Differences in *Chlamydia trachomatis* growth rates in human keratinocytes among lymphogranuloma venereum reference strains and clinical isolates

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The pathogenesis of the primary stage of lymphogranuloma venereum (LGV) is poorly understood. There is no skin cell model and LGV pathogenesis studies are therefore carried out on cells of different origin. Moreover, such studies usually use reference strains, which may have evolved over the years in culture. In this study, a model was developed in which *Chlamydia trachomatis* enters and grows in human keratinocytes at 37 and 33 °C. Keratinocytes were infected with fresh clinical isolates and the three LGV reference strains L1, L2 and L3. Growth was monitored for 5 days post-infection using fluorescence microscopy and image analysis software. Chlamydial replication was quicker at 37 than at 33 °C, despite 33 °C being the temperature of human skin. The serovar L2 reference strain grew significantly faster than the other strains, although the fresh clinical isolates were also serovar L2. When grown in keratinocytes at 33 °C, the L2 and L3 reference strains produced much larger inclusions than the other strains tested. This model, which utilizes keratinocytes, better simulates the conditions present at the initial site of infection in LGV than previously published literature, making it a useful tool for future LGV pathogenesis studies. In addition, the results indicate that fresh clinical isolates should be included in LGV pathogenesis studies.

Received 11 March 2011

Accepted 20 June 2011

INTRODUCTION

Pathogenesis of the primary stage of lymphogranuloma venereum (LGV) is poorly understood. There is no skin cell model and LGV pathogenesis studies are usually carried out with characterized reference strains, which may have evolved over the years in culture and may no longer reflect the isolates infecting patients today. The primary site of infection for LGV is the skin and mucosa of the genital tract. Because keratinocytes are the dominant cell type in this epithelium, we used keratinocytes in this study.

Traditionally, the species *Chlamydia trachomatis* has been subdivided into two biovars, oculogenital (OG) and LGV biovars, based on the diseases they cause (Ward, 2002). However, clinical disease and the plasticity zone suggest that the species *C. trachomatis* should be divided into three biovars, the trachoma biovar, the genital biovar and the LGV biovar (Carlson *et al.*, 2005). LGV patients typically present in the secondary stage of the disease with inguinal lymphadenopathy after resolution of a painless genital ulcer (Schachter & Osoba, 1983); however, in our setting, patients usually present with a painful chancroid-like ulcer before the disease progresses to inguinal lymphadenopathy

(Sturm *et al.*, 2005). LGV is the second most common cause of genital ulcers in Durban, South Africa, and occurs in 13.6% of men (O'Farrell *et al.*, 2008) and 15.4% of women (Moodley *et al.*, 2003) with genital ulcers.

C. trachomatis is an obligate intracellular human pathogen with a biphasic life cycle. The infectious elementary body (EB) binds and enters a suitable host cell, matures to form a reticulate body that undergoes binary fission (Miyairi *et al.*, 2006) and then recondenses to form EBs, which are released upon host-cell lysis. Historically, LGV pathogenesis studies have been conducted on host cells that are different to those affected *in vivo*.

In order to understand better the pathogenesis of LGV, we created a model that simulated the initial site of infection for LGV organisms. Entry and growth in human keratinocytes was demonstrated for the first time by constructing a keratinocyte model. We used two temperatures: the core temperature (37 °C) and surface temperature (33 °C) of the human host. Cell metabolism inhibitors were not used, as these are not found *in vivo* at the site of infection and the absence of these would strengthen this model's utility in studies investigating the effect of *C. trachomatis* on keratinocytes. Additionally, we used fresh clinical isolates from our setting together with previously characterized reference strains.

Abbreviations: EB, elementary body; FBS, fetal bovine serum; LGV, lymphogranuloma venereum; OG, oculogenital; p.i., post-infection; SPG, sucrose/phosphate/glutamate.

