Inhibition of Human Neutrophil NADPH Oxidase by Chlamydia Serovars E, K, and L₂

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Received 11 October 1988/Accepted 27 December 1988

The effects of Chlamydia trachomatis (serovars E, K, and L₂) on human neutrophil activation were examined with respect to the organisms both as primary agonists and as agents that modulate cell responses to a second stimulus. Unopsonized chlamydiae alone, at ratios of 1.5 to 100 organisms per cell, failed to elicit changes in intracellular calcium or membrane depolarization or to stimulate the respiratory burst or degranulation during 60 min of incubation. Each of these functions except the respiratory burst was also normally activated when chlamydia-infected neutrophils were subsequently stimulated with formylmethionyl leucine phenylalanine or phorbol myristate acetate; the respiratory burst was inhibited 30 to 65%. Inhibition was dependent on live organisms and was maximal within 5 min of incubation. The organisms had no effect on the superoxide (O₂⁻) assay, and the site of chlamydial inhibition was determined at the level of the NADPH oxidase itself, not at an intermediate step in the activation cascade. The mechanism of enzyme inactivation could not be determined. These results show that unopsonized chlamydiae do not elicit responses from infected neutrophils and suggest that microbial mechanisms other than those dependent on elaboration of toxic oxygen-derived species are required to inactivate chlamydiae.

The ability of neutrophils to respond to physiological agonists depends in large measure on their prestimulated state (A. I. Tauber, A. B. Karnad, K. L. Hartshorn, J. B. Myers, and J. H. Schwartz, in A. I. Tauber, B. Wintrouble, and A. Simons, ed., Biochemistry of the Acute Allergic Reaction, in press). The activity whereby phagocytes may exhibit augmented functional responses when preexposed to subthreshold concentrations of activating ligands is termed priming. The counterpart of priming is deactivation, in which phagocytes are rendered incapable of a normal response after being preexposed to an agonist that, in eliciting a response, limits the subsequent functional activities. We have sought to establish the metabolic basis of deactivated cells and have shown that in influenza virus-infected neutrophils, depressed Ca²⁺ responses upon formylmethionyl leucine phenylalanine (FMLP) stimulation correspond to a deactivated response, with arachidonic acid release and O₂⁻ generation impaired 40 to 70% (8). In the study reported here, we extended this research by using three serovars of Chlamydia trachomatis. These experiments were initiated because of reported findings that fusion of macropore granules to vacuole-containing chlamydiae (Chlamydia psittaci) is impaired (7, 27), a phenomenon also noted in influenza virus-infected neutrophils (1). The chlamydia-neutrophil model was therefore studied to contrast and compare this model with virus-infected phagocytes, which are dysfunctional because of abnormal calcium metabolism, and gain insight into the mechanism by which chlamydiae may evade phagocyte killing. Although both C. trachomatis and C. psittaci elementary bodies are efficiently internalized by human neutrophils in the absence of antibody and complement, a small percentage nevertheless escape the microbial mechanisms of the cell and remain infectious (17). The basis of this escape has not been elucidated but is a likely source of persistent infection.

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MATERIALS AND METHODS

Reagents. FMLP, cytochalasin B, superoxide dismutase, ferricytochrome c, and ethylene glycol-bis(β-aminoethyl ether)-N,N′,N″,N″-tetraacetic acid (EGTA) were obtained from Sigma Chemical Co. (St. Louis, Mo.). Ficoll-Paque and dextran citrate were purchased from Pharmacia Fine Chemicals (Piscatway, N.J.). Fura-2/AM, pentaacetylmyristyl ester, was purchased from Calbiochem-Behring (La Jolla, Calif.). 3,3'-Dipentyloxacarbocyanine [Di-O-C₃(3)] was obtained from Molecular Probes (Eugene, Ore.).

Chlamydia preparation. C. trachomatis strains E/WW-5/OX and K/WW-31/CX (from R. Barnes, Centers for Disease Control, Atlanta, Ga.) and L2/434 (from the American Type Culture Collection, Rockville, Md.) were grown in McCoy monolayers in 150-cm² tissue culture flasks. Serovars E and K were grown in cells pretreated with DEAE-dextran (30 μg/ml) in HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-sucrose-calcium (HSC) buffer (10 mM HEPES, 1 mM MgCl₂, 90 mM NaCl, 1.5 mM CaCl₂, 0.2 M sucrose) (10). The organisms were purified by differential centrifugation followed by centrifugation through 32% Renografin (Squibb Diagnostics, New Brunswick, N.J.) in HSC buffer (24). Infectivity titers were determined by titration of chlamydiae in 1-dram (ca. 1.8-g) vials containing McCoy cells. Direct immunofluorescence microscopy (Microtrak, culture confirmation test; Syva) was performed to identify inclusion bodies, which were expressed as inclusion-forming units as a measure of infectivity. The purified elementary bodies had a specific infectivity of 1 × 10⁷ to 4 × 10⁷ inclusion-forming units per μg of protein (protein was measured by the method of Lowry et al. [11]). Additional purification with discontinuous gradients of Renografin (26) was not required, since such preparations exhibited the same effects as did less purified chlamydiae, as assessed in parallel experiments (data not shown).

