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# Deoxyribonucleic Acid Synthesis, Cell Cycle Progression, and Division of *Chlamydia*-Infected HeLa 229 Cells

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The fate of lymphogranuloma venereum strain *Chlamydia*-infected HeLa 229 cells was examined by determining the rate of deoxyribonucleic acid synthesis and the kinetics of entry into and progression through S phase and by time-lapse cinemicrography. At an input multiplicity of 5 or less, *Chlamydia*-infected cells showed no inhibition of host deoxyribonucleic acid synthesis or cell cycle progression. Cinemicrography showed division of inclusion-containing cells, with one or both daughters receiving chlamydial inclusions. Analysis of the family trees indicated that the generation times of infected HeLa 229 were not altered relative to those of the uninfected cells.

There is conflicting evidence with regard to the effect of chlamydial infection on the synthesis of host deoxyribonucleic acid (DNA). Although some studies have shown inhibition (7, 9, 13), other reports have indicated that *Chlamydia*-infected cells continued to synthesize DNA and divide (4, 8). More recently, Horoschak and Moulder (6) have examined the development of clones of L cells infected with varying multiplicities of *Chlamydia psittaci* and *Chlamydia trachomatis*. Photomicrographs of Giemsa-stained clones of infected L cells showed that the mean number of cells in colonies of *C. psittaci*-infected cells, representing successive cell divisions, was decreased but not zero. This analysis indicated that at a multiplicity of infection (MOI) of about 1, most of the infected cells divided once, and the infected daughter cells divided one or more times at a significantly slower rate than uninfected controls. At a high MOI, a much lower fraction of infected cells divided to produce colonies.

In this report we present direct observation by time-lapse cinemicrography of HeLa 229 cells infected with the lymphogranuloma venereum LGV $L_2$/434/Bu strain of *C. trachomatis*. Analysis of films has enabled us to assess directly the effect of chlamydial infection on cell division and generation time. Corroborating evidence is presented with respect to the rate of DNA synthesis and the cell cycle progression of infected HeLa cells.

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## MATERIALS AND METHODS

**Cells.** HeLa 229 cells obtained from C. C. Kuo, Department of Pathobiology, School of Public Health and Community Medicine, University of Washington, Seattle, were grown as monolayer cultures in minimal essential medium supplemented with 5% heat-inactivated fetal calf serum (K. C. Biologicals, Lenexa, Kans.) and 40 μg of gentamicin per ml. Cells were counted with a Celloscope particle counter (Particle Data, Elmhurst, Ill.).

***Chlamydia.*** The LGV $L_2$/434/Bu strain of *C. trachomatis*, supplied by C. C. Kuo, was propagated in HeLa 229 cells. Stock cultures were prepared by infecting monolayers with a small volume of inoculum containing a sufficient number of chlamydiae to infect >90% of the HeLa cells. After a 1-h adsorption period at 37°C, unadsorbed inoculum was removed, and monolayers were fed with growth medium containing 2 μg of cycloheximide per ml. After 48 h at 37°C, infected cells were detached, centrifuged at 1,000 × *g*, and resuspended in growth medium. The cell suspension was distributed in screw-capped vials and stored at −70°C. Before the chlamydiae were used for infection, the cell suspension was thawed and rapidly passed several times through a 25-gauge needle to disrupt the HeLa cells and release infectious chlamydiae.

**Infectivity titrations.** Inclusion-forming units (IFU) were estimated in the chlamydial stocks by the method of Furness et al. (5).

**[$^3$H]thymidine incorporation into HeLa nuclear DNA.** Rates of incorporation of the radioactive precursor into cold trichloroacetic acid-insoluble material located in the nuclear fraction of uninfected and LGV-infected HeLa cells was measured at different times after infection by using 10 μCi of [$^3$H]thymidine (New England Nuclear Corp., Boston, Mass.) per dish in appropriate medium, with incubation at 37°C for 1 h. Washed cells were detached and quantitatively

transferred to centrifuge tubes, 0.1 volume of 10% Triton X-100 was rapidly added, and the solution was mixed. Nuclei were sedimented at 2,000 × *g* in 5 min and dissolved in 1% sodium dodecyl sulfate, and trichloroacetic acid-insoluble radioactivity was determined (3).

