The Persistence of *Chlamydia trachomatis* Elementary Body Cell Walls in Human Polymorphonuclear Leucocytes and Induction of a Chemiluminescent Response

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(Received 11 February 1988; revised 13 July 1988; accepted 17 October 1988)

Human polymorphonuclear leucocytes (HPMN) were incubated with [³⁵S]methionine-labelled *Chlamydia trachomatis* (serovar L2/434/Bu) elementary bodies (EB) and EB cell walls. No net loss in the TCA-precipitable radioactivity was observed over 24 h in the HPMN that had taken up EB cell walls. SDS-polyacrylamide gel electrophoresis of the labelled *C. trachomatis* EB and EB cell wall proteins extracted from the HPMN at 2 and 24 h after infection demonstrated the persistence of most of the chlamydial cell wall polypeptides. Analysis of extracts of the HPMN that had taken up either EB or EB cell walls on Urografin density gradients at 2 and 24 h after infection, and electron microscopic observations on fractions representing the peaks, demonstrated the persistence of the EB cell walls in the HPMN. Electron microscopic observations of HPMN that had taken up EB or EB cell walls demonstrated EB cell walls in the HPMN phagosomes at 24 h after infection. The HPMN exposed to EB and EB cell walls of *C. trachomatis* gave chemiluminescent (CL) responses with peaks respectively 12 and 7 times greater than the peak value of the control. The significance of the persistence of the EB cell wall polypeptides and cell walls in the HPMN and activation of the HPMN to produce oxygen radicals (i.e. a CL response), and its possible relation to rheumatic diseases, is discussed.

INTRODUCTION

Chlamydia trachomatis is an obligate intracellular Gram-negative bacterium with a genome of 660×10^6 Da (Sarov & Becker, 1969; Moulder, 1982). A total of 15 serovars of *C. trachomatis* have been described which cause hyperendemic trachoma (serovars A, B, Ba and C) in developing countries and sexually transmitted infections (serovars D to K) such as non-gonococcal urethritis, cervicitis, endometritis, salpingitis, perihepatitis, epididymitis, conjunctivitis and pneumonia. The three serovars L1, L2 and L3 cause lymphogranuloma venereum (reviewed by Ladany & Sarov, 1985). Considerable evidence has been accumulated regarding the possible role of chlamydiae in the pathogenesis of arthritis and other rheumatic diseases (reviewed by Keat, 1986). Recently, typical chlamydial elementary bodies (EB) were seen in joint material from patients with different sexually acquired non-gonococcal genital-tract infections (Keat *et al.*, 1987) and in the synovial tissue of a patient with Reiter's syndrome (Ishikawa *et al.*, 1986). Approximately 5% of patients with lymphogranuloma venereum develop a remitting large-joint arthritis, with a higher proportion experiencing polyarthralgia and erythema nodosum (Abrams, 1968). In cattle and sheep, systemic chlamydial infection has frequently been associated with polyarthritis. In this condition chlamydiae are demonstrated

Abbreviations: CL, chemiluminescence; EB, elementary bodies; HBSS, Hanks' balanced salt solution; HPMN, human polymorphonuclear leucocytes; IFU, inclusion-forming units; MOMP, major outer-membrane protein; PLF, phagosome-lysosome fusion; PMA, phorbol 12-myristate 13-acetate.

within the joint, at least initially, although subsequently synovitis may persist without detectable micro-organisms (Storz, 1967).

