described previously (24). Mock-infected cell lysates were prepared in the same way and used for inoculation of controls. Chlamydiae with known IFU number were suspended in 10% FBS (Seromed-Biochrom, Berlin, Germany) as described previously (24). Mock-infected monolayers to determine the inclusion-forming units (IFUs) by staining with a Chlamydia LPS-specific, FITC-coupled mAb (clone RR402, IgG3; DAKO, Hamburg, Germany) as described previously (24).

Preparation of human peripheral blood neutrophils and coincubation with Cp

Neutrophil granulocytes were isolated from buffy coat blood obtained from healthy adult volunteers as previously described (25). Briefly, buffy coat was diluted 1/6 with sterile PBS and layered on a Histopaque gradient (Sigma-Aldrich, Taufkirchen, Germany) and stored in small aliquots at −80°C until used. Throughout the manuscript the amount of chlamydiae used is depicted as IFUs of Cp.

Preparation of human peripheral blood neutrophils and coincubation with Cp

After centrifugation in PBS for 45 min at 300 × g, followed by 15 min at 800 × g without brake. The PMN-rich layer of Histopaque 1119 was collected and washed twice in complete RPMI 1640 medium (Seromed-Biochrom) containing 50 µM 2-ME, 2 mM L-glutamine, 10 mM HEPES (all from Seromed-Biochrom) complemented with 10% FCS (Life Technologies), after which the cells were further fractionated on a discontinuous Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient consisting of layers with densities of 1.105 g/ml (85%), 1.100 g/ml (80%), 1.093 g/ml (75%), 1.087 g/ml (70%), and 1.081 g/ml (65%). After centrifugation for 25 min at 800 × g, the interface between the 80 and 85% Percoll layers was collected and washed twice in RPMI 1640. All procedures were conducted at room temperature. The purity of granulocytes achieved by this isolation technique was always >99% as determined microscopically by May-Grünwald-Giemsa staining of cytocentrifuge (Shandon, Pittsburgh, PA) slides. The viability of cells was >98% as assessed by trypan blue dye exclusion.

PMN (1 × 10^6/ml) were coincubated at 37°C in a volume of 1 ml of complete RPMI 1640 medium with Cp at a Cp IFU to PMN ratio of 1:1 or with heat-killed HeP-2 cell lysates as negative controls in a humidified atmosphere containing 5% CO2. In addition, coincubation experiments were conducted by using heat-killed Cp (30 min 100°C) or different concentrations of purified Chlamydia trachomatis LPS (26). Further coincubations were conducted with various concentrations of recombinant human IL-8 (rIL-8; PeproTech, Offenbach, Germany), either with supernatants from PMN cocultures or with the supernatants from HeP-2 cells after 8 days. IL-8 was depleted from the supernatant using immunoprecipitation with a murine anti-human IL-8 mAb (clone 94.1; gift from Dr. E. Brandt, Borstel, Germany) and protein G-agarose (BD Biosciences, Heidelberg, Germany) or an isotype-matched control mAb and protein G-agarose as control.

Immunohistochemistry and electron microscopy of infected granulocytes

Human PMN were infected with Cp as described above and cultivated for 1–4 days. For Cp-specific staining cells were cytocentrifuged and fixed in methanol. Subsequently, cells were stained using an FITC-conjugated anti-Cp mAb (clone RR402, IgG3; DAKO) or an FITC-conjugated isotype-matched control mAb (DAKO), followed by counterstaining with hematoxylin.

Immunolabeling on ultrathin sections for electron microscopy was performed as previously described (27). Briefly, human granulocytes were fixed with 2% paraformaldehyde and 0.1% glutaraldehyde in PBS, pH 7.4, for 1 h at 4°C and contrasted with 2% uranyl acetate in cacodylate buffer. After dehydration in a graded acetone series, cells were embedded in LR White (London Resin Co., Reading, U.K.). Ultrathin sections were mounted on 300-nm nickel grids and were blocked for 30 min with 0.5% BSA (Sigma-Aldrich) in TBS (pH 7.4). The sections were incubated for 16 h with the Cp LPS specific CF-2 mAb (Washington Research Foundation, Seattle, WA) diluted 1/250 with TBS. After rinsing with TBS, sections were incubated for 2 h with donkey anti-mouse IgG coupled to 12-nm gold particles (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1/100 with TBS. Sections were contrasted with uranyl acetate and lead citrate (Ultrastainer Carlsberg System; LKB, Bromma, Sweden) and examined with a Philips EM 400 electron microscope.