Equal portions of purified elementary bodies were stored at −70°C. Before the experiments, the stored organisms were thawed, washed once with phosphate-buffered saline

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(PBS; 0.01 M phosphate [pH 7.2]), and resuspended in PBS at a concentration of 10^6 inclusion-forming units per ml for use in functional assays. Killed organisms, used as controls, were prepared by heating elementary bodies to 60°C for 30 min.

**Neutrophil function assays.** Neutrophils were isolated from normal volunteers by erythrocyte sedimentation in dextran-citrate followed by hypotonic lysis and Ficoll-Paque (Pharmacia) density gradient centrifugation as previously described (3). The isolated neutrophils were then suspended in PBS containing calcium and magnesium (140 mM NaCl, 3 mM KCl, 1 mM K_2HPO_4, 1 mM CaCl_2, 1 mM MgCl_2, 5 mM glucose [pH 7.4]) and maintained at 4°C until studied. Counts of viable cells were obtained by using trypan blue exclusion. Chlamydial infection had no effect on cell viability during the time span of these experiments.

**Superoxide production.** Superoxide generation was measured in all cases by assay of the superoxide dismutase-inhibitable reduction of ferricytochrome c as previously described (8). In some experiments, chlamydiae (at various concentrations as specified, ranging from 100 to 1.5 organisms per neutrophil) were incubated directly with neutrophils (10^6 cells per ml) and ferricytochrome c to monitor release of O_2^- in direct response to chlamydiae. This assay, conducted in a 1.0-ml final volume, is a continuous monitor of cytochrome c reduction in a cuvette placed in a thermostatted (37°C) spectrophotometer, with a superoxide dismutase control in the reference position. The time course was examined for 60 min. The effect of chlamydial incubation on subsequent FMLP or phorbol myristate acetate (PMA) stimulation was studied similarly by preincubating organisms (number as specified for each experiment) with neutrophils (10^6/ml) for 20 min. In studies of FMLP stimulation, cytochalasin B (final concentration, 5 μg/ml) was then added to the cell mixture (10^6 cells per ml) for 5 min before addition of FMLP (final concentration, 5 x 10^-7 M), and O_2^- was measured as described above for 10 min; PMA (400 nM) stimulation was assayed in parallel in the absence of cytochalasin B. Various ratios of organisms to cells (1.5 to 100) were used as indicated.

**Degranulation.** Degranulation was assessed under parallel conditions by measurement of lysozyme released as previously described (5). Chlamydia (2.5 to 25 organisms per cell) were incubated for 20 to 60 min (as indicated) with 10^6 neutrophils in 1.0 ml of PBS. For studies in which chlamydia were preincubated with cells before FMLP stimulation, organisms were added 20 min before the addition of cytochalasin B (5 μg/ml), followed 5 min later by addition of FMLP (5 x 10^-7 M), as in the O_2^- assay. Released lysozyme was quantitated after 20 min of cell stimulation (5).

**NADPH oxidase assay.** Assessment of NADPH oxidase was done with a membrane preparation obtained from PMA-stimulated neutrophils as previously described (21). The oxidase was preincubated with organisms for 3 min at 37°C before the addition of NADPH (0.1 mM unless otherwise indicated); reduction of cytochrome c was monitored at an optical density of 550 nm as previously described (21). To assess the effect of chlamydiae on the cytochrome c assay, xanthine oxidase was added to a mixture of purine (0.1 mM) and organisms corresponding to the 50:1 chlamydia/cell ratios used in the intact cell assay, and the rate of O_2^- generation was compared with the xanthine oxidase-purine reaction rate in the absence of chlamydiae (6).

