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In a Transmission Electron Microscope (TEM) energised electrons are used to highlight the morphology and composition on samples under test. With a potential magnification of 1 nanometre, and a high resolution, two dimensional images are produced thereby, allowing for a wide range of applications in both science and technology. TEM consists of the following components: an electron source, thermionic gun, electron beam, electromagnetic lenses, vacuum chamber, two condensers, a sample stage, a fluorescent/phosphor screen and a computer. The basic principal is similar to an optical microscope, however in TEM the photons are replaced by electrons with electromagnetic lenses whilst the images are viewed under a screen projected onto the computer screen. For TEM analysis, samples need to be sliced thin enough for electrons to pass through. The principal of transmission occurs as follows: the speed of electrons is directly correlated to the wavelength of the electron which produces the quality and detail of the image. The lighter areas represent an area of a greater number of electrons passing through the sample and the darker areas reflect the dense areas of the sample. With these differences, information on the structure, texture, shape and size of the sample is provided.

Using the principal of TEM, a study of the life cycle of the organism Chlamydia trachomatis was undertaken. Comparison of the structures of C trachomatis, scanned by TEM and light microscopy, was investigated. McCoy cell line monolayers were prepared and co-cultivated with C trachomatis. Samples were collected and processed for the presence of C trachomatis using an iodine stain technique. Similar samples were prepared for transmission electron microscopy (TEM). Prepared slides from samples incubated at 24, 48, 60 and 72 hours post infection were viewed and data was documented. All electron microscopy samples were prepared using the following technique: cryofixation, fixation, dehydration, embedding, sectioning, staining, freeze-fracture, freeze-etch and sputter coating. TEM produced a high-resolution, black and white image from the interaction with prepared samples. The developmental process of C trachomatis using TEM was compared to that using light microscopy. The findings suggested that the co-infected McCoy cells with C trachomatis was detectable intracellular in parallel cultures viewed by using light microscopy and TEM. In both TEM and light microscopy observation of infected cells revealed that the developmental cycle of the organism corresponded at 24, 48, 60 and 72 hours post infection.

Key words: TEM technique, C trachomatis, McCoy cells, tissue culture, electromagnetic lens

Introduction

Transmission Electron Microscope (TEM) uses energetic electrons to highlight differentiate morphology and composition of an organism in medical laboratories. With a potential magnification of 1 nanometre, and a high resolution, two dimensional images are produced thereby, allowing for a wide range of applications in both science and technology.[1,2] TEMs consist of the following components: an electron source, thermionic gun, electron beam, electromagnetic lenses, vacuum chamber, two condensers, sample stage, phosphor/fluorescent screen and a computer. The basic principal is similar to an optical microscope, however in TEM the photons are replaced by electrons with electromagnetic lenses whilst the images are viewed under a screen projected onto the computer screen. For TEM analysis, samples need to be sliced thin enough for electrons to pass through. The principal of transmission occurs as follows: the speed of electrons is directly correlated to the wavelength of the electron which produces the quality and detail of the image. [3] The lighter areas represent an area of a greater number of electrons passing through the sample and the darker areas reflect the dense areas of the sample. With these differences, information on the structure, texture, shape and size of the sample is provided. [4] Therefore, TEM produces a high resolution image between the prepared sample and the energetic electron within the TEM vacuum chamber. The image formed allow researcher to view samples on a molecular level thereby, making it possible to analyse the structure and texture [1, 3]
McCoy cell lines are widely used in culture techniques for medical diagnostics in the study of microorganisms and their interactions with host cells. Amongst the most popular cell lines used for experimental use, McCoy cells aptly demonstrates the interactions between the host cells death and infecting pathogens. The fibroblastic cell line was developed in 1955. These cells were taken and propagated in culture using the synovial fluid from a patient with degenerative arthritis [5]. Thereafter, the data was made available on McCoy cell lines concerning the morphological and immunological criteria used for characterization of these fibroblasts [6]. Different media can be used to culture McCoy cells such as Eagles Minimum Essential Medium (EMEM), Roswell Park Memorial Institute (RPMI) and Dulbecco’s Modified Eagle’s Medium (DMEM). The media vary according to the addition of foetal calf serum and other supplements such as antibiotics, glutamine and HEPES. [7]

McCoy cell lines and Chlamydia trachomatis Culture

The pathogen C. trachomatis infects the fibroblast similar to the eukaryotic host cell interaction, usually leading to the disruption of the infected cell. This interaction was substantiated by Gordon et. al, who confirmed that the introduction of McCoy cell as a method for diagnosis of both genital and ocular infections caused by C. trachomatis. [8] The cell susceptibility to infection with chlamydial strains was increased by pretreating McCoy cell with diethylaminoethylhextran, cycloheximide, cytohalasine B, 5-iodo-2-deoxyuridine, cycloheximide and centrifugation [9,10,11,12,13]