The growth rate of *C. trachomatis* varies among strains. Strains of the invasive LGV biovar replicate more rapidly than those of the non-invasive OG biovar (Miyairi *et al.*, 2006). Chlamydial replication time is also affected by the type of host cell used (Miyairi *et al.*, 2006). As there is no information on the behaviour of chlamydia in keratinocytes, the growth pattern of the organisms in this cell type may differ from that in published literature. Additionally, there is no published work that compares chlamydial inclusion size among different chlamydial strains.

The purpose of this study was to answer the following questions. (i) Are chlamydia able to enter and replicate within human keratinocytes *in vitro*? (ii) Do chlamydia of the LGV biovar replicate more rapidly in keratinocytes at 33 or 37 °C? (iii) Are there differences in the chlamydial growth rates among reference strains of different biovars compared with fresh clinical isolates? (iv) Is there a difference in the size of the chlamydial inclusions among strains?

METHODS

Cell lines. McCoy (ATCC CRL-1696) mouse fibroblasts were used to propagate *C. trachomatis*, and HaCaT human keratinocytes were used in the experiments. The HaCaT cell line was donated by Professor Norbert E. Fusenig (German Cancer Research Centre, Heidelberg, Germany). This cell line has retained most of the characteristics of its wild-type counterparts (Boukamp *et al.*, 1988). McCoy cells were cultured in Eagle's minimum essential medium (BioWhittaker) supplemented with 2 mM L-glutamine, 25 mM HEPES and 2–10% heat-inactivated fetal bovine serum (FBS) (Gibco). HaCaT cells were cultured in RPMI 1640 (BioWhittaker) with 2 mM L-glutamine, 10 mM HEPES and 2–10% (v/v) heat-inactivated FBS. All cells were cultured at 37 °C unless stated otherwise.

Bacterial strains. Three LGV reference strains were used, as well as three fresh serovar L2 clinical isolates and one serovar E clinical isolate. The three LGV reference strains L1 strain 440 (ATCC VR-901B), L2 strain 434 (ATCC VR-902B) and L3 strain 404 (ATCC VR-903) were donated by Mr Frans Radebe (National Institute of Communicable Diseases, Johannesburg, South Africa). These strains were initially described by Schachter *et al.* (1969). The fresh L2 clinical isolates were collected from patients presenting in the primary stage of LGV (genital ulcers) at the Prince Cyril Zulu Communicable Diseases Clinic in Durban, South Africa, and were designated Ulcer Study 151 (US151), US162 and US197. They were all shown to be serovar L2 using sequence analysis techniques. The OG serovar E strain was isolated by Maleka *et al.* (1996) in our laboratory from a male patient presenting at the same clinic with urethritis. This strain was typed in the Division of Allergy and Infectious Diseases, Department of Medicine, University of Washington, Seattle, USA (by Dr M. Lampe), using mAb microimmunofluorescence.

Propagation of bacteria and standardization of inoculum. *C. trachomatis* strains were propagated in McCoy cell monolayers using standard techniques (reviewed by Black, 1997). Briefly, McCoy cells grown on glass coverslips within Trak vials (Redhill Surgical) were infected by centrifugation for 1 h at 1200 g and cultured at 37 °C for 2 days in Eagle's minimum essential medium containing 10 mM HEPES, 2 mM L-glutamine, 1 µg cycloheximide ml⁻¹, 10 µg gentamicin ml⁻¹, 5 µg amphotericin B ml⁻¹, 5.4 mg glucose ml⁻¹ and 10% (v/v) FBS. After 2 days' growth, infection was confirmed

using a MicroTrak *C. trachomatis* Culture Confirmation Test kit (Trinity Biotech) and fluorescence microscopy. Infected host-cell monolayers were lysed and the chlamydia were harvested in sucrose/phosphate/glutamate (SPG) buffer, aliquotted and frozen at -80 °C.

The infectious titre was determined by infecting McCoy cell monolayers grown in 96-well flat-bottom microtitre plates in triplicate with tenfold serial dilutions of the frozen inoculum. The number of chlamydial inclusions was enumerated and the number of inclusion-forming units ml⁻¹ was calculated.