**Cell cycle progression of LGV-infected HeLa 229 cells.** Mitotic cells were detached (10) from exponentially growing monolayer cultures of HeLa cells and seeded into 35-mm culture dishes at $5 \times 10^3$ cells in 1 ml per dish. After 3 h a series of cultures was infected with LGV at a MOI of 3, allowing 1.5 h at 38°C for adsorption. Rates of DNA synthesis in the synchronous HeLa cells were measured in duplicate dishes by using [2-$^{14}$C]thymidine (0.2 μCi/dish) for a 0.5-h pulse. After washing and acid treatment, bottoms of culture dishes were punched out, and the incorporated radioactivity was counted in a low-background (1.2 cpm) Geiger counter (10).

**Time-lapse cinemicrography and film analysis.** HeLa cells, suspended at a concentration of $10^6$ cells per ml in growth medium, were infected with a volume of LGV suspension sufficient to provide an input MOI of 1 IFU/cell and shaken for 2 h at 37°C to allow attachment. A volume of the cell suspension, adjusted to yield 20 to 30 cells per microscope field (×10; phase-contrast objective) was then delivered into a 35-mm plastic culture dish containing 3 ml of medium. Dishes were fixed in position in a special filming chamber (12) which was maintained at 38°C and equilibrated with a humidified atmosphere of 5% $CO_2$ in air. Exposures were made through a Nikon inverted microscope at 5-min intervals for 65 to 70 h. Computer-aided analysis of the developed 16-mm films was carried with a variable-speed projector (Tagarno, Horsens, Denmark) modified by the addition of a shaft angle encoder to its mechanical frame counter; the encoder was interfaced to a minicomputer (11). Events of interest, such as the time of appearance of an inclusion body, mitosis, and cell disintegration, were entered on the keyboard of the computer as single-character codes (see legend to Fig. 3), and the frame number at which the event occurred was automatically recorded. Data were analyzed with the aid of a computer program that yields time-proportional family trees and lists generation times (11).

## RESULTS

**Effect of LGV infection on HeLa DNA synthesis.** Over a 64-h period after LGV infection at an input MOI of 5 IFU/cell, the rate of incorporation of [$^3$H]thymidine into trichloroacetic acid-insoluble material in the nuclei of HeLa 229 cells was essentially the same as that in uninfected cells (Fig. 1). That the synthesis measured in LGV-infected cells represented only host DNA synthesis was ascertained by the observation that cycloheximide, a selective inhibitor of eucaryotic protein and, indirectly, of DNA synthesis (2), was as effective in inhibiting incorporation of [$^3$H]thymidine in the nuclear material of uninfected HeLa cells as it was in inhibiting incorporation in LGV-infected cells

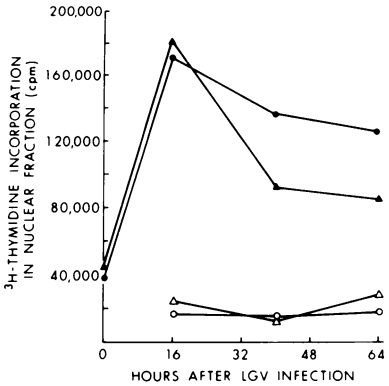


FIG. 1. *Effect of LGV infection on the rate of incorporation of [$^3$H]thymidine into cold trichloroacetic acid-insoluble material in the nuclei of HeLa 229 cells. Replicate cultures ($10^6$ cells per 5-cm dish) were infected with LGV at an input multiplicity of 5 IFU/cell. One-half of the cultures, both uninfected and infected, were incubated with growth medium containing 2 μg of cycloheximide per ml. Rates of [$^3$H]thymidine incorporation were determined as described in the text. Symbols: ●, control uninfected HeLa 229; ▲, LGV-infected HeLa 229; ○, uninfected, with 2 μg of cycloheximide per ml; △, LGV infected, with 2 μg of cycloheximide per ml.*

(Fig. 1). Replication of chlamydiae occurs in the cytoplasm of host cells and is not inhibited by cycloheximide (1). Thus, at an MOI of 5 IFU per cell, there appeared to be no effect of LGV infection on the rate of nuclear DNA synthesis in HeLa 229 cells. Phase-contrast microscopy at 24 h postinfection showed that virtually every cell was infected.