Several models have been proposed to explain the development of chronic arthritic diseases (Ginsburg, 1977). Ginsburg & Lahav (1983) suggested that while 'professional phagocytes' possess bactericidal and bacteriolytic factors capable of killing and depolymerizing the complex cellular constituents of bacteria, certain micro-organisms possess surface and wall components which are extremely resistant to degradation by serum complement, polymorphonuclear leucocytes (PMN) and macrophages. This may, under certain conditions, trigger active release of lysosomal enzymes which may cause inflammatory responses, tissue destruction and rheumatoid arthritis. Cell wall components of streptococci and staphylococci have been shown to trigger chronic destructive arthritis in laboratory animals (reviewed by Ginsburg, 1979). An additional hypothesis was recently put forward on the role of superoxide and oxygen radicals released by activated human PMN (HPMN) (Quie *et al.*, 1977), in the development of chronic (Fanburg *et al.*, 1986), arthritic (Biernond *et al.*, 1986; Lunec *et al.*, 1981; Pasquier *et al.*, 1984) and neoplastic diseases (Cerutti, 1985). Superoxide anions (O_2^-) and related oxygen radicals ($\text{O}_2^{\cdot-}$, $\cdot\text{OH}$) have been shown to oxidize biomembranes (Taffel, 1973), to depolymerize hyaluronic acid (Babior, 1978), and to serve as effectors of cartilage destruction (Burkhardt *et al.*, 1986; Keiser, 1980). Furthermore, these active molecules could denature IgG molecules in tryptophan-rich areas of the molecule, leading to generation of IgG complexes detected in the joint fluid of patients with rheumatoid arthritis (Lunec & Hill, 1984). O_2^- has also been shown to participate in the formation of a potent chemoattractant (Petrone *et al.*, 1980), thereby raising the concentration of HPMN in the synovial cleft (Mohr *et al.*, 1981). HPMN have been shown to be the predominant inflammatory cells which infiltrate the site of chlamydial infection (Kunimoto & Brunham, 1985; Monnickendam & Pearce, 1983; Monnickendam *et al.*, 1980; Woodland & Darougar, 1986). EB of *C. trachomatis* have been shown to activate complement and to stimulate chemotaxis (Megran *et al.*, 1985).

In the present study the fate of the EB (L2/434/Bu) of *C. trachomatis* in HPMN at various times after infection was studied by using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Urografin gradients (employing [^{35}S]methionine labelled EB and EB cell walls) and by electron microscopy. The ability of the EB and EB cell walls to activate the HPMN oxygen burst to produce superoxide anions (O_2^-) and related oxygen species was investigated by using the luminol-enhanced chemiluminescence (CL) assay.

METHODS

Growth conditions, radiolabelling and purification of chlamydial EB. EB of *C. trachomatis* biotype lymphogranuloma venereum (L2/434/Bu) were grown on BGM cells (Flow Laboratories) and purified 48–72 h post-infection by modification of the method of Caldwell *et al.* (1981) as previously described (Zvillich & Sarov, 1985). Radiolabelling of *C. trachomatis* EB was performed in chlamydial growth medium containing 20 mM instead of 100 mM-methionine, and 10 μCi [^{35}S]methionine ml^{-1} (500 μCi mmol^{-1} ; 18.5 MBq mmol^{-1} ; Amersham). For the CL assay and Urografin gradient experiments, EB were washed by centrifugation (43000 g , 45 min, 4 °C in an SW-28 rotor), resuspended in Hanks' balanced salt solution (HBSS; Biological Industries, Beth Haemek, Israel) and sonicated for 20 s in a Branson B-12 sonifier.

Immunoperoxidase assay for titration of *C. trachomatis*. *C. trachomatis* was titrated on BGM cells as described by Shemer & Sarov (1984). The final results of the titration were expressed as inclusion-forming units (IFU) ml^{-1} .

Preparation and radiolabelling of chlamydial EB cell walls. Cell walls of *C. trachomatis* were prepared by the method of Jenkin (1960). Levy & Moulder (1982) showed that envelopes prepared in this way retained some native activity with respect to their ability to attach to susceptible host cells. EB of *C. trachomatis* were suspended in 0.01 M-sodium phosphate buffer (pH 7.4) containing 1% (v/v) sodium deoxycholate (Sigma) and shaken for 4 h at 45 °C. The detergent-treated organisms were centrifuged at 10000 g for 10 min in a Beckman SW-28 rotor, rinsed twice with 0.01 M-sodium phosphate buffer (pH 7.4), incubated for 1 h at 37 °C with 1 mg crystalline trypsin ml^{-1} (Sigma), washed three times with phosphate-buffered saline and stored at -70 °C. Protein content of the preparations was 1–1.5 mg ml^{-1} and represented 10% of the starting material. Protein was determined by the Bio-Rad protein assay kit, with bovine serum albumin as standard. The number of cell walls in a given volume was evaluated by titration of the number of viable EB in the starting material used for the cell wall preparation and protein determination. [^{35}S]Methionine-labelled EB cell walls of *C. trachomatis* were prepared by subjecting labelled EB to the procedure described above.

