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Factors Affecting Viability and Growth in HeLa 229 Cells of *Chlamydia* sp. Strain TWAR

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Two prototype isolates (TW-183 and AR-39) of *Chlamydia* sp. strain TWAR were used to study factors affecting growth of this organism in HeLa 229 cells. The results showed that an incubation temperature of 35°C was better than one of 37°C for growth. The burst size after 3 days of incubation at 35°C was found to be small (13 to 52), which partially explains the difficulty of serial passage in cell culture. Application of a higher centrifugal force (1,700 × *g* versus 900 × *g*) at the time of inoculation enhanced growth 2.2 to 3.6 times. Infectivity was enhanced by treatment of cells with DEAE-dextran (2.4 times) or poly-L-lysine (1.6 times), but not with Polybrene or polyethylene glycol. The viability of the TWAR organism in chlamydia transport medium SPG was also studied. It was shown that the organism was rapidly inactivated at room temperature (22°C); only 1% remained viable after storage for 24 h. The viability was preserved at 4°C, and 70% remained viable after storage for 24 h. Freezing at −75°C inactivated 23% of the organisms when the organisms were frozen within 4 h after harvesting and stored at 4°C before freezing.

The TWAR strain of *Chlamydia* sp. is a newly described organism that causes acute respiratory disease (4, 6, 10). Recent studies suggest that it is a unique chlamydia not belonging to either recognized species, *Chlamydia trachomatis* or *Chlamydia psittaci* (1, 2, 6). Both embryonated chicken egg yolk sac and HeLa 229 cell culture have been used to isolate TWAR organisms (4, 6). However, TWAR has been more difficult to grow than other chlamydiae (6). We have reported preliminary studies showing that cell culture infectivity of TWAR organisms was enhanced by pretreatment of HeLa cells with DEAE-dextran, centrifugation of inoculum onto cell monolayers, and incubation of infected cells with medium containing cycloheximide (6). To further improve the cell culture isolation and passage of TWAR organisms, we have studied several chemical and physical treatments that might affect their viability and growth. The results from these studies are presented in this report.

MATERIALS AND METHODS

***Chlamydia* sp. strain TWAR.** Two prototype TWAR isolates were used. They were TW-183, a conjunctival isolate, and AR-39, a pharyngeal isolate (6). Inocula were either HeLa cell or egg-grown organisms. The titers of the stock inocula were 10^8 inclusion-forming units (IFU) or 10^4 50% egg infective doses per ml, respectively. Cell culture-adapted organisms were used for all experiments except chemical treatment studies, which used egg-grown organisms. Egg inoculum was used for chemical treatment studies to avoid possible bias, since DEAE-dextran was used in adapting TWAR organisms to grow in cell culture.

Infectivity assays in HeLa 229 cell culture. The cell culture tube used was a disposable, flat-bottomed, 1-dram (4-ml) glass vial shell containing a 12-mm-diameter glass cover slip. The vial was capped with a no. 0 rubber stopper or a plastic lid for experiments requiring a higher centrifugation speed (3,000 rpm, 1,700 × *g*). A 1-ml suspension of 2×10^5 HeLa cells was inoculated into each tube, and tubes were incubated overnight to obtain a confluent cell layer. The culture medium used was Eagle minimum essential medium (GIBCO Laboratories, Santa Clara, Calif.) containing 10% heat-inactivated fetal calf serum and antibiotics, including 100 μg each of streptomycin and vancomycin per ml (MEM). Unless specified, inoculation of TWAR organisms was done by first removing the MEM and treating the cell layer with DEAE-dextran (30 μg/ml). A 0.1-ml amount of organisms suspended in SPG medium (8; see below) was added, and the inoculated vial was centrifuged at 2,200 rpm (900 × *g*) for 60 min at room temperature. The inoculum was removed. One milliliter of MEM containing 0.5 μg of cycloheximide per ml was added. The vials were incubated at 35°C. When the vial with a plastic lid was used, the cultures were incubated in a humidified incubator at 5% CO_2 atmosphere. After incubation for 3 days, the cover slips were removed, fixed with methanol, and stained with fluorescein-conjugated genus-specific monoclonal antibody CF-2 (6), and the inclusions were counted. The inoculum for the second passage was obtained by scraping the infected cells off the cover slip and breaking the cells by repeated pipetting with a capillary pipette, followed by Vortex mixing. All inclusion counts were done on triplicate cover slips to obtain an average.

Burst size. To obtain the burst size (number of infectious units contained in an inclusion), the first-passage cell monolayers were harvested and their infectivity titers were assayed by passage into new cell layers (second passage). The titer of the inoculum for the first passage (inclusion count) was obtained in separate vials inoculated at the same time as those used to harvest the inoculum for the second passage. The burst size was calculated by dividing the second-passage IFU titer by the first-passage IFU titer.

Treatments used in the study. The following treatments which have been shown to enhance infectivity of *C. trachomatis* were evaluated: (i) DEAE-dextran (7, 8); (ii) poly-L-lysine (7); (iii) Polybrene (14); and (iv) polyethylene glycol (11). DEAE-dextran (molecular weight, ~50,000; Pharmacia, Inc., Piscataway, N.J.), poly-L-lysine (Sigma Chemical Co., St. Louis, Mo.), and Polybrene (1,5-dimethyl-1,5-diazaundecamethylene polymethobromide; hexadimethrine bromide; Sigma Chemical Co.) were dissolved in Hanks balanced salt solution. Before inoculation, HeLa cells were

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TABLE 1. Cell culture growth of two TWAR isolates at 35 versus 37°C incubation temperature

Strain and expt[a]	Incubation temp				Ratio 35/37°C	
	35°C		37°C			
	Inclusion count[b]	Burst size[c]	Inclusion count	Burst size	Inclusion count	Burst size
TW-183						
1	1,335	9	400	10	3.3	0.9
2	135	8	130	5	1.0	1.6
3	80	21	25	18	3.2	1.2
Avg		13		11	2.5	1.2
AR-39						
1	980	68	440	21	2.2	3.2
2	180	35	135	9	1.3	3.9
3	90	53	80	8	1.1	6.6
Avg		52		13	1.5	4.6

[a] Each experiment represents a separate test with different inoculum dilutions.
[b] Average IFU from triplicate cover slips.
[c] IFU per milliliter, second passage/IFU per milliliter, first passage.

incubated with these reagents for 15 min. Polyethylene glycol (PEG) (molecular weight, 1500; crystal form; BDH Chemicals Ltd., Poole, England; distributed in the United States by Gallard-Schlesinger Chemical Mfg., