Degradation of *Chlamydia trachomatis* in Human Polymorphonuclear Leukocytes: an Ultrastructural Study of Peroxidase-Positive Phagolysomes

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We have previously shown that human polymorphonuclear leukocytes (PMNs) killed organisms belonging to both human biovars of *Chlamydia trachomatis*. However, the mechanism of destruction was still unclear. We therefore conducted an ultrastructural and cytochemical study to investigate the mechanism of chlamydial degradation. PMNs were inoculated with the trachoma serovar B (B/TW-5/OT) or with the lymphogranuloma venereum serovar L2 (L2/434/Bu) for 15, 30, 60, or 120 min and then fixed and processed for transmission electron microscopy. Diaminobenzidine, a cytochemical marker, was used to demonstrate the localization of intracellular peroxidase. Ultrastructural evidence is presented showing the progressive degradation of chlamydiae over a 2-h period within peroxidase-positive phagolysosomes. Pretreatment of organisms with normal or immune serum was not required for the process of degradation.

**Materials and Methods**

*C. trachomatis* strains. Two serovars from both human biovars of *C. trachomatis*, the trachoma serovar B/TW-5/OT and the lymphogranuloma venereum serovar L2/434/Bu, were studied. As previously described, these organisms were grown in HeLa 229 cell cultures (9), purified through a Renografin linear gradient (7), and stored at −70°C until used.

PMN system. PMNs were obtained from normal human volunteers. Venous blood was collected in EDTA (0.2 ml of 10% dipotassium EDTA in 10 ml of blood), and the PMNs were purified by centrifugation through a Ficoll-Paque gradient (Pharmacia, Piscataway, N.J.), followed by dextran sedimentation and hypotonic lysis to eliminate erythrocytes (1). PMN suspensions containing 10⁶ cells in 1.0 ml of Hank's balanced salt solution and 5 × 10⁶ C. trachomatis organisms were incubated together with or without serum as specified in a polypropylene tube in a 37°C water bath for 15, 30, 60, or 120 min before processing for electron microscopy.

TEM. PMNs with or without *C. trachomatis* were processed by a previously described procedure (3). Briefly, the cells were fixed for 15 min in 1% glutaraldehyde buffered with 0.1 M cacodylate at pH 7.4, washed with cacodylate buffer, postfixed with OsO₄, dehydrated by treatment with a series of alcohols, embedded in Epon, and sectioned for transmission electron microscopy (TEM). To demonstrate the cytochemical localization of peroxidase, after glutaraldehyde fixation, the cells were incubated at room temperature for 15 min with 0.5 mg/ml per ml of 3,3’-diaminobenzidine HCl (DAB) (10 mg/ml; Scientific Instruments, Huntington Beach, Calif.), with 1% H₂O₂ added to start the reaction (5). H₂O₂ was omitted from control samples. Uranyl acetate and lead citrate were omitted from most of the samples treated with DAB. The preparations were viewed on a JEOL electron microscope at 60 kV.

Human sera. Human sera were obtained from the serum bank of S.-P. Wang, Department of Pathobiology, University of Washington. These sera were either from patients with confirmed *C. trachomatis* infections or from control normal volunteers. The anti-chlamydia antibody titers of the patients’ sera as measured by the microimmunofluorescence test (12) were 1:128 (serovar B sera) and 1:512 (serovar L2 sera). Normal sera contained no antibody against any of the 15 known serovars of *C. trachomatis*. Chlamydial organisms were incubated either with normal serum or with the immune serum of their respective serovars for 30 min at 35°C before they were used to inoculate PMNs. The sera were not heat inactivated.

**Results**

Control PMNs. Uninoculated PMNs treated with DAB, a substrate for peroxidase, reacted by demonstrating the localization of peroxidase through the appearance of densely stained granules (Fig. 1). They did not change in appearance over the 2-h experimental period.

Inoculated PMNs. PMNs were inoculated with either tra-
cholera or lymphogranuloma venereum elementary bodies (EBs) without serum and prepared for ultrastructural observation at various intervals after infection. Both trachoma and lymphogranuloma venereum serovars produced the same results. At 15 min postinoculation (Fig. 2A and B), many chlamydiae were intracellular, with approximately half of the intracellular EBs within phagosomes and the other half within phagolysosomes. Most phagosomes and phagolysosomes contained a single EB, although some contained more than one. The intraphagosomal EBs were well circumscribed within their respective phagosomes. Moreover, EB membranes were discernible and distinguishable from the phagosomal membranes in singly or multiply occupied phagosomes. The intraphagolysosomal chlamydiae were in various early stages of degeneration. At these stages the chlamydiae were still intact within a phagosome. Many peroxidase-positive granules were located around the phagosome, and peroxidase was detected around chlamydial membranes and in the phagosomal vacuoles surrounding the chlamydia. At 30 min, the results were similar to those at 15 min, although more chlamydiae appeared to be in phagolysosomes than in phagosomes. At 1 h (Fig. 2C), most organisms were in phagolysosomes and many were at later stages of disintegration where only debris could be seen. Some phagolysosomes appeared to be similar to the ones observed at 15 and 30 min; however, some phagolysosomal vacuoles were now empty. At 2 h (Fig. 2D), all PMNs contained only phagolysosomes and most of the phagolysosomal vacuoles were empty. Some empty vacuoles contained peroxidase-positive dots and blebs.

**Serum-treated organisms.** To study the effects of antibody and opsonin on uptake and toxicity, EBs were pretreated with normal or immune serum before inoculating the PMNs. Similar events were observed at the various time intervals regardless of the pretreatment. However, phagosomes and phagolysosomes containing more than one organism were seen more frequently when the EBs had been pretreated with normal immune serum.