Cell culture serves as a standard for comparative detection of C. trachomatis from genital specimens by molecular techniques such as polymerase chain reaction (PCR) and ligase chain reaction (LCR) [13, 14]. With the current DNA amplification techniques the methodology of cell cultures are applied less frequently when isolating C. trachomatis. [12]. However, the McCoy cell line has advantages which are not duplicated by DNA techniques. Cultural techniques may allow: microorganisms to multiply and propagate and preserve microorganisms and even allow multiplication to occur; examination of the interactions between the host cell and the bacteria; the pathological effects which results from bacterial metabolism; Growth for antimicrobial susceptibility testing. [12,13]

Amongst all cell lines utilised such as, HEP2, HeLa 229, BGMK, and McCoy cells remain the leading cell line in investigations, as an in vitro model for infectious processes.
The interaction between bacteria and host cells takes place in the following sequence: adhesion of microorganisms to the eukaryotic cell and entering it, intracellular development with bacterial amplification and releasing of new chlamydial bodies out of the cell.

Cell structure and cell cycle

Existence of *C. trachomatis* is essentially parasitic with a lack a system for electron transport Cytochrome is absent and the organism cannot utilize adenosine triphosphate (ATP). Therefore the organisms are dependent upon host cell ATP and nutrients for their growth and replication. The cell wall constitutes a major outer membrane protein (MOMP) which contains cysteine peptidoglycan that inhibits phagolysosome fusion in the phagocytes. It can also allow for intracellular division as well as cell survival. [6] The method of growth and replication within the host cell is exclusive to *Chlamydiaceae*. [7, 8] The development cycle consists of infectious and non - infectious stages which are morphologically distinct cell types [9, 10, 11, 12]. The small (0.2um) elementary bodies (EBs), which are metabolically inactive infective particles, have a rigid cell wall, with a genomic structure of approximately 600 genes, and a plasmid with 7,498 base pairs (bp).

The inactive, intra cellular elementary bodies (EBs) differentiate into metabolically active reticulate bodies (RBs). These RBs (0.8-1.0 um), known as chlamydial inclusions, replicate by binary fission for several hours, until mature enough in the developmental cycle to repeat the release of small EBs. The developmental cycle takes approximately eight hours post entry to 18-24 hours [13]. At some time between 48-72 hours the cell membrane ruptures and releases a new generation of infective elementary bodies.

The infectivity and influence of the organisms appears to be controlled by the culture conditions and the nature of the host cells [14]. An epithelial environment and the anatomical cell line appear to be both optimal and crucial to chlamydial growth. With these factors in mind the aim of the study was to utilise these conditions and view the growth of *C. trachomatis* by applying an ultra- structural technique to view the developmental cycle.

### Materials and Methods

#### Preparation of Cell Culture

McCoy cell lines were prepared prior to culture. A “starter” culture, at 90% growth rate, was split into a number of cell culture flasks (Corning). The cells were grown in Eagles Minimum Essential Medium (EMEM) with 10 % foetal calf serum and antibiotics (penicillin, amikacin and amphotericin B). All procedures were carried out aseptically and flasks with a confluent growth of 90%-100% of McCoy cells were used. The medium was removed and the cells washed twice with phosphate buffered saline (calcium and magnesium free solution). A volume of 2 mls of trypsin solution was added and the flask gently rocked to spread the solution evenly over the cell sheet. Most of the trypsin mixture was poured off (1.5 mls) and digestion was allowed to continue at 37°C until the cells were separated. Media (10 mls) was added to the flask and by mechanically pipetting the cell/media mixture, the cell clumps were broken up. Dilutions of the re-suspended cells were made up as required e.g. 1:3. Volumes of 2 mls were dispensed into shell vials and grown at 37°C for over 24 hours. A volume of 10 mls of re-suspended cells in media, were seeded into new flasks and maintained as ‘starter’ cultures for further tissue culture work. McCoy cells were passaged each third day of incubation at 37°C.

#### Direct Fluorescent Antibody and Iodine Staining of Cell Culture

Staining and mounting of slides for the Direct Fluorescent Antibody (DFA) was done according to manufacturer’s instruction. Monolayers were examined immediately after staining the fluorescence microscope. Optimum clarity was obtained with the use of 100x magnification for scanning and 250x magnification for confirming the morphology of the organism. The positive and negative controls were used as a reference in evaluating culture specimens. Control slides were included in the viewing. Positive control slides showed inclusion bodies with characteristic apple-green fluorescent stains. The negative controls did not take up the stain. Similarly cell culture slides were stained using the Iodine method. Slides were viewed using light microscopy at 10x magnification and morphological confirmation at 100x magnification. *C. trachomatis* was observed as dark brown inclusions within the McCoy cell.