Live/dead staining to visualize viable Cp and PMN

Granulocytes were infected with Cp as described above and cultivated for 1–4 days. After 18, 42, 66, and 90 h, cells were stained with a combination of SYTO-16 (Molecular Probes, Leiden, The Netherlands) and ethidium bromide. Staining with 5 µmol SYTO-16 for 10 min at room temperature was used to visualize viable intracellular Cp and PMN. This dye penetrates cell membranes and stains DNA. Consequently, the nuclei of viable cells/bacteria appear green. Subsequent to SYTO-16 staining, the cells were stained with 5 µmol ethidium bromide to reveal nonviable PMN and Cp.

Detection of Cp LPS by ELISA

Neutrophil granulocytes were coincubated with Cp and collected after 18, 42, 66, and 90 h. Chlamydia LPS was detected by using an ELISA kit (IDEA PCE Chlamydia, DAKO, Ely, U.K.) according to the manufacturer’s instructions. In short, Cp-infected PMN (1.0 × 10^6) were suspended in 1.0 ml of triethanolamine buffer and heated at 95°C for 15 min to extract Chlamydia LPS. The LPS content was determined by sandwich ELISA using anti-Chlamydia and alkaline phosphatase-conjugated anti-Chlamydia mAbs, supplied in the kit. As a standard for Chlamydia LPS, a series of dilutions (10^3–10^4 IFUs) was added and centrifuged (3,000 × g for 45 min) upon HeP-2 cell cultures, and then Chlamydia LPS was extracted from the cultures. The relative number of Chlamydia organisms was calculated from the standard curve. The lower detection limit was 5 × 10^3 IFUs/assay, as described previously (28).

Assessment of PMN apoptosis

Morphological assessment of apoptosis. In neutrophil granulocytes morphological changes in apoptosis include separation of nuclear lobes and darkly stained pyknotic nuclei (13–15). Accordingly, the morphological criteria for neutrophil apoptosis were one or more densely stained nuclear fragments and the absence of chromatin within nuclear lobes/fragments. Nuclear morphology was assessed on Giemsa-stained cytocentrifuge slides. Cell morphology was examined under oil immersion light microscopy, and a minimum of 200 cells/slide were examined and graded as apoptotic/nonapoptotic.

Annexin V binding. Annexin V exhibits calcium-dependent binding to phosphatidylserine expressed in the outer membrane leaflet of apoptotic PMN (29). Labeling of apoptotic cells with annexin V-FITC and counterstaining with propidium iodide (PI) for necrotic cells (both from Roche, Mannheim, Germany) were performed as recommended by the manufacturer. Labeled cells were...
analyzed by flow cytometry using a FACSCalibur with CellQuest software (BD Biosciences, San Diego, CA).

**TUNEL assay of chromatin fragmentation.** The TUNEL assay (In Situ Cell Death Detection Kit; Roche) was used to detect apoptotic cell death by enzymatic labeling of DNA strand breaks with fluorescein-dUTP and TdT (30). Briefly, $1 \times 10^5$ cells were fixed in 4% formaldehyde/PBS (pH 7.4) for 60 min at room temperature, washed in PBS, and then suspended in permeabilization solution (0.1% Triton X-100/0.1% sodium citrate) for 3 min on ice. Cells were washed again, resuspended in 50 µl of TUNEL reaction mixture or in 50 µl of label solution alone (negative control), and incubated in a humidified dark chamber at 37°C, followed by washing in PBS. The green fluorescence of apoptotic nuclei was detected by flow cytometry as described above.

**Caspase-3 Western blot.** Neutrophil granulocytes ($10^6$) were cocultured with or without *Cp* as described above, transferred into prechilled tubes containing ice-cold RPMI 1640, and centrifuged at $800 \times g$ for 5 min at 4°C. Cell pellets were washed and resuspended in 400 µl of lysis buffer (100 mM HEPES and 1% Nonidet P-40, pH 7.5) containing a protease inhibitor mixture (1 mM EDTA; 0.5 mM EGTA; 1 mM PMSF; 0.5 mM DTT; 2 mM levanosil; 0.5 mM benzamidine (all from Sigma-Aldrich); 10 µg/ml each of aprotinin (Sigma-Aldrich), leupeptin, and pepstatin A (Calbiochem, Bad Soden, Germany); and one protease inhibitor tablet (Roche)). Cytosolic extracts were obtained by centrifugation of lysed PMN at 13,000 × g. The content of active caspase-3 was analyzed by Western blot after separation of 25 µg of protein/lane on a 12% SDS-PAGE and blotting to polyvinylidene difluoride membrane. The mAb recognizing both active caspase-3 (17 kDa) and procaspase-3 (32 kDa; clone 31A1067, mouse IgG1) was purchased from Biocarta (Hamburg, Germany).