**DISCUSSION**

We have presented ultrastructural evidence showing that over a 2-h period the peroxidase-positive lysosomal granules

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**FIG. 1.** TEM of uninoculated PMNs. The cells were incubated with DAB. Uranyl acetate and lead citrate were omitted from the TEM preparations. Numerous peroxidase-positive granules appear as a result of the reactions to DAB. Bar, 10 μm.

**FIG. 2.** TEMs of the progressive degradation of serovar B *C. trachomatis* EBs in PMNs. Inoculated PMNs were incubated with DAB. Uranyl acetate and lead citrate were omitted from the preparations. (A and B) Chlamydiae in early stages of degeneration at 15 min postinoculation. Approximately half of the intracellular EBs were in phagosomes (black arrows), and the other half were in phagolysosomes (white arrows). Peroxidase-positive granules surrounded the chlamydial phagolysosome, and peroxidase was detected around chlamydial membranes and in the phagosomal vacuoles surrounding the chlamydia (white arrows). Panel B is a photomicrograph of a portion of the PMN shown in panel A but at a higher magnification. (C) Chlamydiae in intermediate stages of degeneration at 1 h postinoculation. Most EBs were in phagolysosomes (white arrows). (D) Chlamydiae in late stages of degeneration at 2 h postinoculation. Most phagolysosomal vacuoles were empty (V). Some empty vacuoles contained peroxidase-positive blebs and dots. Magnifications: A, ×12,000; B, ×24,000; C, ×20,000; D, ×18,000. Bars, 10 μm.
of inoculated PMNs degranulated into phagosomes containing chlamydiae and that progressive degradation of the organisms followed. Phagolysosomes are commonly utilized by PMNs for microbial degradation following degranulation (6). However, observations based on a HeLa cell assay of viable chlamydiae in PMNs suggested that few organisms survived (15). The ultrastructural observations presented here support these observations, as do our in vitro observations showing that the cell-free myeloperoxidase (MPO) system, which consists of MPO, H2O2, and a halide, killed chlamydiae. The localization of peroxidase within chlamydial phagolysosomes demonstrated peroxidase involvement in the degradation process. Furthermore, the MPO system was probably also involved. The involvement of peroxidase did not adequately explain the data obtained with MPO-deficient PMNs and with PMNs from chronic granulomatous disease (CGD) patients. The former lack MPO, and the latter lack H2O2 and other oxygen-dependent antimicrobial mechanisms (8). However, both types of PMNs were chlamydicidal. Although the exact bactericidal mechanisms employed within the phagosomes were not studied, our ultrastructural observations were also compatible with chlamydial degradation by oxygen-independent systems, since these systems are composed of the enzymes which reside in the lysosomal granules of the cells (8). It therefore seems likely that phagolysosomal fusion had to occur in PMNs from patients with CGD since these cells employ only oxygen-independent antimicrobial systems. In addition, PMNs from MPO-deficient patients probably also utilized oxygen-dependent systems, since they were more chlamydicidal than the CGD PMNs with organisms from the trachomatis biovar (15). However, our experiments conducted with PMNs from both types of patients, intracellular chlamydial events within these cells will remain unknown.

Both our previous findings and our current findings agree with those of Zvillich and Sarov (16). They found that at a PMN/chlamydia ratio of 1:1 (lymphphgranuloma venereum biovar), a 10^-3 reduction in viable count was obtained when PMNs were allowed to interact with chlamydial organisms for 24 h either in the presence or in the absence of specific antibody. At that ratio, we observed a 10^-1 reduction after an interaction time of only 1 h (15). Zvillich and Sarov also observed ultrastructurally progressive degradation of intraphagosomal organisms over time. EBs appeared to be degenerating in phagocytic vacuoles 30 min after inoculation. After 24 h, many vacuoles appeared to be empty; this observation is indicative of completely degraded organisms. The issue of phagolysosomal fusion was not addressed, nor was a cytochemical marker used. Zvillich and Sarov also observed more multiply occupied vacuoles in cells which had been infected with serum-treated EBs than in those cells which had been infected with untreated organisms. However, multiple occupation did not alter the kinetics of toxicity or the amount of killing which occurred. Zvillich and Sarov (16) and Soderlund et al. (11) observed increased chemiluminescent response in chlamydial organisms treated with antibody. However, Zvillich and Sarov also found that the stronger chemiluminescent response did not correlate with toxicity. The lack of requirement for opsonin for uptake and killing observed by Zvillich and Sarov and ourselves differs from the findings of Hammerschlag et al. (6), who obtained a stronger chemiluminescent response in PMNs when EBs had been treated with serum. Neither specific antibody nor complement was required for the enhanced response. Furthermore, they found that chlamydial entry into PMNs seldom occurred without serum. However, in the absence of data on cytotoxicity the significance of the ultrastructural evidence they presented cannot be assessed. It is possible that a higher chemiluminescent response could occur independently of phagocytosis or killing. Moreover, chlamydiae may be different from other bacteria in not requiring opsonins, since they are known to stimulate a parasite-directed phagocytosis (2).

The present study clearly demonstrates the degradation of C. trachomatis in the phagolysosomes of human PMNs. Prior to this study, only chlamydial organisms which had been pretreated with heat or antibody were unable to evade phagolysosomal fusion in both nonphagocytic (2, 4) and phagocytic (13, 14) cells. Human PMNs are the only cells reported to date in which viable chlamydiae do not evade the fusion of phagosomes with lysosomes.

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LITERATURE CITED