Isolation of HPMN. This was done by the method of Boyum (1968). Heparinized whole blood was obtained from healthy human donors, diluted twofold with RPMI-1640 medium, layered onto Ficoll-Hypaque (Pharmacia; 1.077 g ml⁻¹ at 25 °C), and centrifuged at 400 g for 30 min. The pellet was resuspended in an equal volume of 6% (v/v) dextran in RPMI-1640 medium. The erythrocytes were allowed to sediment at 37 °C for 30 min and the HPMN-enriched supernate 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 and resuspended in RPMI-1640 containing 10% (v/v) foetal calf serum. For the CL assay, HPMN were suspended in HBSS without phenol red. The viability was 99% as determined by 1% trypan blue staining. After 24 h incubation, viability of the HPMN was 85%.

Electrophoretic and TCA analysis of radiolabelled EB and EB cell walls in the HPMN. HPMN (1.6×10^7) were incubated for 2 h (37 °C, in a humidified 5% v/v, CO₂/air mixture) in 1.0 ml RPMI-1640 medium containing 10% FCS, 50 µg streptomycin ml⁻¹, 5 µg gentamicin ml⁻¹ and 8×10^7 radiolabelled EB or cell walls from 8×10^7 EB (100 µg protein). The HPMN were then washed three times by centrifugation (400 g, 10 min) with RPMI-1640 medium and further incubated at 37 °C in a humidified 5% (v/v) CO₂/air mixture, in a volume of 1 ml of the same medium. At 2 and 24 h after the end of the infection period, samples (250 µl) were solubilized by boiling in the presence of SDS and 2-mercaptoethanol and resolved on a 15% (w/v) polyacrylamide slab gel with the discontinuous buffer system of Laemmli (1970, using [¹⁴C]methylated protein size markers (Amersham). After SDS-PAGE, gels were treated with a solution containing 22% PPO, destained and dried under vacuum. Autoradiograms of dried gels were analysed by an LKB Ultrosan XL laser densitometer. Also, samples (250 µl) of HPMN 'infected' with EB cell walls, taken at 2 and 24 h after infection, were treated with 5% (w/v) cold TCA on ice for 20 min and then transferred by filtration to glass-fibre filters (Schleicher & Schüll GF/C filters) washed with 70% (v/v) ethanol, dried, put into scintillation fluid, and their radioactivity measured in a Packard Tri-Carb scintillation spectrophotometer, model 3310.

Urografin gradients and fractionation of infected HPMN samples. HPMN (8×10^6) were incubated for 2 h (37 °C, in a humidified 5% v/v, CO₂/air mixture) in 1 ml RPMI-1640 medium containing 10% FCS, 100 µg streptomycin ml⁻¹ and 2 mM-L-glutamine, with [³⁵S]methionine-labelled EB (4×10^7 IFU ml⁻¹) or cell walls from 4×10^7 EB (50 µg protein). The HPMN were then washed three times (400 g, 5 min) with RPMI-1640 medium and further incubated at 37 °C in a humidified 5% (v/v) CO₂/air mixture, in a volume of 1 ml of the same medium. At 2 and 24 h after the end of the infection period samples (250 µl) of HPMN that had been incubated with EB or EB cell walls, and samples of EB or cell walls alone, were sonicated for 20 s in a Branson-B 12 sonifier. The samples were loaded on 20–65% (v/v) continuous Urografin gradients [solutions containing meglumine diatrizoate and sodium diatrizoate 76% for injection (Schering) in 0.01 M-HEPES buffer pH 7.2, containing 0.15 M-NaCl] and then centrifuged at 43000 g for 16–18 h at 4 °C in a Beckman SW-28 rotor. Fractions were collected, treated with 5% cold TCA on ice for 20 min and then transferred by filtration to GF/C glass-fibre filters, washed with 70% ethanol, dried, put into scintillation fluid and counted. Fractions representing the peaks obtained after Urografin gradient-centrifugation as described above were also observed by electron microscopy: fractions were diluted, sedimented by centrifugation (10000 g, 20 min in Beckman SW-28 rotor), suspended in PBS, mounted on a carbon-coated grid, stained with 1% (w/v) uranyl acetate and observed.

