Original Article

Immuno-gold Labelling of *Chlamydia trachomatis'* Elementary Bodies

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Abstract

Background: *Chlamydia trachomatis* is considered as an important cause of preventable sexually transmitted diseases, worldwide. It is known to be of an obligate intracellular nature and enters its target cells via an endocytic process. As major outer membrane protein (MOMP) is one of the main candidates for the attachment and entry of chlamydia to the host cells, we have tried to label the epitopes by using different techniques.
Materials and Methods: McCoy cells were experimentally inoculated with 10^4 elementary bodies (EBs) followed by 24 hours incubation at 37ºC. The infected cells were then processed for direct fluorescent antibody (DFA) and transmission electron microscopy (TEM) using anti-MOMP antibody and pre- and post-embedded labelling techniques.
Results: DFA was able to detect 11/11 (100%) of the infected cells. These values were recorded as 9/11 (81.81%) and 8/11 (72.72%), using pre- and post-embedded techniques, respectively.
Conclusion: MOMP is proposed to be one of the most important adhesion molecules for chlamydial attachment and entry into host cells.

Keywords: *Chlamydia trachomatis*, Major Outer Membrane Protein, Immuno-gold labelling, Adhesion Site, Direct fluorescent antibody

Introduction

Immuno-gold labelling is one of the most rapidly emerging fields of study for biological and medical research. Protein A-gold and antibody-gold conjugates are usually stable for at least three months. Sensitive methods such as labelling cells using gold-conjugate are needed to differentiate cells (1).
Members of the genus Chlamydia are characterised by a unique developmental cycleconsisting of two distinct morphological forms. Although Chlamydia were originally categorised as protozoa and later viruses, it became clear that they have all the properties of bacteria. The chlamydial envelope lacks conventional peptidoglycan, the membrane possesses penicillin binding proteins (2, 3) however genes required for peptidoglycan synthesis have been shown in the genome of *C. trachomatis* serovar D (4).
Chlamydiae are characterised by a unique obligate intracellular developmental cycle (Fig 1) and morphologically alternate between two distinct forms; the small infectious elementary body (approximately ~ 300 nm in diameter) and a larger dividing intracellular reticulate body (RB) (~1000 nm). The cycle was firstly described by Bedson and Bland (5). The morphology of elementary bodies (EBs) and RBs, the intermediate form and the inclusion membrane in which the complete developmental cycle takes place, have been studied by electron microscopy (6). The EB is a small, dense and spherical body, which is adapted to an extracellular environment. The EB is mainly responsible for attachment to host cells. Therefore, the main function of EBs is to survive in the extracellular environment for a sufficient time to invade susceptible host cells (7). The aim of this work is to focus on chlamydial surface antigens possibly taking part as major chlamydial adhesions to its site of attachment.

Materials and Methods

Growth and maintenance of the McCoy cell line

The McCoy cells were grown at 37°C in 5% CO_2 in 25 cm^3 plastic disposable culture flasks (Sterilin Ltd., Feltham, UK), 1ml Trac bottles (with coverslips) or in 24-well tissue culture plates in McCoy growth medium.

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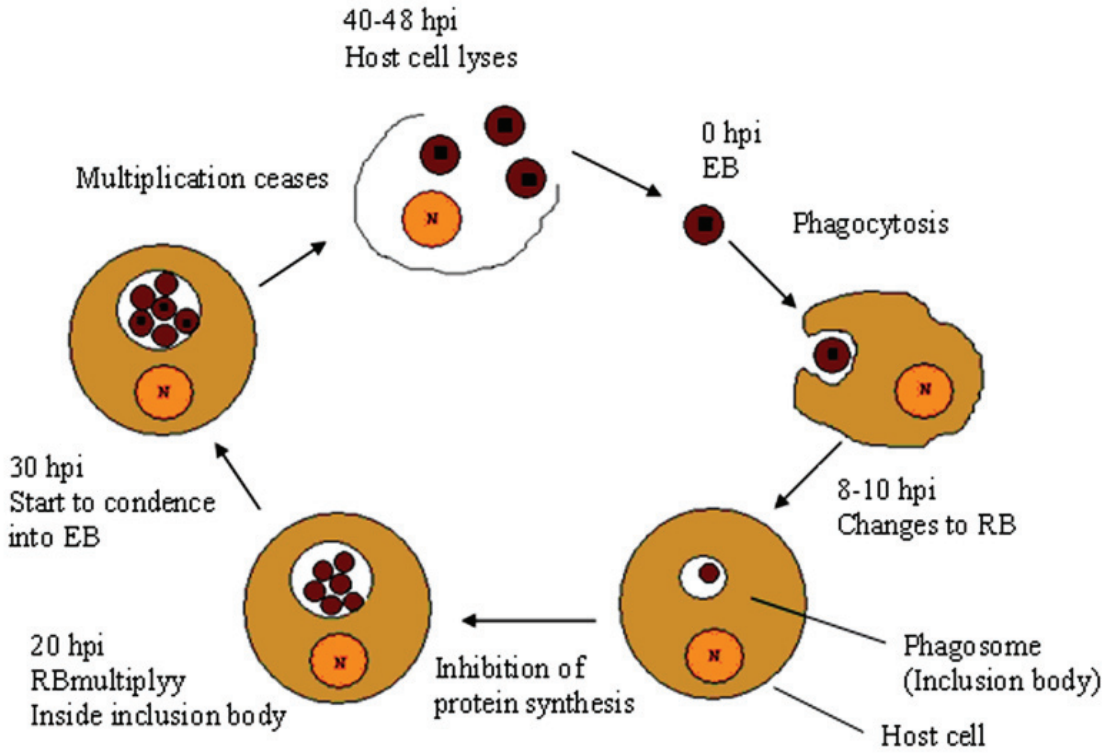

