

## Use of HL Cells for Improved Isolation and Passage of *Chlamydia pneumoniae*

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**We compared growth of the recently discovered respiratory pathogen *Chlamydia pneumoniae* in McCoy, HeLa 229, BHK-21, and HL cells. When cells were not pretreated with DEAE-dextran, HL cells had significantly higher mean numbers of inclusion-forming units (IFUs) on initial inoculation than the other cell lines. When cells were pretreated with DEAE-dextran, HeLa 229 and HL cells had equivalent mean numbers of IFUs on initial inoculation. HL cells had strikingly higher mean numbers of IFUs in passage than HeLa 229, BHK-21, or McCoy cells. In addition, HL cells did not require pretreatment with DEAE-dextran and could be used from 2 to 4 days after seeding. We conclude that HL cells are an excellent cell culture system for laboratory propagation of *C. pneumoniae* and may be a more sensitive cell line for initial isolation.**

In January of 1989, a new species of the genus *Chlamydia* was described by Grayston et al. (5). *Chlamydia pneumoniae*, formerly known as the TWAR agent, was first isolated in 1965 but has only recently been distinguished from *Chlamydia trachomatis* and *Chlamydia psittaci*. Previous studies have demonstrated its unique biological characteristics, its genetic distinction from other *Chlamydia* species, and its importance as a respiratory pathogen (1, 2, 4, 6, 7).

*C. pneumoniae* has proved to be a difficult organism to propagate in cell culture. Kuo et al. compared the ability of HeLa 229, McCoy, and L cells to support the growth of two laboratory strains of *C. pneumoniae* and found HeLa 229 cells superior (8). Kappus et al. presented similar findings when comparing HeLa 229 and McCoy cell lines (E. W. Kappus, M. Walsh, and T. C. Quinn, Program Abstr. 27th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 729, 1987). However, even when initial isolation has been achieved, the ability to propagate *C. pneumoniae* in cell culture has been less than satisfactory and little work comparing the effectiveness of various cell lines for passage of *C. pneumoniae* has been done. We report here a comparison of HL cells (a heteroploid line with a slow life cycle that is used for propagating respiratory viruses) with HeLa 229, McCoy, and BHK-21 cells for the isolation and passage of *C. pneumoniae* (3).

### MATERIALS AND METHODS

**Chlamydia strains.** One *C. pneumoniae* reference strain, TW-183, previously cultivated in HeLa 229 cells, and one *C. trachomatis* reference strain, E/UW-17, previously cultivated in McCoy cells, were studied. Three 10-fold dilutions of each stock strain were prepared in sucrose-phosphate-glutamate transport medium.

**Cell lines.** The four cell lines tested were HeLa 229 (obtained from C.-C. Kuo, Department of Pathobiology, School of Public Health, University of Washington, Seattle), McCoy (from our own laboratory), BHK-21 (ATCC CCL 10; American Type Culture Collection, Rockville, Md.), and HL (obtained from H. F. Maassab, Department of Epidemiology, School of Public Health, University of Michigan, Ann Arbor).

**Cell culture of *Chlamydia* type strains.** Our methods for inoculation and growth of *Chlamydia* strains have been previously described in detail (8, 9, 12, 15). In brief, McCoy, HeLa 229, and BHK-21 cells were seeded into 96-well microtest plates and 15-mm shell vials 24 h prior to inoculation. HL cells were seeded 48 h prior to inoculation. Cell monolayers were examined for confluency and were pretreated with DEAE-dextran (molecular weight,  $5 \times 10^5$ ) (D-9885; Sigma Chemical Co., St. Louis, Mo.; 30  $\mu\text{g}/\text{ml}$ ) in Hanks balanced salt solution for 15 min before inoculation. Comparison plates without DEAE-dextran were treated with Hanks balanced salt solution only. For each experiment, 100  $\mu\text{l}$  of each dilution of *Chlamydia* spp. was inoculated into six wells of a microtest plate and into three shell vials. Cycloheximide (Sigma C-6255; 1.5  $\mu\text{g}/\text{ml}$ ) was incorporated into the culture medium (Eagle minimal essential medium [Flow Laboratories, Inc., McLean, Va.] with 10% fetal bovine serum). Microtest plates and vials were centrifuged at  $1,200 \times g$  at 35°C for 60 min (J6-M centrifuge, 4.2 rotor; Beckman Instruments, Inc., Fullerton, Calif.), fresh medium was applied, and the plates were incubated at 36°C in 3% CO<sub>2</sub> for 48 h (*C. trachomatis*) and 72 h (*C. pneumoniae*) prior to staining. Vials were incubated for 3, 5, and 7 days and then sonicated for 10 s, and 2 ml of fresh medium was added. Three microtest wells containing cell monolayers of each of the four test cell lines were inoculated with 100  $\mu\text{l}$  from each of the four primary passage vials.

