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Use of HEp-2 Cells for Improved Isolation and Passage of *Chlamydia pneumoniae*

PATRICIA M. ROBLIN,* WILSON DUMORNAY, AND MARGARET R. HAMMERSCHLAG

Division of Infectious Diseases, Departments of Pediatrics and Medicine, State University of New York Health Science Center at Brooklyn, Brooklyn, New York 11203

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Chlamydia pneumoniae **has proved to be difficult to isolate and propagate in cell culture. We compared the growth of three strains of *C. pneumoniae*, TW-183 and two clinical isolates from Brooklyn, N.Y., in five cell lines, including HeLa 229, McCoy, HL, HEp-2, and HTED, an immortalized human tracheal cell line. HEp-2 was the most sensitive cell line tested. When 10-fold dilutions of three *C. pneumoniae* strains at known titers were inoculated into the different cell lines, the mean number of inclusion-forming units per milliliter was 1 to 2 log units higher in the HEp-2 than in the other cell lines. This difference was statistically significant. Omission of pretreatment with DEAE-dextran resulted in larger inclusions than those seen in pretreated cells, with the exception of McCoy and HTED cells. Retrieval of clinical specimens previously cultured on HeLa 229 cells and comparison of mean inclusion counts in fresh clinical specimens simultaneously inoculated on HeLa 229 and HEp-2 cells suggested that culture in HEp-2 cells may require only the initial inoculation and one passage, compared with three to four passages, as required by culture in HeLa 229 cells.**

Chlamydia pneumoniae, the newly recognized third chlamydial species, appears to be a primary human respiratory pathogen accounting for as many as 10% of cases of community-acquired pneumonias (2, 4). *C. pneumoniae* has proved to be a difficult organism to isolate in cell culture. Kuo and Grayston (5) compared the abilities of HeLa 229 and McCoy cells to support the growth of two laboratory strains of *C. pneumoniae* and found HeLa 229 to be superior. In a previous study of *C. pneumoniae* infection in Brooklyn, N.Y. we were able to isolate *C. pneumoniae* from nasopharyngeal specimens of 15 patients (2). Only 2 of the 15 specimens were positive on initial inoculation; the remainder required two or three passages in HeLa 229 cells. The inclusions were difficult to identify because they were very small, and they were often lost during passage. Subsequently, Cles and Stamm (3) examined HL cells (a human cell line) and found them to be more sensitive than HeLa 229 cells for the propagation of *C. pneumoniae*. Even when initial isolation has been achieved, the ability to propagate *C. pneumoniae* in cell culture has been less than satisfactory. There are limited data comparing other cell lines in terms of their effectiveness for passage or their relative diagnostic sensitivities when patient specimens are used. These evaluations have also involved a limited number of strains, mainly TW-183, i.e., the prototype strain, and two clinical isolates from Seattle, Wash. We compared the growth and propagation of three strains of *C. pneumoniae*, including two clinical isolates from Brooklyn, in five cell lines: HeLa 229, HEp-2, HL, HTED, and McCoy.

MATERIALS AND METHODS

Chlamydiae. We examined three strains of *C. pneumoniae*: TW-183 (Washington Research Foundation, Seattle, Wash.), T2023 (a clinical isolate from Brooklyn [ATCC VR-1356]), and T2043 (a clinical isolate from Brooklyn [ATCC VR-1355]).

Cell lines. The five cell lines tested were HeLa 229 (ATCC CCL 2.1), McCoy (ATCC CRL 1696), HL (obtained from L. Cles, University of Washington), HEp-2 (ATCC CCL 23), and HTED, an immortalized human tracheal cell line (courtesy of D. Gruenert, University of California, San Francisco) (6). The latter was grown on flasks coated with a solution containing 1 mg of human fibronectin (Collaborative Research, Inc., Bedford, Mass.), 1 ml of Vitrogen 100 (Collagen Corp., Palo Alto, Calif.), and 10 ml of bovine serum albumin (1-mg/ml stock solution [Biofluids, Rockville, Md.]) made up to 100 ml with base medium (Biofluids).