Fig 1: Developmental cycle of C. trachomatis
Attachment of EBs to specific receptors on host cells. Entry (endocytosis), differentiation of EBs to RBs, multiplication of RBs within inclusions.
hpi: hours post-infection

To avoid any possible bacterial or fungal contamination, the cells were supplemented with antibiotics. The cells were subjected to passage as follows.

A laminar flow cabinet was switched on for 15 minutes before use and all surfaces in the hood were disinfected with methyl alcohol. After checking cell confluency by using an inverted microscope, the growth medium was discarded. Cells were washed twice with phosphate buffer solution (PBS) and treated with trypsin. Following incubation at 37°C for 3 minutes, the cells were easily detached from the surface of the bottles. The detached cells were resuspended in 10-20 ml growth medium and the bottles were then incubated at 37°C for 24-48 hours until they were found to be confluent (8).

Culture of chlamydia on confluent cells

The growth medium, Eagle's Minimum Essential Medium (EMEM, LONZA, USA) from tissue culture flasks was replaced by maintenance medium, and from frozen stocks of chlamydia, 50-100 µl were added to the flasks. The flasks were centrifuged at 2000 x g for 1 hour and incubated at 37°C for 48-72 hours. The tissue culture flasks were then directly checked (without fluorescence staining) under a light microscope in order to find any chlamydial inclusions.

Purification and titration of chlamydial EBs

The elementary bodies were isolated from McCoy cells by density gradient centrifugation as described (9). Infected McCoy cells were detached from the tissue culture flasks using a cell scraper and the resulting suspension disrupted by three bursts of sonication (30 seconds at an amplitude of 12 microns), interspersed by 1 minute intervals. The suspension was then centrifuged at 500 x g for 15 minutes and transferred to a plastic centrifuge tube before recentrifuging at 30,000 x g for 1 hour at 4° C. The resulting pellet was then resuspended in 1 ml PBS and sonicated as before and was increased to a volume of 8 ml with PBS. The chlamydia suspension was then placed on the top of an 8 ml urografin 150 in a Beckman centrifuge tube and the suspension centrifuged again as above. The final pellet was

gently washed by adding 1 ml PBS to remove residual urografin before being resuspended in 2-3 ml of 2 sucrose phosphate buffer (2SP). After purification of EBs, they were diluted 1/10, 1/100, 1/1000 with PBS. Each dilution was subsequently used to infect the track bottles that contained the confluent cells and cover slips were prepared for fluorescent microscopy after 24-48 hours of incubation at 37ºC. The numbers of inclusions were then recorded and the mean values of inclusions in three dilutions were then used as the final titration of each preparation (8). The solution was finally vortexed and sonicated as above, titrated to give a final concentration of 1 x 10^5 EBs, and kept at -70° C for further use.

Direct Immunofluorescence

Cells, 24 hours post-infected, that were in the track bottles containing coverslips were put on the slides, dried overnight and fixed in methanol for 30 minutes followed by incubation with a *C. trachomatis* monoclonal antibody (fluorescent conjugated anti-MOMP antibody, Abcam, UK) in a wet chamber at 37°C for 30 minutes. The slides were then gently washed using PBS and mounted with coverslips. The slides were examined using a fluorescence microscope at x100, x400 and x1000 magnification (under oil immersion) to detect any chlamydial inclusions. The work was performed on total of 11 samples.