Growth curves. Unpolarized HaCaT cells were cultured overnight in 96-well flat-bottomed microtitre plates at a seeding density of 1.5 × 10⁴ cells per well. Before infection, the culture medium was removed and replaced with RPMI 1640 containing 10 mM HEPES, 2 mM L-glutamine, 10 µg gentamicin ml⁻¹, 5 µg amphotericin B ml⁻¹, 5.4 mg glucose ml⁻¹ and 10% (v/v) FBS. Cycloheximide was not used in any of the experiments, because it inhibits eukaryotic cell replication by interfering with the 60S ribosomal subunit (Abou Elela & Nazar, 1997). This would prevent eukaryotic host cells from behaving as they would in an *in vivo* situation. Cells were infected by centrifugation for 1 h at 1200 g in triplicate at an m.o.i. of 0.025 or with sterile SPG buffer as a negative control. After centrifugation, the 96-well plates were incubated at 37 or 33 °C for 1 h, and then the culture medium was replaced with fresh culture medium and the plates were returned to their respective incubators for 5 days. On each day post-infection (p.i.), one plate incubated at each temperature was fixed and stained using a MicroTrak *C. trachomatis* Culture Confirmation Test kit. Stained cell monolayers were visualized and the number of chlamydial inclusions per field of view was enumerated at low power using an Olympus BH2-RFCA fluorescence microscope. The mean number of inclusions per field of view was used to calculate the number of inclusions per well. Data were normalized across three experiments by dividing by the starting number of cells.

Photography and image analysis. The stained cell monolayers were photographed (×10 objective lens) in colour using a Nikon ColourView Soft Imaging System digital camera attached to a Nikon Eclipse E600 fluorescence microscope. The same exposure time and red, green and blue balance was used for all pictures captured. Images were analysed using the Soft Imaging System version 3.2. The area occupied by chlamydia (green) and the area occupied by cells (red) was calculated using the phase analysis function on each image captured. The chlamydial inclusion size (µm²) was measured for HaCaT cells at 33 °C for days 2–5 p.i.

Statistical analyses. Each experiment was conducted three times in triplicate. Statistical analyses were performed using one-way analysis of variance (ANOVA) with Tukey's post-test, or Kruskal–Wallis non-parametric ANOVA with Dunn's multiple comparisons test. SPSS version 15.0 and GraphPad Instat version 3.00 were used for statistical analyses. Significance was set at $P \leq 0.05$.

Ethics approval. The study was approved by the BioResearch Ethics Committee of the University of KwaZulu-Natal, South Africa (reference number H184/04).

RESULTS AND DISCUSSION

Previous studies have utilized cells that were not the native host cell for LGV chlamydia, conditions that did not resemble those at the initial site of infection, and reference strains rather than fresh clinical isolates. In this study, we utilized conditions that resembled more closely the initial site of infection for LGV, which is the skin. We used a

keratinocyte cell line and no cell growth inhibitors, because these are not present *in vivo*, together with a low m.o.i. to ensure the presence of sufficient uninfected cells for progeny organisms to infect at the end of the first 48 h chlamydial life cycle.

Replication of *C. trachomatis* in human keratinocytes *in vitro*

C. trachomatis entered and replicated in human keratinocytes *in vitro*, but replication was more efficient at 37 °C than at 33 °C for all strains tested ($P<0.001$), even though LGV begins as a genital ulcer on skin, which has a temperature of 33 °C.

Chlamydial growth over time, expressed as the number of inclusions per 10 000 cells and area occupied by chlamydia,

is shown in Fig. 1. Inclusion counts is a typical method used to quantify chlamydial growth, but we also measured the area occupied by chlamydia, as this takes into account not only the number of inclusions but also their size, and is therefore more representative of the actual number of organisms present.

At 37 °C, the L2 reference strain replicated more rapidly than the other strains, including the three serovar L2 clinical isolates (Fig. 1a, b). This marked difference in behaviour between the L2 reference strain and fresh L2 clinical isolates indicates a need for fresh clinical isolates for use in pathogenesis studies.