**Inclusion stain.** We used CF2, a genus-specific, fluorescein isothiocyanate-conjugated monoclonal antibody to *Chlamydia* lipopolysaccharide (C.-C. Kuo, Department of Pathobiology, School of Public Health, University of Washington, Seattle; available from the Washington Research Foundation, Seattle), to stain inclusions. Inclusions were counted by inverting the plate and scanning each well at a magnification of  $\times 10$  with a Neofluor lens and a Zeiss epifluorescence microscope. A 40 $\times$  long-working distance lens was used for confirmation.

**Statistical procedures.** *t* tests for the differences between means were used for comparison of groups.

### RESULTS

**DEAE-dextran enhancement of cell culture.** To determine the effect of DEAE-dextran pretreatment on the four cell

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TABLE 1. Comparison of the mean number of *C. pneumoniae* IFUs on initial inoculation by using four different cell lines with and without pretreatment with DEAE-dextran

Cell line	Dilution	Mean no. of IFUs <sup>a</sup>		Mean ratio <sup>b</sup>
		DEAE -	DEAE +	
HeLa 229	10 <sup>-1</sup>	20	37	1.6 <sup>c</sup>
	10 <sup>-2</sup>	140	195	
	10 <sup>-3</sup>	1,500	2,075	
McCoy	10 <sup>-1</sup>	17	20	1.3 <sup>d</sup>
	10 <sup>-2</sup>	117	137	
	10 <sup>-3</sup>	1,104	1,583	
BHK-21	10 <sup>-1</sup>	7	9	1.1
	10 <sup>-2</sup>	71	74	
	10 <sup>-3</sup>	742	725	
HL	10 <sup>-1</sup>	31	32	1.0
	10 <sup>-2</sup>	210	232	
	10 <sup>-3</sup>	2,483	2,325	

<sup>a</sup> Represents mean of six replicates for each inoculum.

<sup>b</sup> Mean ratio of number of IFUs with DEAE-dextran (+) to that without DEAE-dextran (-) for all dilutions.

<sup>c</sup> *P* < 0.001, pretreated versus untreated HeLa 229 cells (all dilutions).

<sup>d</sup> *P* = 0.02, pretreated versus untreated McCoy cells (all dilutions).

lines tested, *C. pneumoniae* and *C. trachomatis* were inoculated onto pretreated and untreated monolayers of each cell line. Significant increases in *C. pneumoniae* inclusion counts were seen with pretreated HeLa 229 cells and to a lesser extent with pretreated McCoy cells, but not with pretreated BHK-21 or HL cells (Table 1). In cells not pretreated with DEAE-dextran, the mean number of inclusion-forming units (IFUs) per milliliter of inoculum observed after initial inoculation of *C. pneumoniae* was  $2.6 \times 10^4$  for HL cells versus  $1.6 \times 10^4$  for HeLa 229 cells (*P* < 0.001). Significant increases in *C. trachomatis* inclusion counts were seen in DEAE-dextran-treated HeLa 229 cells but not in the other cell lines (Table 2).