In vitro experiments to detect surface-exposed epitopes on *C. trachomatis* using transmission electron microscopy

A variety of conditions for fixation and labelling of the antibodies as well as embedding were employed to achieve a clear background and to perform a successful labelling of *C. trachomatis*.

Pre-embedded labelling of C .trachomatis

In this method, 200 µl infected McCoy cells were incubated with a chlamydia monoclonal antibody for 2 hours. The samples were then centrifuged at 9780 x g for 3 minutes followed by incubation with gold-conjugated antibody after washing 4-5 times with PBS. Paraformaldehyde 2% (v/v) in PBS was used to fix the pellet for 1 hour or overnight. The fixed samples were then washed for 4-5 times with PBS followed by dehydration steps in 50% (v/v) and 70% (v/v) ethanol, respectively, for 10 minutes each. A pure solution of LR-white resin was added to the samples for 2 hours on the roller, followed by embedding and polymerisation at 55°C overnight. Ultra-thin sections of 70 nm thickness were taken and post-stained using uranyl acetate and lead citrate solutions. Grids were then observed with a Philips EM 400 transmission electron microscope at magnifications between 6,000 and 21,000 (8).

Post-embedded labelling of C. trachomatis

The samples (200 µl) formed a pellet at 9780 x g and were fixed with paraformaldehyde 2% (v/v) in PBS. The fixed cells were then washed 4-5 times with PBS followed by dehydration steps in 50% (v/v) and 70% (v/v) ethanol respectively for 10 minutes each. A pure LR-white resin was then added to the pellet for 2 hours on a roller, followed by embedding and polymerisation at 55°C overnight. Ultra-thin sections of 70 nm thickness were taken. The grids were incubated with a chlamydia monoclonal antibody conjugate after washing 4-5 times with PBS. Grids were then observed with a Philips EM 400 transmission electron microscope at magnifications between 6,000 and 21,000 (8).

Results

Direct fluorescent antibody

EBs of *C. trachomatis* were detected using direct fluorescent antibody. In this commercial method, monoclonal antibodies against the MOMP of *C. trachomatis* produce a specific fluorescent brightness of EBs. All eleven samples checked with direct fluorescent antibody (DFA) were positive (Table 1), showing the high specificity of the technique as it was previously described (10). The sensitivity and specificity of such DFA tests were comparable with the nucleic acid amplification test (NAAT, ligase chain reaction) to detect *C. trachomatis* (11).

Table 1: Comparison between three tests: DFA, pre-, and post-embedded labelling of C. trachomatis.

Sample Nos	Tests		
	DFA	Pre-embedded labelling	Post-embedded labelling
1	+	–	–
2	+	–	–
3	+	+	–
4	+	+	+
5	+	+	+
6	+	+	+
7	+	+	+
8	+	+	+
9	+	+	+
10	+	+	+
11	+	+	+

DFA: Direct fluorescent antibody

Transmission electron microscopy

To detect the surface exposure of epitopes located on chlamydia major outer membrane including (MOMP) and chlamydia lipopolysaccharide (LPS), an anti-chlamydia antibody in an immunocytochemical system for transmission electron microscopy was used. A thin section transmission electron micrograph of urografin purified EBs from serovar lympho granuloma venereum (LGV) is shown in Fig 2. Analysis of the specimen preparations revealed that antigenicity was preserved and that localisation of the gold label was specific for EBs, as was previously reported (12).

A transmission electron microscopy result presented here was employed to suggest the adherence of EBs to the host cells which were previously shown in direct fluorescent antibody (Fig 3). In an in vitro model, the pre-embedded method was successfully used (Fig 2).

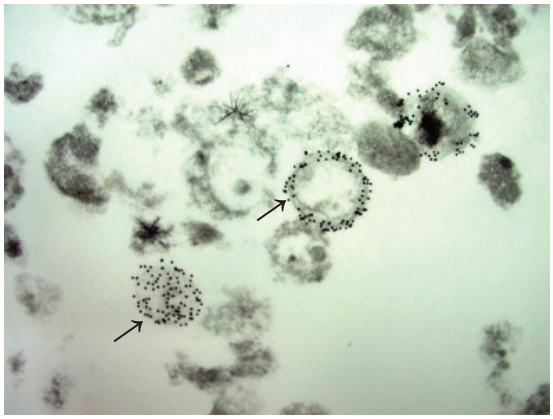

Fig 2: Gold – labelled conjugates selectively attached to the membrane of EB's (arrows) (×17000).