**Effect of LGV infection on cell cycle progression of synchronized HeLa 229 cells.** We investigated the possibility that data previously obtained by others suggesting inhibition of DNA synthesis in *Chlamydia*-infected cells (7, 9, 13) could have resulted from the existence of a discrete phase in the cell cycle of host cells, at which time DNA synthesis was impaired. Therefore, the kinetics of entry into the S phase of HeLa 229 was studied in uninfected and LGV-infected cultures. Mitotic cells detached by the method of Terasima and Tolmach (10) were plated, and early G1 cells were infected with LGV at an input MOI of 3 IFU/cell. Kinetics of entry into S phase by uninfected and *Chlamydia*-infected cells were measured by pulsing the cultures with [$^{14}$C]thymidine at hourly intervals. The data shown in Fig. 2 clearly indicate that both cultures entered S phase 7 h after mitosis and reached the peak of DNA synthetic phase at the same time (13 h). Thus, infection of early G1 HeLa 229 cells with LGV produced no alteration in the entry into and progression

through S phase of the host cell cycle. Parallel infected cultures were examined 24 h postinfection. About 85% of the cells had the distinctive cytoplasmic inclusion.

On the basis of the biochemical evidence summarized in Fig. 1 and 2, it is clear that at these multiplicities of LGV infection host cell DNA synthesis was not inhibited.

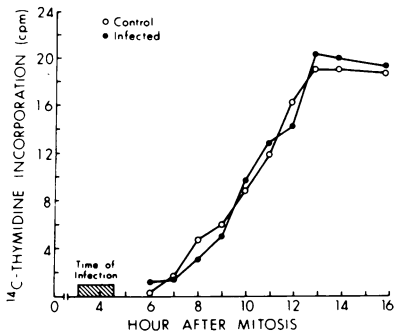


FIG. 2. *Progression through G1 and entry into S phase by uninfected and LGV-infected HeLa 229 cells. Mitotic cells (10) were plated and infected during early G1 with LGV at an input multiplicity of 3. Rates of DNA synthesis were determined as described in the text.*

**Time-lapse cinemicrography of LGV-infected HeLa 229 cells.** Proliferative behavior of cells in culture can be documented by time-lapse cinemicrography. The fate of individual cells in a given field—all residing in the same microenvironment, some infected and some uninfected, the latter serving as an internal control—were recorded and analyzed with respect to time of appearance of chlamydial inclusion bodies, fate of cells containing the inclusion bodies, generation time of uninfected and infected cells, and incidence of cell death. Such data were used to construct time-proportional family trees of individual cells and list generation times (11). An example of a family tree is shown in Fig. 3A, illustrating the spectrum of events observed in LGV-infected HeLa cell cultures.

Cell 4 divided at 457 min after infection, giving rise to daughters whose behavior is shown by branches a and b (Fig. 3A). In branch a, a chlamydial inclusion body was visible at 894 min, and this cell divided (a → k → m → o and p). The inclusion body was transmitted to one daughter at each division (a → l; k → n), and no inclusion developed in subsequent generations; i.e., apparently infection-free clones developed (o, p. q, r, u, v, y, and z). The lack of discernible