**Cytokine assays**

Neutrophil granulocytes were coincubated with *Cp* (see above), and culture supernatants were collected after 18, 42, 66, and 90 h. Supernatants were stored at −20°C until cytokine determination. IL-8 and GM-CSF were measured using ELISA (R&D Systems, Wiesbaden, Germany) according to the manufacturer’s instructions.

**Results**

**Neutrophil granulocytes internalize *Cp* in vitro**

Purified human PMN were coincubated in vitro with *Cp*. Fig. 1A shows that numerous bacteria were taken up after 42 h of coincubation. The morphology of intracellular *Cp* in PMN was analyzed using immunoelectron microscopy. Immunogold staining with anti-*Cp* LPS Ab clearly showed that *Cp* was intracellular (Fig. 1C). Infected PMN showed morphologically intact, pear-shaped elementary bodies after 42 h of coincubation (Fig. 1D).

**Survival and replication of *Cp* in human PMN**

Immunofluorescent staining with FITC-conjugated anti-*Cp* mAb was applied to demonstrate the intracellular presence of *Cp* 18 and 90 h after infection. After 18 h of PMN-*Cp* coincubation, 81 ± 11% of PMN contained one or more positively stained structures, with an average of 12 intracellular *Cp* (n = 6; range, 6–30) per cell. During prolonged culture, the ratio of *Cp*-positive PMN remained constant; however, the intensity of intracellular fluorescence appeared to increase during the 90-h incubation (Fig. 2).

To quantify the bacterial load, the amount of *Cp* LPS was measured in infected PMN by ELISA (28). Infected PMN were lysed 18 and 90 h after infection and analyzed for *Cp* LPS. A 5-fold increase in chlamydial load was observed in PMN 4 days after infection (Fig. 2). This finding indicates that *Cp* not only survives, but also multiplies in PMN.

**FIGURE 1.** Neutrophil granulocytes internalize *Cp* in vitro. Freshly isolated human neutrophil granulocytes were coincubated in vitro with *Cp* in a 1:1 ratio (A) or in medium alone (B) for 42 h, cells were fixed with methanol, stained with FITC-labeled *Cp* LPS-specific mAb and counterstained with Evans blue. Original magnification, ×1,000. Shown is an electron micrograph of PMN coincubated with *Cp* for 42 h. Cells were fixed with 0.1% glutaraldehyde and 2% paraformaldehyde, and ultrathin sections were stained using an *Cp* LPS-specific mAb and Immunogold. Bars = 0.2 µm (C). Electron micrograph of PMN coincubated with *Cp* for 42 h were fixed with 2.5% glutaraldehyde and 1% OsO4 (D). Representative micrographs of three independent experiments are shown.

**FIGURE 2.** *Cp* multiplies inside PMN. Freshly isolated PMN were coincubated in vitro with *Cp* in a 1:1 ratio or in medium alone. Upper panels, PMN were coincubated with *Cp* for 18 or 90 h, fixed with methanol, and stained with FITC-labeled *Cp* LPS-specific mAb and counterstained with Evans blue (magnification, ×1000). Lower panel, Number of *Cp* in PMN after 18 and 90 h of coculture with *Cp*, as measured with *Cp*-specific LPS ELISA. The data represent the mean ± SD for three independent experiments.
A two-color immunofluorescent staining (live/dead staining) was used to investigate Cp viability in PMN. After staining with SYTO 16 (green) and ethidium bromide (red), viable cells and bacteria show green staining, whereas dead cells with compromised membranes stain red. This staining demonstrated that after 90 h of coincubation, both PMN and the ingested bacteria were viable (green staining; Fig. 3A) In contrast, all PMN cultured in medium alone were dead and stained red after 42 h (Fig. 3B).