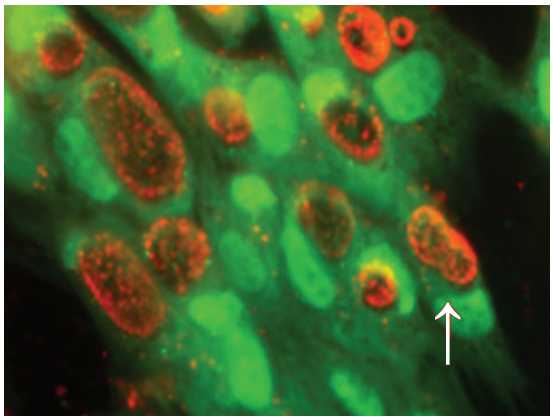

Fig 3. Direct fluorescent antibody – C. trachomatis using Chlamydia monoclonal antibody conjugate (×1000). Red spots representing EBs. The arrow points to an inclusion body.

Out of 11 DFA positives, 9 (81.81%) and 8 (72.72%) were found to be positive in pre- and post-embedded TEM, respectively.

Discussion

A number of adhesins have been proposed including MOMP. By immunoelectron microscopy of infected endometrial epithelial cells and in isolated cell secretory compartments, MOMP, LPS and the inclusion membrane protein A (IncA) were localized to the endoplasmic reticulum (ER) and co-localized with multiple ER markers, but not with markers of the endosomes, lysosomes, Golgi nor mitochondria (13). Such findings may lead us to the theory that MOMP can play a role in the attachment process and thus could induce a signal transduction pathway inside the infected cells.

Using the trachoma strain *C. trachomatis* serovar B and hamster kidney (HaK), MOMP displayed at least two independent adhesion functions. Involvement of negatively charged hydrophilic residues exposed in MOMP variable domains II and IV were involved in initial electrostatic interactions to bring the negatively charged EB close to the host cells (14, 15). Moreover, a heparan sulphate-like glycosaminoglycan (GAG) (16), chlamydial cytadhesin (CCA) (17), chlamydial heat-shock protein (hsp 70) (18-20), a 28 kDa grpE protein (21), and the cysteine rich protein OMP2 (22, 23) have been shown to function as chlamydial adhesins. Zhang and Stephens proposed that *C. trachomatis* serovar L2 synthesises a unique heparan sulphate-like GAG required for attachment to HeLa cells (16, 24). The same heparan sulphate-like mechanism has also been shown in trachoma serovar infectivity (25). However, an alternative view has suggested that the heparan sulphate-like molecules originate from host cells rather than Chlamydia (26).

The attachment site of chlamydia remained unknown, although a number of studies have suggested that the heparan sulphate-like molecules function as an active attachment site. Detecting, localizing and counting ultra small particles and nanoparticles in sub- and supra-cellular compartments are subjects of interest. For particles with sufficient contrast (e.g. colloidal gold), visualization requires high resolutions that are achievable by TEM (27). Therefore, we have developed the technique to follow the major outer membrane proteins at its site.

Conclusion

In this study a gold conjugate antibody has

been successfully used to confirm that the firm attachment sites of EBs to the host cells is an active process which continued to remain intact even following a few fixative processes during the transmission electron microscopy procedures.