**Determination of intracellular calcium levels.** Intracellular Ca^2+ levels were determined by using the fluorescent indicator Fura-2 as previously described (8). Isolated neutrophils were incubated with the acetoxymethyl ester of Fura-2 (2.5 μM) in PBS for 1.2 h at 37°C. In some experiments, the cells were suspended in fresh buffer and fluorescence was measured after the indicated addition of chlamydiae. Alternatively, cells were suspended at 10^7/ml in fresh medium containing chlamydiae or in control buffer and incubated for 0.5 h in a 37°C water bath with slight shaking. Viable cell counts were again determined after the washing step, and cells were then resuspended in PBS at a concentration of 2 x 10^9/ml. Immediately before each assay, cells were again centrifuged within the assay cuvette and suspended in fresh buffer to remove extracellular probe. After equilibration, cells were incubated with cytochalasin B (5 μg/ml) for 5 min and then stimulated with 10^-3 M FMLP (as in the O_2^- assay described above). Base-line fluorescence determinations were made before and 5 min after addition of cytochalasin B. After addition of FMLP, the peak fluorescence level was noted. Ca^2+ determinations were made by using the ratio of the peak fluorescence at excitation wavelengths of 340 nm and 380 nm as read on a spectrofluorometer (model LS-5; The Perkin-Elmer Corp., Norwalk, Conn.). Emission wavelength was 510 nm. Equal proportions of cells treated in the same way but not loaded with probe were used to determine autofluorescence; 0.1 mM manganese was added to quench extracellular probe. The maximum quench observed was less than 2% of the signal. The Ca^2+ concentration was calculated by using a K_d (the dye-Ca^2+ dissociation constant) of 224 nM. Chlamydial preparations alone did not fluoresce at the wavelengths used in this assay. Either two or three assays were done for each group of cells incubated with chlamydiae or buffer, and the results were averaged. These mean values were then treated as single datum points in statistical calculations, in which P values were determined by the paired Student t test.

**Membrane depolarization.** The cell membrane potential was estimated from the fluorescence of neutrophils equilibrated with the probe Di-O-C_5(3) in a modification of a method used previously (15). The neutrophils were washed and preequilibrated for 5 min in the medium to be used and then added to a cuvette containing Di-O-C_5(3) to yield a final concentration of 10^6 cells per ml and 2.5 x 10^-7 M Di-O-C_5(3). The fluorescence was monitored at an excitation of 470 nm and an emission of 500 nm in a Perkin-Elmer spectrophotometer (model LS-5). The cells in the cuvette were constantly stirred with a magnetic stirrer. The stable increased fluorescence (F) after the addition of neutrophils is an indicator of cell membrane potential; once a stable value was obtained, FMLP (5 x 10^-7 M) was added. The rate of decrease in fluorescence was monitored, and the rate of change in the first 90 s was expressed as percent ΔF per minute; the maximum decrease in fluorescence was expressed as percent ΔF.

**RESULTS**

Neutrophils, when incubated with low concentrations of either dead or live chlamydiae (ratio of organisms to cells, 1.5 to 100), did not generate a respiratory burst as measured by oxygen consumption or O_2^- generation (data not shown) for up to 60 min of incubation. However, when neutrophils were preincubated with chlamydiae for 20 min and then stimulated with FMLP (at the maximal activating concentration of 5 x 10^-7 M), O_2^- production was inhibited by live organisms (Table 1). Maximal inhibition occurred by 5 min after chlamydiae were added to neutrophil suspensions and remained constant for up to 60 min. Somewhat less but
significant inhibition was noted with PMA stimulation: with a chlamydia-to-cell ratio of 25:1, serovars E, K, and L2 inhibited PMA-stimulated O$_2^-$ generation by 62 ± 9%, 60 ± 11%, and 65 ± 3% (mean ± standard deviation, n = 3), respectively. These data show that (i) inhibition was not specific to FMLP activation and (ii) that PMA bypasses the proximal activation steps of the FMLP-receptor coupled reaction (22), inhibition likely occurred at a distal site.

The ability to inhibit O$_2^-$ production initiated by FMLP stimulation was most potent with serovars E and K, whereas higher concentrations of organisms were required to achieve significant inhibition with the L2 serovar (Table 1). Dead organisms had no significant effect on O$_2^-$ production, which suggested that these findings did not result from nonspecific inhibitory effects of the organisms on the O$_2^-$ assay. We confirmed that chlamydiae had no effect on the assay per se by demonstrating that at the same chlamydial concentrations that inhibited 50% of neutrophil O$_2^-$ production, no inhibition was seen with the enzyme assay of O$_2^-$, using a xanthine oxidase-purine system (6), under conditions that generated O$_2^-$ rates comparable to those seen with FMLP-stimulated cells (data not shown).