Culture of *C. pneumoniae*. In order to assess the sensitivities of the five cell lines, the titers of 10-fold dilutions of a known titer of each strain of *C. pneumoniae* (10^5 inclusion-forming units [IFU]/ml) were determined in each cell line in duplicate 96-well microtiter plates. McCoy and HeLa 229 cells were seeded 24 h prior to inoculation. HTED, HEp-2, and HL cells were seeded 48 h prior to inoculation. All cell monolayers were examined on the day of inoculation for confluence. Cell lines were pretreated for 10 min either with 30 μg of DEAE-dextran (Sigma Chemical Co., St. Louis, Mo.) per ml in phosphate-buffered saline (PBS) or with PBS alone before inoculation. For each experiment, 100 μl of each dilution (10^{-1} to 10^{-10}) of both strains was inoculated onto eight wells of each cell line. All plates were centrifuged at 1,700 × *g* for 1 h and then incubated for 1 h at 35°C. Fresh overlay medium, Iscove's modified Dulbecco's medium (GIBCO) containing 1 μg of cycloheximide (Sigma) per ml and 10% fetal calf serum, was added, and plates were incubated for 48 and 72 h at 35°C. At 48 and 72 h, the first plate was fixed and stained with an antilipopolysaccharide (i.e., genus antigen) monoclonal antibody (Pathfinder Chlamydia Culture Confirmation System; Kallestad Diagnostics, Chaska, Minn.). The second plate was frozen at −70°C and then thawed and passed onto fresh monolayers, as described above. After 48 and 72 h of incubation, the cells were fixed and stained. The inclusions per well were counted, and the titer (IFU per milliliter) was calculated by averaging the number of inclusions in each well and then multiplying by the dilution factors.

Retrieval of *C. pneumoniae* from previously cultured patient

* Corresponding author.

TABLE 1. Comparison of five cell lines for growth of *C. pneumoniae*[a]

Strain	Passage	Highest mean IFU/ml detected				
		HEp-2[b]	HL	HeLa 229	McCoy	HTED
TW-183	1	2.4×10^7	2.6×10^6	4.8×10^5	2.8×10^4	5.9×10^6
	2	2.8×10^8	1.3×10^6	5.1×10^6	2.4×10^5	3.3×10^5
T2023	1	2.8×10^7	6.4×10^6	6.3×10^5	4.4×10^4	4.5×10^4
	2	1.4×10^8	8.0×10^6	6.5×10^6	3.3×10^5	6.3×10^5
T2043	1	1.2×10^7	6.6×10^6	5.9×10^5	5.1×10^4	5.1×10^4
	2	1.5×10^8	4.5×10^6	6.6×10^6	5.0×10^4	5.9×10^4

[a] Eight replicates at each well for 10 dilutions for each cell line and *C. pneumoniae* strain tested.
[b] $P = 0.05$; the results of the Duncan multiple-range test show that cell line HEp-2 is significantly superior to the other four cell lines. The Scheffe test of uniform variance shows that the variance of the concentration measured in HEp-2 cells is significant.

specimens. Nasopharyngeal specimens which were initially positive on HeLa 229 cells and which were stored at −70°C for up to 7 months were retested on HEp-2 cells. All these isolates were confirmed as *C. pneumoniae* by staining with a fluorescein-conjugated species-specific monoclonal antibody (Washington Research Foundation). Additional specimens selected were from patients who had serologic evidence of acute infection but whose cultures were negative on HeLa 229 cells. All specimens were inoculated on HEp-2 cells in duplicate plates, centrifuged at 1,700 × *g* for 1 h, incubated for 1 h, and overlaid with Iscove's Dulbecco modified Eagle medium with 1 μg of cycloheximide per ml. After 72 h, each specimen was fixed and stained as described above. The specimens were passaged an additional three times.

Comparison of mean IFU per milliliter in fresh clinical specimens simultaneously cultured in different cell lines. Fresh clinical specimens from patients with suspected *C. pneumoniae* infection were inoculated on different cell lines and cultured as described above. The specimens were passaged three times.

Statistical methods. A two-way analysis of variance examining the factors of cell line, isolates of *C. pneumoniae*, and passage number was performed. The Scheffe test of uniform variance was used to compare cell lines. Simultaneous cultures (mean IFU) were compared by using the Wilcoxon signed rank test (1), as the data were distributed nonparametrically.