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References

1. Fossmark R, Martinsen TC, Qvigstad G, Bendheim M, Kopstad G, Kashima K, Waldum HL. Ultrastructure and chromogranin: A immunogold labelling of ECL cell carcinoids. APMIS. 2005; 113(7-8): 506-512.
2. Barbour A, Amano K, Hackstadt T, Perry L, Caldwell HD. Chlamydia trachomatis has penicillin-binding proteins but not detectable muramic acid. J Bacteriol. 1982; 151: 420-428.
3. Fox A, Rogers J, Gilbart J, Morgan S, Davis CH, Knight S, et al. Muramic acid is not detectable in Chlamydia psittaci or Chlamydia trachomatis by gas chromatography mass spectrometry. Infect Immun. 1990; 58: 835-837.
4. Stephens R, Kalman S, Lammel C, Fan J, Marathe R, Aravind L, et al. Genome sequence of an obligate intracellular pathogen of humans: Chlamydia trachomatis. Science. 1998; 282: 754-759.
5. Bedson S, Bland J. Morphological study of psittacosis virus with a description of the developmental cycle. Exp Pathol. 1932; 13: 461-466.
6. Matsumoto A. Electron microscopic observations of surface projections on Chlamydia psittaci reticulate bodies. J Bacteriol. 1982; 150: 358-364.
7. Hackstadt, T. Cell Biology. In Chlamydia: intracellular biology, pathogenesis, and immunity. Stephens R (ed). Washington DC USA American; Society for Microbiology; 1999; 101-138.
8. Hosseinzadeh S. Molecular basis in the interaction of Chlamydia trachomatis with human spermatozoa. Presented for th Ph.D. Sheffield. The University of Sheffield. 2002.
9. Caldwell HD, Kromhout J, Schachter, J . Purification and partial characterization of the major outer membrane protein of Chlamydia trachomatis. Infect Immun. 1981; 31: 1161-76.
10. Cles L, Bruch K, Stamm W. Staining characteristicsof six commercially available monoclonal immunofluorescence reagents for direct diagnosis of Chlamydia trachomatis infections. J Clin Microbiol. 1988; 26: 1735-1737.
11. Renton A, Filatova E, Ison C, Meheus A, Dmitriev G, Akovbian V, et al. Performance of direct fluorescent antibody tests for routine diagnosis of Chlamydia trachomatis in Russian sexually transmitted disease clinics. Int J STD AIDS. 2009; 20(4): 292-294.
12. Collett BA, Newhall WJ, Jersild RA, Jones RB. Detection of surface-exposed epitopes on Chlamydia trachomatis by immune electron microscopy. J Gen Microbiol. 1989; 135: 85-94.
13. Giles DK, Wyrick PB. Trafficking of chlamydial antigens to the endoplasmic reticulum of infected epithelial cells. Microbes Infect. 2008; 10(14-15): 1494-1503.
14. Su H, Watkins N, Zhang Y-X, Caldwell HD. Chlamydia trachomatis-host cell interactions: role of the chlamydial major outer membrane protein as an adhesin. Infect Immun.1990; 58: 1017-1025.
15. Su H, Raymond L, Rockey DD, Fischer E, Hackstadt T, Caldwell HD. A recombinant Chlamydia trachomatis major outer membrane protein binds to heparan sulphate receptors on epithelial cells. Proc Natl Acad Sci USA. 1996; 93(20): 11143-11148.
16. Zhang JP, Stephens RS. Mechanism of Chlamydia trachomatis attachment to eukaryotic host cells. Cell. 1992; 69: 861-869.
17. Joseph T, Bose S. A heat-labile protein of Chlamydia trachomatis binds to HeLa cells and inhibits the adherence of chlamydiae. Proc Natl Acad Sci USA. 1991; 88: 4054-4058.
18. Raulston JE, Davis CH, Schmiel DH, Morgan MW, Wyrick PB. Molecular characterization and outer membrane association of a Chlamydia trachomatis protein related to the hsp 70 family of proteins. J Biol Chem. 1993; 268: 23139-23147.
19. Raulston JE, Terry RP, Stephen TR, Wyrick PB. Localization of Chlamydia trachomatis heat shock proteins 60 and 70 during infection of a human endometrial epithelial cell line in vitro. Infect Immun. 1998; 66(5): 2323-2329.
20. Raulston JE, Davis CH, Paul, TR, Wyrick PB. Heat shock protein 70 kDa and the chlamydial envelope: an entry level position? In International Chlamydia Symposium. San Francisco: CA, USA. 1998; 83-86.
21. Schmiel DH, Raulston JE, Fox E, Wyrick PB. Characterization, expression and envelope association of Chlamydia trachomatis 28 kDa protein. Microb Pathogen. 1995; 19 (4): 227-236.
22. Chang JP, Stephens RS. Mechanism of Chlamydia trachomatis attachment to eukaryotic host cells. Cell. 1992; 69: 861-869.
23. Ting LM, Hsia RC, Haidaris CG, Bavoil PM. Interaction of outer envelope proteins of Chlamydia psittaci GPIC with the HeLa cell surface. Infect Immun. 1995; 63 (9): 3600-3608.
24. Stephens RS. Molecular mimicry and Chlamydia trachomatis infection of eukaryotic cells. Trends Microbiol. 1994; 2: 99-101.
25. Chen J, Stephens R. Trachoma and LGV biovars of Chlamydia trachomatis share the same glycosaminoglycan-dependent mechanism for infection of eukaryotic cells. Mol Microbio. 1994; 11: 501-507.

26. Taraktchoglou M, Pacey AA, Turnbull JE, Eley A. Infectivity of Chlamydia trachomatis serovar LGV but not E is dependent on host cell heparan sulfate. Infect Immun. 2001; 69: 968-976.
27. Mayhew TM, Mühlfeld C, Vanhecke D, Ochs M. A review of recent methods for efficiently quantifying immunogold and other nanoparticles using TEM sections through cells, tissues and organs. Ann Anat. 2009; 191(2): 153-170.