Preparation for thin-section electron microscopy. Samples were prepared according to the method of Biberfeld (1971), as previously described (Zvillich & Sarov, 1985). Electron micrographs of thin section were taken with a Philips 201 C transmission electron microscope.

Chemiluminescence assay. Luminol-enhanced CL 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 CL assay were prepared by adding 0.8 ml HBSS without phenol red and without antibiotics, containing 2 µM-luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma), 0.1 ml cell suspension (10^6 HPMN) and 0.1 ml EB or EB cell wall suspension 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 and placed in the scintillation counter, and the light emission was recorded for 0.2 min at intervals of 6–10 min. Zymosan and PMA (phorbol 12 myristate 13-acetate; Sigma) were used as controls and were prepared as previously described (Zvillich & Sarov, 1985). The specificity of the assay was controlled by addition of 400 U superoxide dismutase ml⁻¹ (EC 1.15.1.1; Sigma) to the reaction mixture containing activated HPMN.

RESULTS

Fate of [³⁵S]methionine-labelled EB and EB cell wall polypeptides in the HPMN

HPMN were incubated with [³⁵S]methionine-labelled EB cell walls of *C. trachomatis* at a ratio of 1:5. In both cases at 2 h following absorption, about 30% of the TCA-precipitable

Fig. 1. SDS-PAGE of [^{35}S]methionine-labelled EB (a) and EB cell walls (b) of *C. trachomatis* extracted from the HPMN at 2 h (lanes 2) and 24 h (lanes 3) after infection. Lanes 1 contain EBs (a) and EB cell walls (b) alone. Molecular masses of EB cell wall polypeptides are marked on the right. M, molecular mass standards.

Fig. 2. Continuous 20–65% (v/v) Urografin density gradients loaded with: (a) [^{35}S]methionine-labelled EB (○) and EB cell walls (□), serving as position standards for (b) and (c). (b) Extracts of HPMN 'infected' with EB cell walls. Samples taken at 2 h (□) and 24 h (■) after infection. (c) Extracts of HPMN infected with EB. Samples taken at 2 h (○) and 24 h (●) after infection. Samples were sonicated for 20 s and loaded on Urografin density gradients. Fractions were collected after centrifugation from the bottom (fraction no. 1) to the top (fraction no. 30).

Fig. 3. Transmission electron micrographs of peak fractions obtained after Urografin gradient centrifugation and fractionation. Fractions representing the peaks of (a) EB cell wall preparation, (b) extract of EB-infected HPMN 24 h after infection, and (c) extract of HPMN 'infected' by EB cell walls 24 h after infection were collected, concentrated by centrifugation, mounted on carbon-coated grids and stained with uranyl acetate. The bar markers represent in 0.2 μm (a) and 0.1 μm (b, c).

radioactivity was associated with the HPMN while 70% was recovered in the medium. No significant net loss in TCA-precipitable radioactivity was observed in samples of HPMN 'infected' by EB cell walls at 24 h after 'infection' (for example: 13 305 c.p.m. were detected in an HPMN sample 2 h after infection and 12 820 c.p.m. were detected in a sample taken 24 h after infection).