We next examined other neutrophil functions that may be dissociated from the respiratory burst response, depolarization of the plasma membrane (15) and degranulation (5) (Table 2). In neither case was infection with chlamydiae associated with altered functional responses. These activities, O$_2^-$ generation, depolarization, and degranulation, are each dependent on mobilization of intracellular Ca$^{2+}$ in FMLP-stimulated cells (14). We confirmed that Ca$^{2+}$ mobilization was in fact unimpaired in chlamydia-infected neutrophils by examining the fluorescent response of the intracellular Ca$^{2+}$ probe Fura-2 on FMLP stimulation. Chlamydiae alone induced no Ca$^{2+}$ response (data not shown). These data and those showing comparable inhibition of O$_2^-$ production with FMLP and PMA suggested that the inhibitory effects of chlamydiae were at a late step in activation of the respiratory burst enzyme, NADPH oxidase, or that the organisms were directly inhibitory to the oxidase itself.

Thus, in an attempt to establish the basis of chlamydia-induced inhibition of O$_2^-$ production, we examined the direct effect of chlamydiae on the O$_2^-$-generating enzyme NADPH oxidase. This activity may be studied in a membrane preparation harvested from activated neutrophils (21). Serovar E, at organism/cell ratios comparable to 25:1, which reduced whole-cell O$_2^-$ production to 36% of the control value (Table 1), yielded an oxidase activity of 22.9 ± 7.8% (mean ± standard deviation, n = 3) of the control value (5.05 ± 0.84 nmol of O$_2^-$/min per 10$^6$ cells). Comparable inhibition was exhibited with serovars K and L2 in single determinations. Serovar E exhibited dose-responsive inhibition of the enzyme (Fig. 1). Inhibition could not be overcome by addition of a 100-fold excess (3 mM) of NADPH above its $K_m$ (21), and therefore we were unable to demonstrate competitive inhibition characteristics; the mechanistic basis for chlamydial inhibition of the oxidase was not examined further. These data do show, however, that O$_2^-$ generation is inhibited at the level of the NADPH oxidase and is consistent with our observation of selective inhibition of the respiratory burst, with no effect on other neutrophil functions. Comparable inhibition of the isolated oxidase and intact cellular O$_2^-$ production confirmed that chlamydiae specifically inactivate the enzyme that generates toxic oxygen-derived species.

**DISCUSSION**

Chlamydiae cause diverse clinical diseases, which are differentiated according to the serovar of the organism: endemic trachoma (serovars A, B, Ba, and C), inclusion conjunctivitis (serovars D, E, F, G, H, I, J, and K), nongonococcal urethritis, epididymitis, and a variety of pelvic inflammatory conditions (serovars D through K), and lymphogranuloma venereum (serovars L1, L2, and L3) (12, 19). These conditions are each uniquely inflammatory, exhibiting a spectrum ranging from occult disease to florid

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**TABLE 1. Effects of chlamydiae on FMLP-stimulated O$_2^-$ generation**

<table>
<thead>
<tr>
<th>Serovar</th>
<th>Chlamydia/cell ratio</th>
<th>% Control (mean ± SD, n = 5)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Live organisms</td>
<td>Dead organisms</td>
</tr>
<tr>
<td>E</td>
<td>2.5:1</td>
<td>45 ± 20, P &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>25:1</td>
<td>36 ± 26, P &lt; 0.002</td>
</tr>
<tr>
<td>K</td>
<td>2.5:1</td>
<td>48 ± 19, P &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>25:1</td>
<td>52 ± 26, P &lt; 0.016</td>
</tr>
<tr>
<td>L2</td>
<td>2.5:1</td>
<td>71 ± 25, P &lt; 0.07</td>
</tr>
<tr>
<td></td>
<td>25:1</td>
<td>55 ± 36, P &lt; 0.05</td>
</tr>
</tbody>
</table>

* Control: 6.06 ± 2.47 nmol of O$_2^-$/min per 10$^6$ cells.

**TABLE 2. Effects of chlamydiae on FMLP-induced depolarization and degranulation**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Chlamydia/cell ratio</th>
<th>Determination (mean ± SD, n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(% ΔF/min)</td>
<td>Lysozyme (% release)</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serovar</td>
<td>E</td>
<td>32.2 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>2.5:1</td>
<td>38.3 ± 9.1</td>
</tr>
<tr>
<td></td>
<td>25:1</td>
<td>34.7 ± 8.4</td>
</tr>
<tr>
<td></td>
<td>L2</td>
<td>32.3 ± 8.0</td>
</tr>
<tr>
<td></td>
<td>2.5:1</td>
<td>26.3 ± 11.9</td>
</tr>
<tr>
<td></td>
<td>25:1</td>
<td>34.0 ± 7.8</td>
</tr>
<tr>
<td></td>
<td>K</td>
<td>30.7 ± 5.5</td>
</tr>
</tbody>
</table>