**Comparative growth of *C. pneumoniae* and *C. trachomatis* by using HeLa 229, McCoy, BHK-21, and HL cells.** Significantly higher *C. pneumoniae* inclusion counts were seen after initial inoculation with both the HeLa 229 and HL cell lines than with McCoy and BHK-21 cells (Table 3). After passage, *C. pneumoniae* propagated for 3, 5, and 7 days in HL cells had strikingly higher inclusion counts than it did in the other cell lines (*P* < 0.001 for each comparison; Table 4). Microscopic evaluation of the HL cell monolayers at days 5 and 7 showed many small inclusions indicative of second-generation reinfection of the monolayer.

For *C. trachomatis*, initial inoculation onto HeLa 229, McCoy, and BHK-21 cells produced significantly higher inclusion counts than inoculation onto HL cells (Table 3). The highest inclusion counts for *C. trachomatis* in passage were seen when McCoy cells were passed after 3 days of growth, BHK-21 cells were passed after 5 days of growth, and HL cells were passed after 7 days of growth (Table 4).

### DISCUSSION

Grayston, Kuo, Wang, and colleagues have recovered *C. pneumoniae* from patients with acute pharyngitis, bronchitis, sinusitis, and pneumonia (6, 8). However, the organism has been considered fastidious, being difficult to recover in initial culture and to maintain and propagate in passage. Thus, improved methods for the isolation and propagation of

TABLE 2. Comparison of the mean number of *C. trachomatis* IFUs on initial inoculation by using four different cell lines with and without pretreatment with DEAE-dextran

Cell line	Dilution	Mean no. of IFUs <sup>a</sup>		Mean ratio <sup>b</sup>
		DEAE -	DEAE +	
HeLa 229	10 <sup>-1</sup>	85	103	1.2 <sup>c</sup>
	10 <sup>-2</sup>	650	746	
	10 <sup>-3</sup>	5,300	6,225	
McCoy	10 <sup>-1</sup>	84	92	1.1
	10 <sup>-2</sup>	623	633	
	10 <sup>-3</sup>	4,475	5,525	
BHK-21	10 <sup>-1</sup>	86	65	0.9
	10 <sup>-2</sup>	650	654	
	10 <sup>-3</sup>	5,075	6,075	
HL	10 <sup>-1</sup>	54	60	1.1
	10 <sup>-2</sup>	446	433	
	10 <sup>-3</sup>	3,525	3,850	

<sup>a</sup> Represents mean of six replicates for each inoculum.

<sup>b</sup> Mean ratio of number of IFUs with DEAE-dextran (+) to that without DEAE-dextran (-) for all dilutions.

<sup>c</sup> *P* = 0.05, pretreated versus untreated HeLa 229 cells (all dilutions).

*C. pneumoniae* would be valuable for both clinical and research purposes.

To date, relatively few studies have compared various cell lines and culture techniques for the isolation of *C. pneumoniae*. By using *C. pneumoniae* initially grown in yolk sacs, Kuo et al. reported that initial isolation of *C. pneumoniae* was better in HeLa 229 cells than in McCoy or L cells (8). No data on the efficiency of growth in these cell culture systems after passage were provided (8). Our results confirm their finding that HeLa 229 cells treated with DEAE-dextran were better than McCoy cells for initial isolation of *C. pneumoniae*. Of greater interest, however, was our finding that HL cells consistently demonstrated strikingly higher inclusion counts both upon initial inoculation and after passage than all the other cell lines tested. HeLa 229 cells were particularly poor in enhancing growth of *C. pneumoniae* in passage. These results are particularly striking in view of the fact that the strain we used, TW-183, has been adapted to HeLa cells. Further experience with the use of

TABLE 3. Mean ratio of IFUs observed on initial culture in HeLa 229, McCoy, and HL cells to IFUs observed in BHK-21 cells<sup>a</sup>

Host cell	Mean ratio <sup>b</sup>	
	<i>C. pneumoniae</i> <sup>c</sup>	<i>C. trachomatis</i> <sup>d</sup>
BHK-21	1.00	1.00
HeLa 229	3.29	1.25
McCoy	2.10	1.10
HL	3.31	0.74

<sup>a</sup> *n* = 18 for each cell line and *Chlamydia* species tested; all experiments used DEAE-dextran pretreatment.