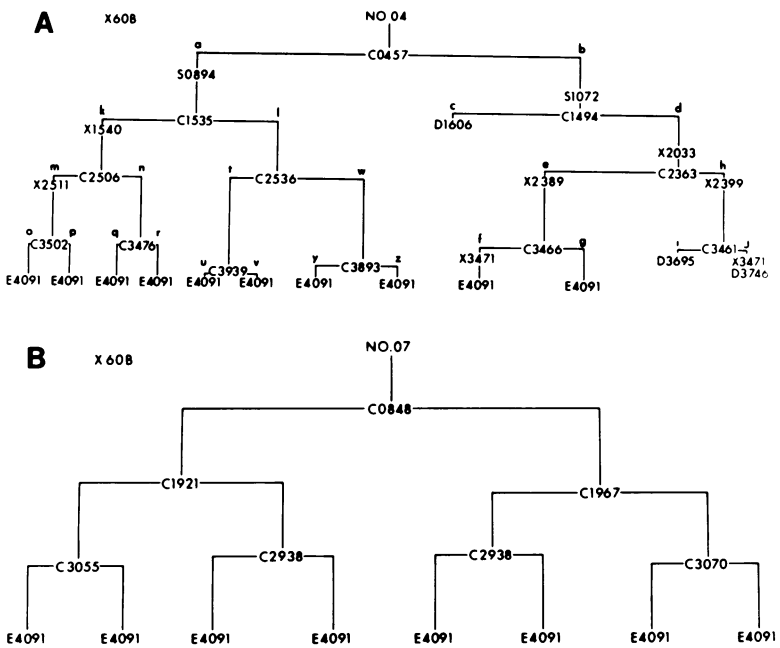


FIG. 3. *Time-proportional family trees of LGV-infected (A) and uninfected (B) HeLa 229 cells, generated by a computer program (11) of data obtained by time-lapse cinemicrography described in the text (experiment X60B). The symbols represent the following: N, beginning of infection, or history in case of uninfected cell; C, division or cytokinesis; S, time in minutes at which an inclusion body was first seen on the film; X, inclusion-containing cell after division; D, death of a cell; and E, end of film. For convenience in tracing the family tree, each cell has been identified with lower-case letters (a, b, c, etc.).*

inclusion bodies in o and p illustrates a phenomenon we have observed in several films; i.e., the inclusion body contained in a cell may not appear in either of the daughters. The basis for this event is not presently understood. In branch b an alternate behavior is seen. An inclusion body became visible at 1,072 min, and after division of this cell, one daughter (cell c) died at 1,606 min. In the other daughter (cell d), a cytoplasmic inclusion became discernible at 2,033 min, and the cell divided at 2,363 min. Both daughter cells contained an inclusion (cells e and h), and both of these cells divided. The progeny cells i and j died a few hours later.

TABLE 1. *Generation times of LGV-infected and uninfected HeLa 229 cells*

| Cell type | No. of individual generations | Generation time (min) | | |
|---|---|---|---|---|
| | | Range | Mean | Standard deviation |
| Uninfected | 43 | 839–1,784 | 1,228 | 230 |
| Infected | 16 | 869–1,560 | 1,142 | 177 |

Figure 3B is a family tree of an uninfected HeLa 229 cell (cell 7) recorded in the same experiment. The cinemicrographic analysis allowed direct measurement of generation times of individual cells present in a given field of view. Table 1 shows that the lengths of the cell cycles of both uninfected and LGV-infected HeLa 229 cells were approximately the same, a conclusion consistent with the biochemical data (Fig. 1 and 2).

The division of an inclusion-containing HeLa 229 cell is documented in the series of time-lapse exposures (Fig. 4).