Infection with Cp leads to delayed apoptosis of PMN

The live/dead staining of infected PMN clearly indicated that not only the bacteria, but also the infected PMN, remained viable and morphologically intact up to 90 h (Fig. 2). This finding was unexpected, because PMN are cells with a short life span, undergoing rapid apoptosis, with classical features, such as cell shrinkage, cytoplasmic condensation, and condensation, of nuclear heterochromatin (13–15). Using these criteria the ratio of apoptotic cells was determined in PMN cultured in vitro in the absence or the presence of Cp. After 18 h, 73 ± 14% (n = 6; range, 54–91%) of PMN cultured in medium alone had apoptotic nuclear morphology (Fig. 4). In contrast, the rate of apoptosis was strongly reduced when PMN were coincubated with Cp. Only 16 ± 6% of the cells (n = 6; range, 8–25%) had apoptotic morphology after 18 h of coincubation (Fig. 4). Mock-infected HEp-2 lysates did not reduce the apoptotic rate of PMN. In PMN-Cp cocultures most of the apoptotic PMN seen after 18 h were those without intracellular Cp (data not shown). These data thus suggest Cp protected neutrophils from apoptosis without evidence of significant necrotic death as assessed by trypan blue exclusion (not shown).

Visible changes of nuclear morphology is associated with the progressed stage of cellular apoptosis. An earlier marker of PMN apoptosis is the appearance of phosphatidylserine (PS) on the outer membrane, a process that is called membrane-flip. PS can be detected by staining with annexin V. Annexin V staining revealed high cell surface PS expression on most neutrophils in noninfected cultures after incubation in vitro for 42 h (Fig. 5A). Coincubation with Cp resulted in a marked decrease in annexin V binding (Fig. 5A). Staining for necrosis using PI showed that Cp infection does not increase the level of necrosis (Fig. 5A).

In addition to the staining with annexin V and PI, the inhibitory effect of Cp on neutrophil apoptosis was investigated by the TUNEL assay, which reveals the apoptotic fragmentation of nuclear DNA. In medium alone, most PMN became TUNEL-positive during the 42-h incubation period (Fig. 5B). In the presence of Cp, most PMN remained TUNEL-negative (Fig. 5B).

In addition to spontaneous apoptosis, PMN can also undergo apoptosis induced by external signals, such as after cross-linking of Fas (CD95) on their surface (31). We could demonstrate that Fas cross-linking with an anti-Fas Ab resulted in 94 ± 1% (n = 4) apoptotic PMN after 18 h. Preincubation of PMN with Cp for 3 h before anti-Fas treatment decreased the ratio of apoptotic cells to 72 ± 2% (n = 4).

Coincubation with Cp results in the inhibition of procaspase-3 processing in PMN

Caspase-3 is one of the key enzymes involved in spontaneous apoptosis of neutrophils (32). Using Western blot techniques, we found that fresh PMN contained high levels of procaspase-3 (32...
kDa; Fig. 6). Processing of procaspase-3 in PMN cultured in medium alone led to the appearance of active caspase-3 (Fig. 6). Coincubation with \( Cp \) resulted in the complete inhibition of pro-caspase-3 processing (Fig. 6).

**Coincubation with \( Cp \) induces the release of IL-8 by PMN**

Chemokine production by PMN is thought to affect the inflammatory process by recruiting or activating various leukocyte populations. As the primary target cells of IL-8 are PMN, the production of this chemokine by inflammatory granulocytes appears to serve as an amplifying loop, attracting more PMN to the site of inflammation. Additionally, IL-8 and GM-CSF have been reported to inhibit the spontaneous apoptosis of PMN (16, 17).

IL-8 and GM-CSF were measured in supernatants of PMN cultures after coincubation with different doses of \( Cp \). Eighteen hours after coincubation, \( Cp \) induced a dose-dependent release of IL-8 (Fig. 7). The time kinetics of IL-8 induction by \( Cp \) were investigated using PMN to \( Cp \) IFU ratio of 1:1, with mock-infected HEp-2 cell lysates, or in medium alone. The IL-8 content of the supernatants was measured at the given time points using ELISA. The figure shows the mean ± SEM of duplicate assays for each condition obtained from three independent experiments.