<sup>b</sup> Mean ratio of IFUs observed on initial culture in HeLa 229, McCoy, and HL cells to IFUs observed in BHK-21 cells.

<sup>c</sup> *P* ≤ 0.001 for all comparisons, with the exception of *C. pneumoniae* growth in HeLa 229 cells compared with that in HL cells (no significant difference).

<sup>d</sup> *P* ≤ 0.001 for all comparisons, with the exceptions of *C. trachomatis* growth in BHK-21 cells compared with that in HeLa 229 cells (*P* = 0.002) and growth in BHK-21 or HeLa 229 cells compared with that in McCoy cells (no significant difference).

TABLE 4. Ratio of *Chlamydia* IFUs after second passage to IFUs on initial inoculation<sup>a</sup>

Host cell line	Days of growth	Ratio <sup>b</sup> (no.) <sup>c</sup>	
		<i>C. pneumoniae</i>	<i>C. trachomatis</i>
HeLa 229	3	15.0 (9)	55.7 (6)
	5	21.9 (9)	30.3 (6)
	7	4.4 (9)	8.9 (9)
McCoy	3	258.9 (9)	1,153.9 (3)
	5	1,051.0 (6)	1,560.0 (3)
	7	274.2 (9)	409.9 (6)
BHK-21	3	60.5 (9)	628.8 (6)
	5	434.0 (9)	7,055.8 (3) <sup>d</sup>
	7	52.3 (9)	324.6 (3) <sup>e</sup>
HL	3	336.4 (6)	144.7 (6)
	5	2,702.9 (3)	2,703.2 (3)
	7	17,252.4 (3) <sup>d</sup>	9,540.6 (3) <sup>d</sup>

<sup>a</sup>  $P \leq 0.001$  for all cell line passage comparisons at 3, 5, and 7 days, with the exception of *C. trachomatis* grown in McCoy cells compared with BHK-21 cells (no significant difference).

<sup>b</sup> Ratio of *Chlamydia* IFUs after second passage to IFUs on initial inoculation.

<sup>c</sup> Represents number of replicates which could be accurately read in second passage plates.

<sup>d</sup> Reported ratios may be higher because of culture counts too high for accurate assessment.

<sup>e</sup> Reported ratios may be higher because of elementary body cytotoxicity.

HL cells for wild-type strains is needed, but initial experience in our laboratory has demonstrated that HL cells permitted initial recovery and rapid growth in passage of two wild-type *C. pneumoniae* strains recovered from throat swabs.

Unfortunately, none of the cell lines tested were optimal for the isolation and propagation of both *C. pneumoniae* and *C. trachomatis*. HeLa 229 and McCoy cells have traditionally been used for culturing *C. trachomatis*, with BHK-21 cells being used less frequently. We found that these three cell lines had about equivalent sensitivity for isolation of *C. trachomatis*. However, growth of *C. trachomatis* in HL cells on initial inoculation was clearly inferior to that in the other cell lines tested. Pretreatment of cell lines with DEAE-dextran enhanced primary inclusion counts of *C. trachomatis* in HeLa 229 cells, was inconsistent in McCoy and BHK-21 cells (i.e., produced small increases or decreases in inclusion counts at various dilutions with no apparent consistent effect), and resulted in no enhancement in HL cells. Similar results have previously been reported by others (10, 11, 13, 14).

In summary, HL cells proved to be the most sensitive of those cell lines tested for the isolation and propagation of *C. pneumoniae* TW-183. In addition, these cells require fewer enhancement procedures, making it easier to work with them. Their sensitivity, for example, was not enhanced by pretreatment with DEAE-dextran, and thus this maneuver can be omitted. HL cell monolayers can also be set up and used from 2 to 4 days after seeding, while HeLa 229 cells must be used within 2 days of seeding. Finally, this cell line allows a specimen with few IFUs to reinfect the same monolayer if it is left intact without repeat centrifugation, thus enhancing recovery in this situation. Further studies with HL cells are needed to determine the optimal concen-

tration of cycloheximide, centrifugation speed, and temperature to be used, but our studies indicate that this cell line will be very useful to those studying *C. pneumoniae* and may well provide a more sensitive cell line for diagnostic confirmation of *C. pneumoniae* infection. Clinical studies with HL cells should be undertaken to assess their relative diagnostic sensitivity by using patient specimens.