#### Processing of Tissue Culture for Transmission Electron Microscopy

Chlamydia infected McCoy cell monolayers in tissue culture flasks were harvested at the time points 24, 48, 60 and 72 hours post infection. Growth was stopped at the respective time intervals. The cultures were fixed in 1% gluteraldehyde, in Eagles Minimum Essential Medium (EMEM with pH 7.2) for 1 hour at 4°C, washed twice in EMEM for 10 minutes and post fixed in 1% osmium tetroxide for 1 hour. Thereafter, the cells were rinsed with distilled water.
After several rinses in distilled water, the samples were dehydrated in (A) grade series of ethanol and embedded in resin. The cells were subsequently processed using conventional techniques.

**Ultramicrotomy**

Semi-thin sections (1µm) were cut with a Reichert ultracut ultramicrotome using glass knives. Sections were collected onto glass slides, heat fixed, stained with 1% alkaline to Toluidine blue and examined with a Nikon Optiphot photomicroscope. Fields of interest were selected and located on the block face and the block trimmed to produce a “mesa” with a trapezoidal shape. Ultra thin sections (50-60 nm) were cut, collected onto uncoated 2000 mesh copper grids and double stained with uranyle acetate of Reynold’s citrate for 2-3 minutes respectively.

**Transmission Electron Microscopy**

Cells for transmission electron microscopy study were grown on Thermanox coverslips to 90% confluence. Sections were viewed on the JEOL 1200X transmission electron microscope at an accelerating voltage of 60-100KV. Images were photographed using Ilford fine grain plate film. Furthermore, the DFA and Iodine tests were also performed on all samples for the presence of EBs. Iodine stains were done to compare the various stages of *C trachomatis* from inoculation to the release of EBs.

**Results in Culture Medium**

Growth of *C trachomatis* in McCoy Cells Monitored by Electron Transmission Microscopy

McCoy cells, which are infected with *C trachomatis*, were examined using light microscopy and transmission electron microscope (TEM). Using a visually identified fluorescent stained culture slides revealed a difference in the minimum and maximum inclusion sizes attained within the McCoy cells (fig. 2). *C trachomatis* structures began as small inclusions which swelled and increased in size as the replication process continued, however, not all inclusions of *C trachomatis* appeared to reach the same maximum size simultaneously. Ultrastructural examination of the co-infected cells showed that between 1 to 24 hours the infective EB’s were adsorbed into McCoy cells for replication. (fig. 3A). At 48 hours formation of RB’s was seen (fig.3B). Examination at 60 hours revealed EB’s causing cell lysis and empty capsules (fig.3C). McCoy cells at 72 hours were empty of reticulate bodies or elementary bodies (fig. 3D).

![Figure 2A: Elementary bodies seen as apple green fluorescent *C trachomatis* structures](image_url)
Figure 2B: Fluorescent stained culture slides revealed a difference in the minimum and maximum inclusion sizes attained within the McCoy cells. *C. trachomatis* structures began as small inclusions which swelled and increased in size through the replication process. At 24 hours the infective EB’s were adsorbed into McCoy cells for replication. (fig. 2A). At 48 hours and 60 hours formation of RB’s and EB’s causing cell lysis and empty capsules respectively (fig. 2B and 2C). McCoy cells at 72 hours were empty of reticulate bodies or elementary bodies (fig. 2D).
Figure 3: McCoy cells infected with *C. trachomatis* were incubated at 37°C for 24 hrs, 48 hrs, 60 hrs and 72 hrs. Intracellular *Chlamydia trachomatis* inclusions are demonstrated with micrograph revealing a number of *C. trachomatis* inclusion bodies. Analysis of culture slides by TEM demonstrates the presence of multiple *C. trachomatis* inclusion bodies. Many of the EBs and RBs appears to adhere to the outer circle of the cell. Some cells reveal multiple inclusion bodies and some appear to have released their EBs at 60 hrs of incubation. At 72 hours the cell is an empty capsule depicting the release of EBs into the environment. The images of McCoy cells monolayer were obtained at a magnification of 200x.

