Interaction between Human Polymorphonuclear Leucocytes and *Chlamydia trachomatis* Elementary Bodies: Electron Microscopy and Chemiluminescent Response

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Incubation of human polymorphonuclear leucocytes (HPMN) with highly purified *Chlamydia trachomatis* serotype L_{2}/434/Bu elementary bodies (EB), in the presence and absence of specific antibody, resulted in a $10^3$-fold reduction of viable count after 24 h incubation. Electron microscopy observations indicated activation of the HPMN by the EB. Attachment of the EB to the HPMN cell membrane, formation of a cytoplasmic cup and EB-containing vacuoles were observed. In addition, two types of phagocytic vacuoles were observed after 30 min incubation; in one type, a single EB was tightly surrounded by the vacuolar membrane, while the other type was enlarged and held one or more intact EB or degenerated EB or both. A fuzzy coat was observed on EB located in the HPMN vacuoles only in the presence of specific antibody. Empty vacuoles containing degenerated EB were observed in the HPMN after 24 h incubation. HPMN exposed to EB of *C. trachomatis* produced a marked chemiluminescent response with a peak 14 times greater than the peak value of the control. A second stimulation with phorbol 12-myristate 13-acetate and zymosan was achieved. The chemiluminescent peak value in the presence of heat-treated EB (56 °C, 20 min) was 50% of that obtained in the presence of untreated EB. The significance of the chemiluminescent response in the killing mechanism of *C. trachomatis* EB by HPMN is discussed.

INTRODUCTION

The role of human polymorphonuclear leucocytes (HPMN) in host defence against microorganisms is well established (Quie & Mills, 1978; Walach et al., 1982). Their microbicidal activity is facilitated by a number of antimicrobial systems, some dependent on oxygen and others operative in its absence (Gabig & Babior, 1981). Phagocytosis and killing of some microorganisms by HPMN requires opsonization of the invading pathogens, while others are ingested without being coated by serum opsonins (Danley & Hilger, 1981). In addition to the initiation of phagocytosis, the HPMN response to stimulation by some micro-organisms is characterized by an increase in oxidative metabolism, and the production of highly reactive, antimicrobial substances (Quie et al., 1977). The metabolic processes can be detected in several ways. Among these is light emission stimulated by the oxygen radicals being generated, which can be detected in a chemiluminescence assay such as that described originally by Allen et al. (1972).

*Chlamydia trachomatis* is an obligate intracellular Gram-negative bacterium with a genome of $660 \times 10^6$ Dal (Sarov & Becker, 1968, 1969; Moulder, 1982; Ward, 1983). The multiplication cycle of *C. trachomatis* involves two distinct forms of micro-organisms: the elementary body (EB) which is the infectious extracellular form and the reticulate body which is the metabolically active, highly permeable intracellular form (Moulder, 1982; Ward, 1983). A total of 15 serotypes

Abbreviations: EB, elementary bodies; HBSS, Hanks' balanced salt solution; HPMN, human polymorphonuclear leucocytes; PMA, phorbol 12-myristate 13-acetate.
of *C. trachomatis* have been described. The A, B, Ba and C serotypes are the cause of hyperendemic trachoma in developing countries (Dunlop, 1983). Strains D to K are a major cause of sexually transmitted infections, non-gonococcal urethritis (Oriel, 1983), cervicitis, endometritis (Hare & Thin, 1983), salpingitis (Westrom & Mardh, 1983), conjunctivitis and pneumonia (MacFarlane & Macrae, 1983). Subclinical infections by *C. trachomatis* appear to be a major cause of mechanical infertility in women (Westrom & Mardh, 1982). The three serotypes L1, L2 and L3 are the causes of lymphogranuloma venereum, a sexually transmitted systemic disease (Schachter & Caldwell, 1980). Current knowledge of the humoral and cellular immune responses generated during chlamydial infections and how these control or exacerbate disease is limited (Yong et al., 1982). It has been demonstrated that chlamydial antigens classified as group antigens can be detected on the membranes of infected host cells (Richmond & Stirling, 1981). The significance of this finding regarding cell mediated immunity needs further investigation.

Polymorphonuclear leucocytes are the predominant inflammatory cells which infiltrate the sites of early infection (Monnickendam & Pearce, 1983) and interaction of these cells with *C. trachomatis* is currently being investigated (Soderlund et al., 1984).