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#### LITERATURE CITED

- Campbell, L. A., C.-C. Kuo, and J. T. Grayston. 1987. Characterization of the new *Chlamydia* agent, TWAR, as a unique organism by restriction endonuclease analysis and DNA-DNA hybridization. *J. Clin. Microbiol.* **25**:1911-1916.
- Campbell, L. A., C.-C. Kuo, R. W. Hissen, and J. T. Grayston. 1989. Isolation of a gene encoding a *Chlamydia* sp. strain TWAR protein that is recognized during infection of humans. *Infect. Immun.* **57**:71-75.
- Cavallaro, J. J., and A. S. Monto. 1971. HL cells, a sensitive line for the isolation and propagation of respiratory syncytial virus. *Proc. Soc. Exp. Biol. Med.* **140**:507-510.
- Chi, E. Y., C.-C. Kuo, and J. T. Grayston. 1987. Unique ultrastructure in the elementary body of *Chlamydia* sp. strain TWAR. *J. Bacteriol.* **169**:3757-3763.
- Grayston, J. T., C.-C. Kuo, L. A. Campbell, and S.-S. Wang. 1989. *Chlamydia pneumoniae* sp. nov. for *Chlamydia* sp. strain TWAR. *Int. J. Syst. Bacteriol.* **39**:88-90.
- Grayston, J. T., C. C. Kuo, S. S. Wang, and J. Altman. 1986. A new *Chlamydia psittaci* strain, TWAR, isolated in acute respiratory tract infections. *N. Engl. J. Med.* **315**:161-168.
- Grayston, J. T., C. Mordhorst, A. L. Bruu, S. Vene, and S. S. Wang. 1989. Countrywide epidemics of *Chlamydia pneumoniae*, strain TWAR, in Scandinavia, 1981-1983. *J. Infect. Dis.* **159**:1111-1114.
- Kuo, C.-C., H.-H. Chen, S.-P. Wang, and J. T. Grayston. 1986. Identification of a new group of *Chlamydia psittaci* strains called TWAR. *J. Clin. Microbiol.* **24**:1034-1037.
- Kuo, C.-C., and J. T. Grayston. 1988. Factors affecting viability and growth in HeLa 229 cells of *Chlamydia* sp. strain TWAR. *J. Clin. Microbiol.* **26**:812-815.
- Kuo, C.-C., S.-P. Wang, and J. T. Grayston. 1977. Growth of trachoma organisms in HeLa 229 cell culture, p. 328-336. *In* D. Hobson and K. K. Holmes (ed.), *Nongonococcal urethritis and related infections*. American Society for Microbiology, Washington, D.C.
- Kuo, C. C., S. P. Wang, B. B. Wentworth, and J. T. Grayston. 1972. Primary isolation of TRIC organisms in HeLa 229 cells treated with DEAE-dextran. *J. Infect. Dis.* **125**:665-668.
- Ripa, K. T. 1982. Microbiological diagnosis of *Chlamydia trachomatis* infection. *Infection* **10**(Suppl. 1):S19-S24.
- Ripa, K. T., and P. A. Mardh. 1977. New simplified culture technique for *Chlamydia trachomatis*, p. 323-327. *In* D. Hobson and K. K. Holmes (ed.), *Nongonococcal urethritis and related infections*. American Society for Microbiology, Washington, D.C.
- Rota, T. R. 1977. Trachoma agent under varying cell culture conditions: infection in McCoy and BHK-21 cells, p. 314-322. *In* D. Hobson and K. K. Holmes (ed.), *Nongonococcal urethritis and related infections*. American Society for Microbiology, Washington, D.C.
- Yoder, B. L., W. E. Stamm, C. M. Koester, and E. R. Alexander. 1981. Microtest procedure for isolation of *Chlamydia trachomatis*. *J. Clin. Microbiol.* **13**:1036-1039.