At 33 °C, there was a steady increase in the area occupied by chlamydia for most strains (Fig. 1c), but the serovar L2

Fig. 1. Chlamydial growth curves as indicated by mean area occupied by chlamydia per 10 000 cells (a, c) and mean number of inclusions per 10 000 cells (b, d) in HaCaT cells at 37 °C (a, b) and 33 °C (c, d). *, $P<0.05$; ***, $P<0.001$.

reference strain was the only strain with an increase in inclusions per 10 000 cells over the 5-day period (Fig. 1d). This indicates that the progeny EBs for this strain were infectious when grown in HaCaT cells at 33 °C. This is in agreement with the results obtained by Fields *et al.* (2002) using the same strain. The number of inclusions produced in monolayers infected with other strains was either stable at the various times tested or declined. As the serovar L2 reference strain was shown to replicate more rapidly than any other strain at 37 °C, and the marked increase in inclusions per 10 000 cells at 33 °C only occurred between days 4 and 5 p.i., it is possible that an incubation period of 5 days is too short for the same effect to be observed with the other strains tested. The decline in inclusions per 10 000 cells observed with serovars L1 and L3 may have been due to the rupture of mature inclusions. The resulting inclusions produced by progeny EBs could be too small to be detected at low magnification at this time point. Although the burst size was not included in the analysis, the increase in the number of inclusions over time is indicative that the progeny EBs were mature and able to escape and infect neighbouring cells.

C. *trachomatis* reference strains produce larger inclusions than fresh clinical isolates in HaCaT cells at 33 °C

The size of chlamydial inclusions was quantified by measuring the area of individual inclusions in HaCaT cells at 33 °C from 2 to 5 days p.i. At 1 day p.i., inclusions in monolayers incubated at 33 °C were smaller than those in monolayers incubated at 37 °C and were too small to visualize and enumerate at low magnification. Van Ooij

et al. (1998) and Fields *et al.* (2002) both reported a smaller inclusion size when infected cells were incubated at 32 rather than 37 °C.

At 2 days p.i., the area of most inclusions was about 100 μm^2 (Fig. 2a). Inclusions of the L2 reference strain grew rapidly from day 2 to 4, and those of L3 from day 3 to 4. The other strains exhibited a more moderate inclusion growth. There was, however, a sharp decline in the median inclusion size from day 4 to 5 for both L2 and L3 reference strains. Despite the low median inclusion size, the maximum inclusion size for these strains at day 5 was still higher than the maximum inclusion size produced by any other strain. The data were skewed by the presence of numerous smaller inclusions at this time point. The range of area values is plotted for each day p.i. in Fig. 2(b).

The overall data indicated an extremely significant difference in inclusion size among the strains ($P < 0.001$), and post-testing revealed extremely significant differences between most pairs of strains. There were also extremely significant differences between the strains at each day p.i. ($P < 0.001$).

Of particular interest is the fact that the fresh clinical isolates were also shown to be serovar L2, but they reached a maximum inclusion size of about 1000 μm^2 compared with 4489 μm^2 for the L2 reference strain. This again indicates a difference in the behaviour of the reference strains compared with the fresh clinical isolates. Either the reference strains have been modified by decades of culture under conditions different to those *in vivo* or the strains present in our setting are different to the reference strains. Either way, these results indicate that fresh clinical isolates are imperative for pathogenesis studies of LGV.

Fig. 2. Median size (a) and measure of dispersion (b) of chlamydial inclusions in HaCaT cells at 33 °C from 2 to 5 days p.i.

In conclusion, our model of infection of keratinocytes by *C. trachomatis* offers a better simulation of the conditions present at the initial site of infection in LGV than previously published literature, making it a useful tool for future LGV pathogenesis studies. In addition, our study demonstrated that fresh clinical isolates should be included in LGV pathogenesis studies.

ACKNOWLEDGEMENTS

We thank Gaetan Kabera from the Medical Research Council, South Africa, for advice with the statistical analyses, and Fatima Khan from the HIV Pathogenesis Program, University of KwaZulu-Natal, South Africa, for determining the serovars of the LGV clinical isolates using sequence analysis techniques.

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