In the present study we examined the interactions between HPMN and highly purified EB of the L1/Bu/434 biotype of *C. trachomatis*. The one step growth curve technique was used to measure loss of infectivity of the EB ingested by the HPMN. Preparations of HPMN incubated with *C. trachomatis* EB, in the presence and absence of specific antibody, were examined by electron microscopy. The ability of the EB to induce HPMN to produce potentially chlamydicidal oxidative metabolites was tested by measuring the chemiluminescence response of the HPMN.

**METHODS**

*Organisms and growth conditions. Chlamydia trachomatis* biotype lymphogranuloma venereum (L1/434/Bu) was grown in MA-104 (embryonic rhesus monkey kidney) cell cultures (Cevenini et al., 1983). The cells were grown in RPMI-1640 medium supplemented with 10% (v/v) foetal calf serum, 100 U penicillin ml⁻¹, 100 μg streptomycin ml⁻¹, 100 U mycostatin ml⁻¹ and 2 mM-L-glutamine (Bio-Lab, Jerusalem, Israel) and were passed every 4 d. Before infection, the cells were washed and the medium was replaced by maintenance medium which contained minimum essential medium supplemented with 5% foetal calf serum, 1% (w/v) glucose, 0.15% (w/v) sodium bicarbonate, 100 μg streptomycin ml⁻¹, 10 μg gentamicin ml⁻¹ and 10 μg Fungizone ml⁻¹. After 2 d the cells were infected and the medium was replaced with maintenance medium supplemented with 1 μg cycloheximide ml⁻¹ (growth medium).

*Purification of chlamydiae.* Elementary bodies (EB) of *C. trachomatis* were purified 48-72 h post-infection from MA-104 monolayers grown in 175 cm² polystyrene culture flasks (Nunc Inc., Denmark) by a modification of the method described by Caldwell et al. (1981). The medium was poured off and the cells were removed with 4 mm glass beads into 10 ml cold Hanks' balanced salt solution (HBSS; Bio-Lab, Jerusalem, Israel). The cell suspensions were pooled from the flasks, and ruptured by vortexing for 2 min. The resulting suspension was centrifuged at 400g for 15 min at 4 °C. The supernate was layered over 8 ml of a 35% (v/v) Urografin solution (diatrizoate meglumine and diatrizoate sodium 76% for injection; Schering AG Berlin/Bergkamen, FRG) in 0.01 M-HEPES containing 0.15 m-NaCl and then centrifuged at 4300 g for 1 h at 4°C in a Beckman SW 27 rotor. The pellets were suspended in 0.01 m-sodium phosphate (pH 7.2) containing 0.25 m-sucrose and 5 mM-L-glutamic acid (SPG), pooled and layered over discontinuous Urografin gradients [13 ml 40%, 8 ml 44% and 5 ml 52% (v/v) Urografin]. These gradients were centrifuged at 43000 g for 1 h at 4 °C in an SW 27 rotor. EB bands located at the 44/52% Urografin interface were collected, diluted with SPG and then centrifuged at 43000 g for 45 min. The EB pellets were suspended in SPG and stored at −70°C. For the chemiluminescence assay, EB were washed by centrifugation (43000 g, 45 min in an SW 27 rotor) and resuspended in HBSS.

*Isolation of HPMN.* This was done by the method of Boyum (1968). Heparinized whole blood was obtained from healthy human donors, diluted twofold, layered onto Ficoll-Hypaque (1.077 g cm⁻³) at 25 °C and centrifuged at 400 g for 30 min. The pellet was resuspended in an equal volume of 6% (w/v) dextran in minimum essential medium. The erythrocytes were allowed to sediment at 37 °C for 30 min, and the HPMN-enriched supernatant was removed and centrifuged for 5 min at 400 g. Contaminating erythrocytes were removed by osmotic shock. The HPMN cells (99% pure) were washed three times in minimum essential medium and resuspended in RPMI-1640 + 10% foetal calf serum. For the chemiluminescence assay, HPMN were suspended in HBSS without phenol red. The viability was 99% as determined by 1% Trypan blue staining. After 50 h incubation, viability of the HPMN was 80%. 

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Inactivation test. HPMN (10⁶) and 10⁷ inclusion forming units (IFU) of C. trachomatis EB were incubated [1 h, 37 °C in 5% (v/v) CO₂] in 1.0 ml RPMI-1640 containing 5% heat-inactivated foetal calf serum, 50 μg streptomycin ml⁻¹, 5 μg gentamicin ml⁻¹, 5 μg Fungizone ml⁻¹, with or without human serum diluted 1:10. The human serum had a chlamydia-specific IgG titre of 512 determined by immunoperoxidase assay, as described by Cevenini et al. (1983). The cells were washed twice with RPMI-1640 medium (100 g, 5 min) and further incubated at 37 °C in an atmosphere of 5% CO₂, in a volume of 1 ml of the same medium. At 0, 4, 24 and 50 h post-infection, samples (200 μl) were taken out and sonicated for 20 s in a Branson-12 sonifier.