RESULTS

Comparison of cell line sensitivity. When 10-fold dilutions of *C. pneumoniae* at known titers were inoculated into the different cell lines, the highest dilutions were detected in HEp-2 cells and were followed by those in HL, HeLa 229, McCoy, and HTED (Table 1). The results of the Duncan multiple-range test ($P = 0.05$) show that the HEp-2 cell line is significantly superior to all the other cell lines tested. The Scheffe test of uniform variance showed that the variance of the concentration measured in HEp-2 cells (mean number of IFU per milliliter) is significantly different from that measured in the other four cell lines tested. The duration of incubation, 48 or 72 h, did not seem to affect the number of the inclusions, but the inclusions were significantly larger at 72 h, which made detection easier. On passage after 48 or 72 h, the mean titer of *C. pneumoniae* detected in HEp-2 cells was 1 to 2 log units higher than those in the other cell lines (Table 1). Pretreatment with DEAE-dextran did not increase the number of inclusions but decreased the sizes of the inclusions in all cell lines except McCoy and HTED (Fig. 1).

Retrieval of *C. pneumoniae* from previously cultured patient specimens. Nine nasopharyngeal specimens that were previously culture positive on HeLa 229 cells were recultured on HEp-2 cells. HEp-2 cells were selected because they were the most sensitive in the initial evaluation and the limited amount of remaining specimen precluded testing in the other

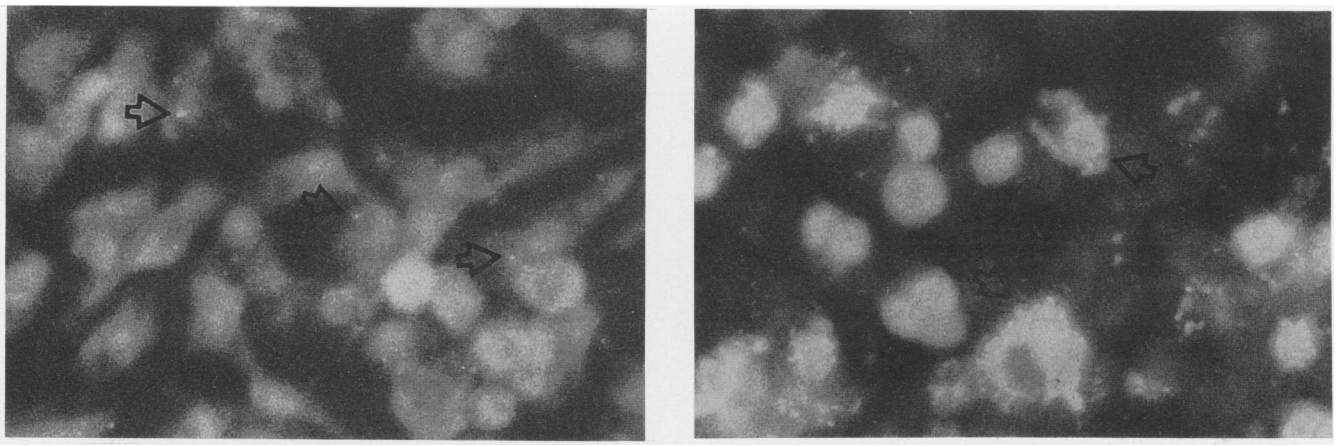

FIG. 1. Photomicrographs of HEp-2 cells infected with *C. pneumoniae* TW-183 and incubated for 72 h. Note the very small inclusions (arrows) in cells pretreated with DEAE-dextran (A) and the larger inclusions in untreated cells (B). Magnification, ×400.

TABLE 2. Reculturing of previously cultured clinical specimens on HEp-2 cells

Specimen	HeLa 229		Mean IFU/ml on HEp-2 (passage 1)
	Passage	Mean IFU/ml[a]	
T2294	2	10	0
T2297	3	15	45
T2303	3	20	2,700
T2306	3	10	175
T2309	3	25	10
T2312	2	10	120
T2319	3	45	10
T2314	3	10	25
T2317	3	15	1,620
T2287[b]	4	0	25
T2313[b]	4	0	40
T2315[b]	4	0	15

[a] $0.02 < P < 0.05$ (Wilcoxon signed rank test), HeLa 229 versus HEp-2 cells.
[b] Patient had serologic evidence of acute infection, but the specimen was initially culture negative on HeLa 229 cells.

cell lines. Eight of the nine specimens were found to be positive upon initial inoculation on HEp-2 cells (without DEAE-dextran pretreatment). All were positive for the first time after the second or third passage in HeLa 229 cells (Table 2). One specimen, T2294, was not retrievable even after four passages in HEp-2 cells. The titers of two specimens, T2303 and T2317, were 2 log units greater on HEp-2 cells than when the specimens were originally cultured on HeLa 229 cells. An additional three specimens, T2287, T2313, and T2315, which were still negative after four passages on HeLa 229 cells were retested in HEp-2 cells. All three patients had serologic evidence of acute *C. pneumoniae* infection (immunoglobulin M antibody titers of ≥1:8). These specimens were positive after the initial inoculation in HEp-2 cells (Table 2).