Viable *C. trachomatis* can be cultured in a medium which can propagate and sustain its growth through the various stages of development. The technique for the isolation of *C. trachomatis* makes possible the study of the processes which occurs during the developing inclusions at different stages of the developmental phase of the cycle. The process can be viewed using Iodine staining techniques (fig 4 A, B and C)
Figure 4: Iodine stained at 24 hrs, 48 hrs and 60 hrs. (A) demonstrates the growth of Elementary Bodies (Ebs) within McCoy cell cultures. (B) Inclusions, seen at 48 hours in 37°C McCoy cell cultures. Reticulate Bodies (Rb) inclusion size appears distorted with a more intense colour. (C) Incubation of *C. trachomatis*-infected cultures at 60 hours post infection. The mechanism of Rbs has been elucidated by the ruptured McCoy cells. The 72 hr sample did not capture any inclusions and was therefore excluded.

Discussion

Collection of adequate clinical specimen is a fundamental prerequisite for the successful recovery of *C. trachomatis*. Various factors affect the formation of intracytoplasmic inclusion bodies in cells. Included among these factors are the quantity of the inoculum, the speed/momentum of centrifugation, temperature at which the inoculum is incubated, inhibitory effects of animal sera and the hydrogen ion concentration of media and buffer solutions. [15,16]. Again, culture conditions and route of inoculation appear to be important [14].

In current study the performance characteristics of TEM on cell cultures was used. The testing of specimens submitted to the laboratory for ultra-structural identification of *C. trachomatis* clearly indicated the growth cycle. Samples were collected and transported as recommended using the appropriate culture conditions and route of inoculation. McCoy cells was used as an *in vitro* culture system to document the growth stages of *C. trachomatis*, using the DFA and Iodine stains in comparison to transmission electron microscopy. Slides were prepared and viewed accordingly. During the initial stages of the investigation the presence of EB’s were verified in smears, using the DFA technique (Microtrak; Syva). The fluorescent labelled antibodies, directed against the major outer membrane for *C. trachomatis*, was used for visualisation. Small elementary bodied were detected within cells. Iodine stained cultures indicated inclusion bodies which were representative of EBs from the initial infection to 24 hours post infection. The development of RBs was viewed from 24-48 hours and as the developmental life cycle continues for *C. trachomatis* the various phases of EBs and RBs were observed. As in other studies positive smears were compared to the inoculum of similarly stained cell cultures (Microtrak, Syva). [17]. Small elementary bodies were detected within cells, changing into the metabolically active RBs within 8 hours post infection.

At 24 hours post-infection the cytoplasm of cells showed a membrane bound chlamydial inclusion. As the RBs appeared larger with maturation, the cell periplasmic space reduced in size. The process indicated that the newly infected McCoy cell line adsorbed the infective EB particles and development of RBs was noticed. Concurrently at 24 hours post infection there were some RBs replicated into EBs at the periphery of the inclusion. Samples at 24 hours post infection typically represented the mid-phase of *C. trachomatis* development, when RB’s were actively dividing. At 48 hours the cell was surrounded by a thin layer of cytoplasm with numerous EBs and RBs occupying most of the nuclear space. At 60 hours the cytoplasm of the uninfected cells showed several vacuoles whilst some inclusion particles in infected cells demonstrated no set pattern of division between the host cell cytoplasm and that of EBs and RBs. This finding is consistent with previous reports where it was demonstrated that the host cell cytoplasm and inclusion matrix with its associated chlamydial particles in some instances there was no distinct division. [18]

Overall the RB’s reorganise into EB’s and within 2-3 days the infected cells ruptured with the release of newly formed EB’s. The process became continuous as long as cells were available. The metabolically active cells propagated the existence of *C. trachomatis* and an indication of the reason on why chlamydial infections required prolonged treatment of the infection during the necessary long growth cycle.
Conclusion

*Chlamydia trachomatis* interaction with its host manifests as a series of complications. TEM offers a clear and precise view of the process of chlamydial growth. The various stages of growth as well as the genetic interactions of the organism are well documented. [19, 20] Similar to another study the ultrastructural view of the attachment and entry of *C. trachomatis* with a cell was used to monitor and observe appropriately the *in vitro* model within a host cell [21, 22]. The mechanism could eventually enable the development of newer and uncomplicated methods of patient diagnosis and treatment. Detection of *C. trachomatis* by cell culture maybe required for additional laboratory characterization of the organisms in any nucleic acid amplification test which is positive. Basically, the reversion to Koch's postulate becomes effective i.e. if we can grow it then it is present for identification. Furthermore, *C. trachomatis* has been found to facilitate the acquisition and transmission of HIV infection. Therefore, as an opportunistic infection in HIV patients' syndromic management of the organism is imperative. Recurrent and latent infections are the major challenges in both the symptomatic and asymptomatic control of the bacteria.

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References