MA-104 cells as a control (10⁶ cells ml⁻¹) were infected at an m.o.i. (multiplicity of infection) of 1 IFU per cell with 1 h adsorption at 37 °C. The cells were washed twice with RPMI-1640 medium (300 g, 5 min), diluted with the same medium as the HPMN and seeded in a 96 microwell plate (2 × 10⁴ cells per well). At 0, 4, 24 and 50 h post-infection, the cells were scraped into the medium inside the well. Triplicate wells were pooled (300 μl) and the sample was sonicated for 20 s. Viable organisms in the HPMN and control cell lysate were titrated on MA-104 cells by a modified procedure of Kimmel et al. (1984). MA-104 cells were seeded (5 × 10⁴ cells per well) in a 96 microwell plate (Nunc). After 48 h the cells were infected, in duplicate with 10-fold dilutions of the control and HPMN lysates. At 48 h post-infection the cells were fixed with ethanol (100%), and subjected to an immunoperoxidase assay (Haikin & Sarov, 1982). The final results of the titration were expressed as IFU ml⁻¹.

Electron microscopy. Samples were prepared according to the method of Biberfeld (1971). Pellets of HPMN were fixed in 2% (w/v) glutaraldehyde in cacodylate buffer (pH 7.2, 0.1 M) for 90 min and washed twice with the same buffer. After fixation samples were treated with 1% (w/v) OsO₄ for 60 min, dehydrated in alcohol, washed three times with propylene oxide and embedded in Araldite. The blocks were sectioned and stained with lead citrate and uranyl acetate. Electron micrographs of thin sections were taken with a Philips 201C transmission electron microscope.

Chemiluminescence assay. Luminol-enhanced chemiluminescence was measured by a modified procedure of Trush et al. (1978), in a liquid scintillation counter (Packard, Tri-Carb liquid scintillation spectrometer, model 3310) kept at ambient temperature (approx. 23 ± 1 °C) and set ‘out of coincidence’ in the tritium mode. Samples for the chemiluminescence assay were obtained by adding 0.8 ml HBSS without phenol red and containing 2 μM-luminol (Sigma), 0-1 ml cell suspension (10⁶ HPMN) and 0-1 ml purified EB, to sterile, disposable 4 ml polypropylene tubes (38 × 12-5 mm; Nunc). The tubes (dark adapted for 24 h before the assay) were put into glass vials, placed in the scintillation counter and the light emission was recorded for 0-1 min at intervals of 6–10 min. Zymosan (Sigma) and PMA (phorbol 12-myristate 13-acetate; Sigma) were used in the second chemiluminescence stimulation assay. Zymosan was prepared by boiling 100 mg in 2 ml saline for 15 min in a water bath and centrifuging at 600 g for 5 min. The pellet was suspended in 2 ml HBSS. Zymosan was used at 2.5 mg ml⁻¹ in the assay. PMA was dissolved in a drop of DMSO and further diluted with HBSS to a concentration of 1 μg ml⁻¹. The final concentration of PMA added to the vials was 0-1 μg ml⁻¹.

RESULTS

Inactivation of C. trachomatis EB by HPMN

The ability of HPMN to inactivate C. trachomatis EB (L₂/434/Bu serotype) is shown in Fig. 1. Incubation of HPMN and the EB at a ratio of 1 : 1 resulted in a 10³-fold reduction in infectivity after 24 h incubation in the presence and absence of specific antibody. The control consisted of MA-104 cells incubated with C. trachomatis EB at a ratio of 1 : 1 in the same conditions as the HPMN. In the control, the infectivity titre assay, using the modified immunoperoxidase assay for detection of C. trachomatis IFU in infected cell samples, showed a decrease in infectivity 4 to 24 h post-infection followed by a 10³-fold increase in IFU ml⁻¹ 50 h post-infection (Fig. 1). This L₂ serotype growth pattern in the control is in accordance with that typically observed by other investigators (Ward, 1983).