FIG. 1. Inhibition of NADPH oxidase by serovar E chlamydiae. Shown is the dose-response (expressed as 10$^6$ organisms per ml) inhibition of a membrane preparation of NADPH oxidase harvested from PMA-stimulated neutrophils (see Materials and Methods for details).
that were unable is normal plasma membrane depolarization, in diverse neutrophils by responsive when stimulated by toxic particle itself (4), as chemical constituents relax from an excited electronic state. We have shown that CL under physiologic conditions is not due to singlet oxygen (a putative product of the respiratory burst [9]) as previously postulated, and the source of light emission is not known.

Although neutrophils are capable of killing both trachoma (B/TW-5/OT) and lymphogranuloma venereum (L2/434/BU) biovars rapidly (3 to 3.5 logs over 60 min at a particle/cell ratio of 1:10), the microbicidal mechanism is not clearly understood but is not likely to depend on the generation of toxic oxidative species (26). Although the myeloperoxidase system kills the organisms in vitro, myeloperoxidase-deficient neutrophils killed chlamydiae at the same rate as did normal, myeloperoxidase-containing cells (26). The neutrophils obtained from patients with chronic granulomatous disease, which do not mount a respiratory burst at all, killed biovars 434 and 434 cells normally and showed a modest reduction of killing of TW-5 cells (2-log drop versus 3 to 3.5 logs over 60 min) (26). A second study, using a lymphokine-enhanced macrophage-killing system, showed that chronic granulomatous disease cells normally killed C. psittaci (13). It should be noted that chronic granulomatous disease patients do not show a higher risk of chlamydial infection than do normal individuals (23); therefore, these studies confirm the clinical data that killing of chlamydiae is not significantly dependent on an oxidative mechanism. Our results support this basic conclusion, since the respiratory burst response was neither activated by low concentrations of chlamydiae nor fully responsive when stimulated by a second agonist (in our studies, FMLP or PMA).

The experimental design of our studies ascertained the initial interaction of diverse chlamydial serovars with human neutrophils at relatively low infective concentrations (2.5 to 100 organisms per cell) and quantitated both initial and subsequent parameters of neutrophil effector functions as elicited by a second agonist, FMLP. Under these conditions, we found no direct activation of the cells by unopsonized chlamydiae in diverse assays: O$_2^-$ production, degranulation of lysozyme (a priming and secondary granule marker), plasma membrane depolarization, and change in free cytosolic calcium concentrations. We relied on the results of previous studies that examined the effective infectivity of chlamydia under these conditions (16). In contrast to studies that showed high organisms/cell ratios to examine the nonspecific, late (30 to 60 min) CL response (18, 20, 28), our results concur with those of Tosi and Hammerschlag; i.e., a respiratory burst is not stimulated by chlamydiae (24). But we were unable to confirm their finding that degranulation is initiated (24). We indirectly confirmed that lysosomal fusion is normal in the early phase of infection (25), as evidenced by a normal degranulation response to FMLP. Each of the three serovars, however, selectively impaired the respiratory burst upon activation with FMLP but differed from influenza virus in the mode of neutrophil deactivation (8).

Oxidative killing is not the principal method of chlamydial inactivation, then, because the enzyme-degrading processes and acidification become essential for normal killing. In this regard, studies to define which neutrophil constituents degrade chlamydiae focus the line of further investigation for establishing the mechanism by which the phagocytes deal with chlamydial infection. Support for this rationale is found in published studies showing that lysozyme is a major degradative effector agent of chlamydiae in neutrophils (16). Both electron microscopy showing normal granule-vacuole fusion (25) and our studies, which show normal degranulation in FMLP-stimulated, chlamydia-infected neutrophils, support this modality as a likely mechanism of killing chlamydiae. Therefore, our findings corroborate clinical and laboratory data suggesting that the respiratory burst in chlamydia-infected neutrophils is not a host defense mechanism used by the phagocytes. How and to what extent, under physiological conditions, various serovars of chlamydiae are destroyed by neutrophils remain to be established. Furthermore, the finding that chlamydia-infected neutrophils are impaired in the oxidative response to a second agonist suggests a possible mechanism for secondary infections in the clinical setting of primary chlamydial infection.

**ACKNOWLEDGMENTS**

We thank Ann Marie Happnie for expert secretarial assistance. This work was funded by Public Health Service grant PO1 AI24760 from the National Institutes of Health.

**LITERATURE CITED**