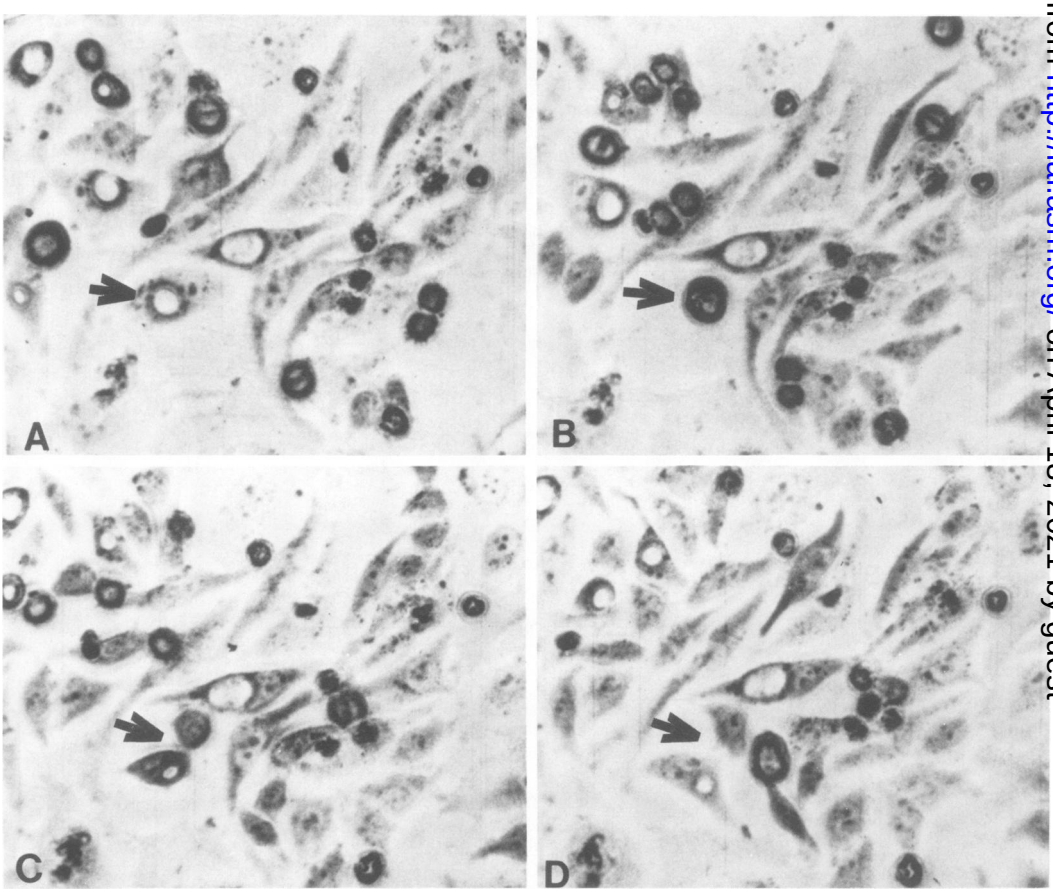


FIG. 4. *Selected frames from a time-lapse cinemicrograph of LGV-infected HeLa cells. (A) At 2,773 min after infection; arrow indicates an inclusion-containing cell. (B) At 2,835 min; the same cell is rounded up preparatory to division. (C) At 2,887 min; arrow indicates the daughter cells (only the lower one contains the inclusion body). (D) At 2,978 min, showing that the upper daughter remained free of the inclusion. A number of inclusion-free as well as inclusion-containing HeLa 229 cells can be seen in these frames.*

## DISCUSSION

Survival of *C. psittaci*- and *C. trachomatis*-infected L and HeLa cells has been documented recently by their colony-forming ability (6). Application of the well-known percent labeled mitosis analyses of *C. psittaci*-infected HeLa cells indicated that a prolongation of S phase and of generation time of the host was induced only at moderate to high MOI and in cells containing large inclusions (4). This elegant study also revealed that little effect was detectable in the early stages of infection. The authors suggested that the lag, before inhibition of HeLa DNA synthesis became apparent, could be due to a requirement of some development of *C. psittaci*. Biochemical evidence comprised of DNA synthetic activity and a direct measurement of the kinetics of entry into and progression through S phase by LGV-infected HeLa cells (Fig. 1 and 2) corroborate data recorded by time-lapse cinemicrography (Fig. 3 and 4 and Table 1). The following facts are apparent: (i) chlamydial inclusions are discernible in the cytoplasm of LGV-infected HeLa cells as early as 15 h after infection; (ii) inclusion-containing cells undergo apparently normal division; (iii) one or both daughter cells may receive the inclusion; (iv) in at least certain cases, a daughter cell without an inclusion appears to be free of chlamydial infection; (v) inclusion-containing daughter cells can divide again; (vi) the mean cell generation time is not altered by infection or by the presence of a visibly growing inclusion in the cells; and (vii) the inclusion-containing cells do exhibit a tendency to disintegrate.

The documentation by time-lapse cinemicrography of the frequent appearance of infection-free daughters from inclusion-bearing cells (Fig. 3A) extends the observations with *C. psittaci*-infected L cells (6). The films clearly show that this phenomenon is not due to a differential loss of inclusion-bearing cells, a possibility considered by Horoschak and Moulder (6).

Based on the data presented here and the recent results (6) of colony-forming ability of *Chlamydia*-infected cells, as well as on the impression derived from at least a dozen time-lapse movies, it seems that low-multiplicity chlamydial infection per se has no inhibitory effect on host DNA synthesis and cell division. Injury and explosive disintegration result from the enlargement of a chlamydial inclusion body, which occurs either in a time-dependent fashion in singly infected cells, or by the fusion of many inclusion bodies in multiply infected ones. Crocker et al. (4) repeatedly noted the significance of multiplicity of chlamydial infection and of inclusion size on the inhibitory effects on generation time, rate of entry into S phase, and its duration. We suggest that no *Chlamydia*-specified inhibitor of host DNA synthesis (and protein synthesis) is produced early in the chlamydial cycle of LGV replicating in HeLa 229 cells. It remains to be seen if other chlamydial strains would behave similarly after infection of HeLa or other susceptible cells.

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