The EB or EB cell wall polypeptides extracted from the HPMN 2 and 24 h after infection were examined by SDS-PAGE followed by autoradiography. Fig. 1(a, b) demonstrates the appearance of the 29, 35–37, 40–43 kDa polypeptides [MOMPs: major outer-membrane proteins (Salari & Ward, 1981)] and 57, 60, 62, 72, 92 and 135 kDa polypeptides, in the HPMN at 2 and 24 h. Densitometry scanning of autoradiograms indicated an insignificant change in the MOMPs (less than 3%) while some differences (in the 5–10% range) in the higher molecular mass polypeptides (≥ 57 kDa) occurred 24 h after infection.

Fig. 2 demonstrates that in continuous, 20–65%, Urografin density gradients, 24 h after infection, the content of extracts of HPMN 'infected' by EB cell walls appeared in the same position as purified EB cell walls (position standard) (Fig. 2a versus 2b), while the content of extracts of EB-infected HPMN appeared as a broad band in a position between those of EB alone and EB cell walls (Fig. 2a versus Fig. 2c).

The content of fractions representing the peaks was also examined by electron microscopy (Fig. 3). Samples of EB-infected HPMN 24 h after infection contained predominantly EB cell

Fig. 4. Transmission electron micrographs of thin sections of HPMN infected with *C. trachomatis* EB and EB cell walls at a ratio of 1:5. (a, b) Attachment of EB (a) and EB cell walls (b) to the HPMN cell membrane 30 min after infection. (c, d) Cell walls in phagosomes of EB-infected HPMN (c) and in HPMN 'infected' by EB cell walls (d), 24 h after infection. The bar markers represent 0.2 μ m.

walls (Fig. 3b) and occasionally EB, while EB cell walls (Fig. 3c) could be observed 24 h after infection in the samples of HPMN 'infected' by EB cell walls.

Electron microscopic observations of HPMN infected by C. trachomatis EB and EB cell walls

HPMN were incubated with *C. trachomatis* EB and EB cell walls at a ratio of 1:5. Cells were fixed 30 min after infection. Attachment of EB (Fig. 4a) and EB cell walls (Fig. 4b) to the

Fig. 5

Fig. 5. Luminol-enhanced CL response of 10^6 HPMN incubated (a) in the presence of *C. trachomatis* EB cell walls at a ratio of 10 (●) and 50 (■), and (b) in the presence of EB at a ratio of 10 (△) and 50 (□) [i.e. 10 or 50 EB IFU and about 10 or 50 EB cell walls (12 or 55 μ g protein) per cell]. The symbol □ in (a) and (b) represents HPMN without stimulant. The results represent one of three experiments that gave similar results.

Fig. 6

Fig. 6. Luminol-enhanced CL response of 10^6 HPMN, which were initially stimulated with *C. trachomatis* EB cell walls (about 50 EB cell walls per HPMN), to PMA (a) and Zymosan (b). (a) PMA ($1 \mu\text{g ml}^{-1}$) was added to the HPMN 60 min after stimulation with EB cell walls (■). (b) Zymosan (2 mg ml^{-1}) was added to the HPMN 80 min after stimulation with EB cell walls (●). The controls consisted of HPMN incubated with medium alone (□), with $1.0 \mu\text{g PMA ml}^{-1}$ (△), or with $2.0 \text{ mg Zymosan ml}^{-1}$ (○). The results represent one of three experiments that gave similar results.

HPMN was observed. We (Zvillich & Sarov, 1985), and Yong *et al.* (1986), have previously shown the partial degradation of chlamydial EB in HPMN after 2 h of incubation. In the present study we observed vacuoles in HPMN infected by either EB or EB cell walls, 24 h after infection, which contained cell walls (Fig. 4c and d, respectively) and occasionally EB.