**Heat-killed \( Cp \) and LPS molecules mediate an early antiapoptotic effect up to 42 h after PMN contact**

LPS has been demonstrated to inhibit the spontaneous apoptosis of PMN (18). To study the effect of *Chlamydia* LPS on apoptosis inhibition, we used both heat-killed \( Cp \) and purified *C. trachomatis* LPS, at present the only biological active purified form of chlamydial LPS available. *C. trachomatis* LPS also reduced the spontaneous apoptosis of PMN in a dose- and time-dependent manner (Fig. 8A). In addition we tested the antiapoptotic activity of LPS from other bacteria. In addition to *Ct* LPS and heat-killed \( Cp \),
Salmonella enterica (Se) LPS also exerted an antiapoptotic effect (Fig. 8B). Similarly to Cp, Se is also an intracellular bacterium. Moreover, Se LPS has structural similarities to Cp LPS (33). To investigate whether the antiapoptotic effect of LPS is specific for intracellular bacteria, we tested LPS from a typical extracellular bacterium Ec. We found that LPS derived from Ec also delayed PMN apoptosis (Fig. 8B). This observation was in concordance with the literature (18). For all the different LPS molecules (tested in the same concentration) and the heat-killed Cp, the antiapoptotic effect was strongest after 18 h (Fig. 8B).

Recombinant human IL-8- and IL-8-containing supernatants of PMN-Cp cocultures inhibit spontaneous PMN apoptosis

IL-8 has been demonstrated to inhibit PMN apoptosis (34). Therefore, we tested whether the amounts of IL-8 produced by PMN after Cp infection were able to inhibit PMN apoptosis in an autocrine fashion. Recombinant human IL-8 reduced the spontaneous apoptosis of PMN in a dose-dependent manner, first apparent at a concentration of 25 ng/ml and maximal at 200 ng/ml (data not shown). Supernatants taken from PMN-Cp cocultures 42 h after infection, containing ~50 ng/ml IL-8 or 90 h after infection containing ~100 ng/ml IL-8, exerted the same antiapoptotic activity on fresh PMN as 50 ng rhIL-8 (Fig. 9A). Supernatants taken from PMN cultured in medium alone had no antiapoptotic effect (not shown).

To investigate whether IL-8 was responsible for the antiapoptotic effect of the supernatants, IL-8 was depleted from supernatants taken from PMN-Cp cocultures 42 and 90 h after infection. Using immunoprecipitation with anti IL-8 mAb and protein G-agarose, the IL-8 concentration was reduced from 51.2 ± 5 to

**FIGURE 8.** Heat-killed Cp and LPS molecules reduce spontaneous PMN apoptosis. A, Freshly isolated PMN were coincubated in vitro with viable Cp at a 1:1 ratio, in medium alone, or with 1, 0.1, or 0.01 μg/ml purified C. trachomatis LPS (Ct LPS). The percentage of apoptotic PMN at the indicated time points was determined by microscopic evaluation of >200 cells on cytocentrifuge preparations stained with Giemsa. B, Freshly isolated PMN were coincubated in vitro with either viable or heat-killed Cp at a 1:1 ratio, in medium alone, or with 0.1 μg/ml Ct LPS, 0.1 μg/ml Se LPS (Se LPS), or 0.1 μg Ec (Ec LPS) of purified C. trachomatis LPS (Ct LPS). The percentages of apoptotic PMN after 18 h (■), 42 h (□), and 66 h (□) were determined by microscopic evaluation of >200 cells on cyt centrifuge preparations stained with Giemsa. The data represent the mean ± SD for three independent experiments.

**FIGURE 9.** Cp-induced IL-8 reduces spontaneous PMN apoptosis. A, Freshly isolated PMN were coincubated in vitro with Cp in a 1:1 ratio, in medium alone, with rhIL-8 (50 ng/ml), or with supernatant taken 42 or 90 h after Cp-PMN coculture (Cp sup). Cytocentrifuge preparations were stained with Giemsa, and the percentage of apoptotic PMN was determined by microscopic evaluation of >200 cells. Data are representative for three independent experiments. B, Freshly isolated PMN were coincubated in vitro in medium alone, with Cp in a 1:1 ratio, with supernatant taken 42 or 90 h after Cp-PMN coculture (Cp-PMN SN), with these same supernatants depleted for IL-8 using a polyclonal anti-IL-8 serum and protein G immunoprecipitation, or with a depletion control. The efficiency of IL-8 depletion was assessed using IL-8 ELISA (inset). The data represent the mean ± SD for three independent experiments. *, p < 0.05; **, p < 0.005 (by paired two-tailed Student’s t test).
In this study we describe that neutrophil granulocytes (PMN) can serve as host cells for \( C. \) pneumoniae. We demonstrated that \( C. \) pneumoniae, after being phagocytosed by human PMN, remain viable and multiply intracellularly. In addition, we showed an inhibitory effect of \( C. \) pneumoniae on the spontaneous apoptosis of neutrophil granulocytes mediated by chlamydial LPS and the autocrine production of IL-8.