Comparison of mean IFU of fresh clinical specimens simultaneously cultured in HeLa 229 and HEp-2 cells. Sixty clinical nasopharyngeal specimens from patients with suspected *C. pneumoniae* infection, obtained between 16 March and 18 July 1991, were simultaneously inoculated onto HeLa 229 and HEp-2 cells. These specimens had been frozen at −70°C for 2 to 7 months. *C. pneumoniae* was isolated from 5 (8.3%) specimens in both cell lines. A comparison of the passage at which the organism was initially detected and the mean IFU per milliliter is shown in Table 3. None of the specimens were positive until the second passage with HeLa 229 cells, whereas three were positive after the first inoculation in HEp-2 cells. The mean number of IFU per milliliter in HEp-2 cells was significantly higher than that in HeLa 229 cells ($P = 0.05$, Wilcoxon signed rank test).

TABLE 3. Comparison of passage number and mean *C. pneumoniae* IFU in clinical specimens simultaneously inoculated in two different cell lines

Specimen	HeLa 229		HEp-2	
	Passage no.	IFU/ml[a]	Passage no.	IFU/ml
T2337	2	5	2	30
T2345	2	5	1	12
T2350	2	6	1	20
T2352	2	2	1	5
T2360	2	10	2	15

[a] $P = 0.05$ (Wilcoxon signed rank test), HeLa 229 cells versus HEp-2 cells.

DISCUSSION

C. pneumoniae has been considered a fastidious organism and a difficult organism to recover in initial culture and to propagate by passage. Improved methods for isolation and propagation of *C. pneumoniae* would be valuable in both clinical and research settings. Many previously published studies of infection with *C. pneumoniae* have depended on serology for diagnosis (4). However, we have reported several patients with culture-confirmed *C. pneumoniae* infection who failed to develop a serologic response (2). Culture is probably the "gold standard" for the diagnosis of this infection.

Thus far, few studies have compared various cell lines and culture techniques for the isolation of *C. pneumoniae*. Kuo and Grayston reported that initial isolation of *C. pneumoniae* was better in HeLa 229 than in McCoy cells (5); TW-183 and AR39 were the only strains tested. Subsequently, Cles and Stamm (3) found that HL cells, which are thought to be a human-lung-derived cell line, were consistently more sensitive on initial inoculation and passage; TW-183 was the only strain tested in that study. Kuo and Grayston (6) subsequently confirmed the finding of Cles and Stamm by using three strains of *C. pneumoniae*: TW-183, AR39, and AR388.

Our results suggest that HEp-2 is the most sensitive cell line tested so far for the isolation and propagation of *C. pneumoniae* for both laboratory and clinical strains. In addition, HEp-2 cells do not require pretreatment with DEAE-dextran, which greatly simplifies the culture procedure. In fact, the use of pretreatment with DEAE-dextran had an adverse effect on the recovery of *C. pneumoniae* in HEp-2 cells, resulting in very small inclusions which were difficult to see. Finally, culture of clinical specimens in HEp-2 cells may require only one passage, versus three to four passages required in HeLa 229 cells. HEp-2 cells are a well-defined cell line and are readily available from several sources, including the American Type Culture Collection.

HTED is a transformed human tracheal cell line that has been demonstrated to be sensitive for the propagation of various respiratory viruses, including parainfluenza virus (7). On the basis of these findings, we thought it might be a sensitive cell line for the isolation of *C. pneumoniae*. However, HTED cells appeared to be very similar to McCoy cells in terms of sensitivity.

Further clinical studies with HEp-2 cells should be undertaken to assess their relative diagnostic sensitivity by using patient specimens. It is possible that there are other cell lines that would also be suitable for the isolation of *C. pneumoniae*. A more efficient culture system will facilitate the diagnosis and help promote investigation and knowledge of this fastidious organism.

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