Effect of C. trachomatis EB and EB cell walls on the luminol-enhanced CL response of HPMN

Incubation of HPMN with *C. trachomatis* EB cell walls or EB, in a ratio of 1:10 and 1:50, in both cases induced a marked CL response (Fig. 5a, b). Activity increased with an increase in the number of EB or EB cell walls. Peak activity was obtained after about 45 and 10 min of incubation respectively. A second stimulation of HPMN with PMA and Zymosan, after challenge with EB cell walls (Fig. 6), could be achieved, indicating the potential of HPMN to be further activated by other stimuli. A second stimulation of EB-activated HPMN, by PMA and Zymosan, was previously shown (Zvillich & Sarov, 1985). The different patterns of the CL response induced by EB and EB cell walls in the HPMN may be due to some differences between the number of EB and EB cell walls employed, and/or due to differences in surface characteristics between the EB and EB cell walls. Differences in the CL response of HPMN to infection with different strains of *Klebsiella pneumoniae*, due to cell wall differences, have been observed (Robinson *et al.*, 1984).

DISCUSSION

In the present study we have analysed the possibility that EB cell wall antigens and cell walls persist in HPMN, and have examined their ability to induce a CL response, i.e. the production of oxygen free radicals which might have a role in the aetiology of rheumatic and chronic diseases (Halliwell & Gutteridge, 1984).

Eissenberg *et al.* (1983), using indirect immunofluorescence, ferritin labelling of secondary granules and radioisotopic labelling, also obtained evidence indicating the persistence of *Chlamydia psittaci* EB envelopes in mouse resident peritoneal macrophages as well as in L cells, in the absence of phagosome-lysosome fusion (PLF). However, Yong *et al.* (1986) demonstrated PLF in chlamydia-infected HPMN by detecting peroxidase-positive phagosomes containing chlamydia. The reason for the lack of degradation of *C. trachomatis* EB cell walls by HPMN after as long as 24 h, in spite of the probable occurrence of PLF, is unknown. It has been suggested that the inability of the HPMN to degrade cell wall components of certain bacteria is due to the presence of lipopolysaccharide, polysaccharide-teichoic acid complexes and certain lipids and waxes which hinder the accessibility of the peptidoglycan to the lysozyme (Ginsburg & Lahav, 1983). In this regard Rozenberg-Arska *et al.* (1985) showed, by an electron microscopic study, the rapid killing (15 min after phagocytosis) and degradation of unencapsulated *Escherichia coli* by HPMN, while encapsulated *E. coli* resisted killing and degradation by HPMN after 1 h of incubation. Tamura & Manire (1968) demonstrated the failure of lysozyme/EDTA to affect the integrity of envelopes of Chlamydia, indicating that they differ fundamentally from those of most bacteria. Further studies are required in order to characterize the degradation-resistant components of *C. trachomatis* cell walls.

Previous studies have demonstrated that EB can induce a CL response in HPMN (Soderlund *et al.*, 1984; Zvillich & Sarov, 1985). In the present study we have demonstrated that EB cell walls can also induce a CL response in HPMN (Fig. 5). Superoxide anions ($\cdot\text{O}_2^-$) and hydrogen peroxide (H_2O_2) are also produced by EB-activated HPMN (Zvillich & Sarov, 1988). Several Gram-negative bacteria, as well as lipopolysaccharide and the synthetic *N*-formylated methionyl oligopeptide analogous to the NH_2 -terminal degradation product of bacterial proteins, can induce the production of H_2O_2 , $\cdot\text{O}_2^-$ and CL by HPMN (Bradley, 1979; Dahlgren & Stendahl, 1982; Robinson *et al.*, 1984). The chlamydial cell wall component(s) involved in the activation of the HPMN need further characterization.

We suggest that undegraded or partially degraded chlamydial cell walls, exocytosed by phagocytosing HPMN, or translocated by macrophages (Ginsburg, 1977), may accumulate in the joints or tissues, attract (Megran *et al.*, 1985; Petrone *et al.*, 1980) and stimulate HPMN to produce oxygen radicals, which may participate in cartilage destruction directly (Burkhardt *et al.*, 1986), or indirectly (Lunec & Hill, 1984; Wickens *et al.*, 1984).

This study was supported by the Middle East Eye Research Institute (MEERI). We thank Dr Moreen Fridman and Galila Green for criticism and help with the manuscript.

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