Effect of HPMN cells on C. trachomatis EB: electron microscopy observations

Several steps in the phagocytosis of the EB were observed. Fig. 2(a) shows an EB attached to the HPMN membrane and formation of a phagocytic cup can be seen in Fig. 2(b). Activation of the HPMN, characterized by the presence of large phagocytic, chlamydiae-containing vacuoles was observed (Fig. 2d). Two types of EB-containing phagosomes were observed after 30 min incubation. One type contained single EB in close contact with the phagosome membrane (Fig. 3a). The other type was enlarged and contained single or a number of intact EB (data not shown) or both intact degenerated EB inside the phagosome (Fig. 2c). A fuzzy coat was observed on EB
inside phagocytic vacuoles only in the presence of specific antibody (Fig. 3b). After 24 h incubation, only empty and degenerated EB-containing vacuoles were observed (Figs 2e and 2f). These results were consistently observed in several experiments.

Effect of C. trachomatis EB on the luminol-enhanced chemiluminescent response of HPMN

Incubation of HPMN with EB of C. trachomatis induced a marked chemiluminescent response. The peak activity was recorded after about 50 min incubation and increased with an increase in the number of IFU per cell in a dose–response relationship (Fig. 4a). Addition of heat-treated EB of C. trachomatis (56 °C, 20 min) resulted in a decrease of 50% in the chemiluminescence peak value (Fig. 4b). The capability of the EB-sensitized HPMN to be further stimulated was investigated using PMA, a phagocytosis-independent stimulant, and zymosan, a phagocytosis-dependent stimulant (Quie et al., 1977). Addition of 0.1 µg PMA ml⁻¹ (Fig. 5b) and 2.5 mg zymosan ml⁻¹ (Fig. 5a) (approx. 3 x 10⁸ particles) after 40 min incubation in the presence of C. trachomatis EB, resulted in an increased chemiluminescent response, with a peak value similar to that obtained when PMA and zymosan were added to the control. Thus, the response of the HPMN to C. trachomatis EB, at the multiplicity described above, indicated that exposure of HPMN to C. trachomatis EB did not reduce the capability of the cells to be further stimulated by PMA and zymosan to produce a second chemiluminescent response.

DISCUSSION

This study describes the results of experiments showing that HPMN inactivate C. trachomatis EB (L₂/434/Bu biotype) in both the presence and absence of specific antibody (Fig. 1). This finding is in agreement with that of Yong et al. (1982) who found that two biotypes of C. trachomatis, TW-5 and L₂/434, were killed by HPMN. Electron micrographs of HPMN exposed to C. trachomatis EB (Fig. 2d) showed EB within phagocyte vacuoles in the HPMN cytoplasm after 30 min incubation. Two types of EB-containing phagosomes were observed. One appeared as a tightly enclosed vacuole containing a single EB (Fig. 3a) while the other was enlarged and...
Fig. 2. Transmission electron micrographs of HPMN infected with *C. trachomatis* EB. Cells were fixed 30 min after chlamydial challenge. *(a)* Attachment of the EB to the HPMN cell membrane; *(b)* formation of a phagocytic cup; *(c)* intact and degenerated EB inside a large vacuole; *(d)* activated HPMN with phagocytic EB-containing vacuoles; *(e)* HPMN with empty vacuoles and with *(f)* degenerated EB-containing vacuoles after 24 h incubation. Bar markers: *(a)* 0.2 μm; *(b)* 0.4 μm; *(c)* 0.4 μm; *(e)* 2 μm; *(f)* 2 μm.
Fig. 3. Transmission electron micrographs of HPMN infected with *C. trachomatis*. Cells were fixed 2 h after chlamydial challenge. (a) EB inside a tightly enclosed phagocytic vacuole; (b) an EB-containing vacuole in the presence of specific antibody. The human serum had a chlamydia-specific IgG titre of 256 determined by the immunoperoxidase assay described by Cevenini *et al.* (1983). A fuzzy coat can be observed on the EB inside the phagocytic vacuole. Bar markers: (a) 0.2 μm; (b) 0.1 μm.

contained a single or a number of intact EB, degenerated EB, or both (Fig. 2c). These two types of vacuoles could represent various stages of digestion of the *C. trachomatis* EB or may indicate the existence of two different mechanisms of entry of the infectious particles into the HPMN. Rikihisa & Ito (1980), using electron-dense tracers during entry of rickettsia into HPMN, characterized two types of vacuoles. Silva *et al.* (1982), studying the mechanism of entry of *Toxoplasma gondii* into mouse macrophages, observed the existence of two types of cytoplasmic
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Fig. 4. Luminol-enhanced chemiluminescent response of $10^6$ HPMN incubated (a) in the presence of C. trachomatis EB at a m.o.i. of 1 (△), 10 (□), and 50 (○) (i.e. 1, 10 or 50 IFU per cell) and (b) in the presence of heat-treated (56°C, 20 min) EB (○). Controls in (a) and (b) consisted of HPMN incubated with medium alone (●). The results represent one of three experiments.