During intracellular growth of \( C. \) pneumoniae, maintenance of host cell integrity is essential not only for supplying nutrients, but also for shielding the intracellular organisms from antimicrobial serum factors. Although \( C. \) pneumoniae is known to preferentially infect the epithelial tissue of the respiratory tract, this bacterium can also multiply in vitro in monocytes/macrophages, T lymphocytes, endothelial cells, and aortic smooth muscle cells (28, 35).

Epithelial cells represent the first cellular barrier for \( C. \) pneumoniae in the lung. In vitro studies demonstrated that \( C. \) pneumoniae can infect both primary human airway epithelial cells and the bronchial epithelial cell line BEAS-2B and induce a massive production of IL-8, a chemokine acting primarily on PMN (8, 36). Consistent with these data, it was demonstrated in animal models that PMN are the first leukocytes recruited to the site of \( C. \) pneumoniae infection in the lung (37, 38). As professional phagocytes, PMN play an important role in the early defense against infections. Both opsonin-dependent and opsonin-independent uptakes of microorganisms by PMN have been described (39–42). PMN have been reported to ingest \( C. \) psittaci and \( C. \) trachomatis elementary bodies, partially by opsonin-independent uptake mechanisms (11). In our study we also observed an opsonin-independent uptake of \( C. \) pneumoniae by PMN, resulting in 80% infected PMN after 18 h.

Upon cell entry, \( C. \) pneumoniae starts the formation of a nonacidified host vacuole, termed inclusion; in this study chlamydial vacuoles have a unique intracellular biphasic life cycle. The spore-like elementary bodies facilitate transfer between cells. The metabolically active reticulate bodies are responsible for intracellular replication (43). In our present study micrographs taken 18, 42, and 90 h after infection demonstrated the presence of such inclusions inside PMN. The inclusions containing reticulate and elementary bodies were, however, atypical compared with inclusions in epithelial cells, but were similar to those found in monocytes (6). This suggests that the formation of inclusions is an important mechanism of \( C. \) pneumoniae to evade killing by neutrophil granulocytes.

After an inclusion is formed in a susceptible target cell, the chlamydial growth cycle can be initiated. Elementary bodies develop into reticulate bodies (44, 45). The length of the complete developmental cycle, as studied in cell culture models, is 48–72 h (46, 47). In concordance with these data, we observed chlamydial survival and multiplication during the extended PMN life span of up to 90 h, suggesting that a complete developmental cycle may take place inside PMN. Our results indicate that \( C. \) pneumoniae has evasion mechanisms capable of escaping elimination by PMN and clearly show that PMN can serve as a host cell for \( C. \) pneumoniae.

Importantly, survival and multiplication of \( C. \) pneumoniae in PMN are accompanied by the survival of host PMN after infection. As PMN normally undergo spontaneous apoptosis, this means that \( C. \) pneumoniae has evolved mechanisms that allow it to actively interrupt the apoptotic process. A number of pathogens are known to alter apoptotic thresholds in neutrophils. Most of them, however, induce apoptosis to escape neutrophil-mediated destruction (22, 23). In contrast, we demonstrated that \( C. \) pneumoniae can manipulate neutrophils, making them suitable host cells for its multiplication. \( C. \) pneumoniae-induced delay of apoptosis extends the life span of neutrophils, which is then used by this obligate intracellular pathogen for its own survival. Recent literature gives several examples of \( C. \) pneumoniae-specific inhibition of host cell apoptosis induced by a wide spectrum of apoptotic stimuli. Fan et al. (48) reported that host cells infected with \( C. \) trachomatis are profoundly resistant to apoptosis induced by the kinase inhibitor staurosporin, the DNA-damaging agent etoposide, and several immunological apoptosis-inducing molecules, such as TNF-\( \alpha \), anti-Fas Ab, and granzyme B/perforin. In addition, \( C. \) pneumoniae was reported to delay staurosporin- or anti-Fas-induced apoptosis of HeLa cells (49) and 8-methoxypsoralen plus hypericin-induced apoptosis of human monocytes (50). In contrast to the above-described host cells that undergo apoptosis only after external induction, PMN are intrinsically short-lived cells that undergo apoptosis without known external stimuli, a process called spontaneous apoptosis. Until now, the intracellular parasite \( L. \) major and the agent of HGE were the only known microorganisms able to inhibit this spontaneous apoptosis of PMN (9, 10). Using morphological analysis and annexin V binding as well as by detecting the apoptosis-associated DNA fragmentation, we demonstrated that infection of PMN with \( C. \) pneumoniae also leads to the inhibition of their spontaneous apoptosis.