Fig. 5. Luminol-enhanced chemiluminescent response of $10^6$ HPMN which were initially stimulated with $3 \times 10^7$ IFU C. trachomatis EB ml$^{-1}$ (○). After 50 min incubation (arrows) zymosan (a) and PMA (b) were added at concentrations of 2.5 μg ml$^{-1}$ and 0.1 μg ml$^{-1}$, respectively. The control (●) consisted of HPMN incubated with medium alone. The control for EB-stimulated HPMN treated with zymosan or PMA was stimulated cells without addition of zymosan or PMA. For clarity of the experimental curve this control is not shown. The results represent one of three experiments.

Vacuoles. Both groups suggested that their findings could be related to a dual mechanism of entry of the parasites. EB of chlamydiae enter phagocytes such as mouse macrophages by two different endocytic pathways. Infectious EB enter via a parasite-specified pathway, whereas heat-inactivated EB are taken in by a host-specified route (Byrne & Moulder, 1978; Zeichner, 1983). The involvement of these two mechanisms in the entry of C. trachomatis EB to HPMN is a subject for further investigations.

A fuzzy coat was observed only on EB treated with specific antibody, in the HPMN phagosome (Fig. 3b). A fuzzy coat was also observed on rickettsia located in phagosomes of the HPMN, in the presence of specific antibody (Rikihisa & Ito, 1983). The role of this coat is unknown but it may serve as a marker for the entry of opsonized chlamydiae to the cells.

It is well recognized that activation of HPMN by some micro-organisms leads to the production of highly reactive molecules in these cells. Superoxide anions, singlet oxygen and hydroxyl radicals are produced as a result of respiratory burst activation. The production of these highly reactive molecules by HPMN can be measured as light emission, termed chemiluminescence (Trush et al., 1978). As shown in Fig. 4(a) the highly purified EB of C. trachomatis induced a chemiluminescent response with a maximum peak value after 40–50 min of incubation, 14 times greater than the peak value of the control. The HPMN retained activity
since a second stimulation by PMA or zymosan could be achieved. Recently, Soderlund et al. (1984) also found that serotypes E and L1 of C. trachomatis induced a chemiluminescent response in HPMN which was increased by type-specific antibody. The lower chemiluminescent production observed in the presence of heat-treated (56 °C, 20 min) EB may be related to modification of some EB surface molecules, possibly responsible for the stimulation of these chemiluminescent responses generated by the HPMN. Bose & Paul (1982) observed a reduced rate of association of heat-treated EB (L2/434/Bu serotype) to HeLa 229 cells and related this to the modulation of ligands (adhesins) present on the surface of the EB.

Yong et al. (1982) suggested that oxygen-dependent antimicrobial systems are not essential for the chlamydial activity of HPMN. This conclusion was based on the fact that the peroxidase inhibitors azide and cyanide had little or no effect on the inactivation of C. trachomatis by normal HPMN. However, HPMN lacking a respiratory burst and with myeloperoxidase (MPO) deficiency obtained from patients with chronic granulomatous disease inactivated C. trachomatis serotype B less efficiently than they did an L2 serotype. Thus, the findings of Yong et al. (1982) do not necessarily exclude the involvement of the MPO system in the chlamydial activity of the HPMN, and it is possible that some C. trachomatis serotypes are killed by oxygen-dependent systems while others are sensitive to other powerful antimicrobial systems present in the HPMN (Gabig & Babior, 1981). It should be noted that activation of the respiratory metabolism does not necessarily lead to engulfment and subsequent phagocytosis and killing (Henson et al., 1972). For example, cytochalasin B-treated HPMN, interacting with aggregated IgG or with opsonized zymosan, respond with enhanced superoxide generation and production of chemiluminescence, even though particles remain on the outer surface of the cells (Goldstein et al., 1975). H2O2 and free radicals at the site of the infection might have a direct effect on the tissue damage caused by C. trachomatis in acute and chronic infections and may even have some role in the pathogenesis of cervical carcinoma (Henry-Suchet et al., 1981; Paavonen et al., 1979; Schachter et al., 1982). Clearly, further studies are required to understand the mechanism of killing and the significance of the C. trachomatis EB-induced chemiluminescent response by HPMN.

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REFERENCES


HAJKIN, H. & SAROV, I. (1982). Determination of
specific IgA antibodies to varicella zoster virus by immunoperoxidase assay. Journal of Clinical Pathology 35, 645-649.