Infection of PMN with \( L. \) major was found to inhibit the activation of caspase-3 (10), which is associated with the spontaneous apoptosis of PMN (51). Freshly isolated PMN contain high levels of procaspase-3, which is cleaved during spontaneous apoptosis, giving rise to the enzymatically active caspase-3 (52). In our present study we demonstrated that \( C. \) pneumoniae affects the survival of PMN via a mechanism that involves the inhibition of procaspase-3 cleavage. The \( C. \) pneumoniae-mediated delay of neutrophil apoptosis is, therefore, associated with a decrease in caspase-3 activity in infected PMN.

The mechanism of how HGE agents can prolong the life span of PMN is not known. It was not ruled out that HGE-derived LPS could be responsible for the observed effect (9). Our present data showed that LPS plays an important role in \( C. \) pneumoniae-mediated inhibition of PMN apoptosis. We observed that not only viable, but also heat-killed, \( C. \) pneumoniae had an antiapoptotic effect. Moreover, chlamydial LPS inhibited the apoptosis of PMN as well. \( C. \) pneumoniae LPS has been characterized as having a rough phenotype that has a genus-specific epitope(s), and it is similar to the LPS of \( S. \) enterica, as it has the core lipid A moiety and 3-deoxy-o-manno-oct-2-ulosonic acid (Kdo) core, but lacks the distal O-polysaccharide region (33). Due to technical difficulties, to date no biologically active \( C. \) pneumoniae LPS has been available. Therefore, we used both \( S. \) enterica LPS and \( C. \) trachomatis LPS and observed a delay in the spontaneous apoptosis of PMN. This LPS-mediated antiapoptotic effect was most prominent up to 42 h after LPS-PMN contact.

Although apoptosis is an intrinsic cell process, it can also be modulated by cytokines such as GM-CSF and IL-8, which were both shown to delay the spontaneous apoptosis of PMN in a dose-dependent fashion (17, 53). Although upon stimulation PMN themselves are able to secrete both these antiapoptotic cytokines, production of GM-CSF was not observed after \( C. \) pneumoniae infection. However, PMN produced high amounts of IL-8 upon coculture with \( C. \) pneumoniae, especially at later time points. Subsequently, we could show that the IL-8-containing supernatants had a strong antiapoptotic effect on freshly isolated neutrophils. Depletion of IL-8 from supernatants taken 90 h after \( C. \) pneumoniae cocultures, containing high levels of IL-8,
increased the antiapoptotic effect of these supernatants significantly. These data indicate that the antiapoptotic effect of Cp is at least partially mediated by the autocrine production of IL-8 by PMN.

Bacterial LPS was reported as a potent inducer of IL-8 production by PMN (54). In the present study Chlamydia LPS induced also IL-8 production by PMN (data not shown). Heat-killed Cp induced IL-8 production of PMN. These data indicate that LPS plays a major role in Cp-induced IL-8 release by PMN. Taken together, the data suggest that Chlamydia LPS effects PMN apoptosis both by acting directly on the cells and by inducing the autocrine production of IL-8. At early time points after infection LPS mediates the antiapoptotic effect on PMN, at later time points LPS-mediated IL-8 release seems to govern the antiapoptotic effect.

Intracellular parasites have evolved diverse mechanisms to enhance their survival and replication in host cells (55). In this study we have shown that the obligate intracellular parasite Cp can infect and multiply in PMN, one of the first line of cellular defense against pathogenic microorganisms. Infection with Cp leads to the extended life span of PMN. These data suggest that PMN serve as host cells for intracellular survival of Cp within the first days/after days of infection.

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References


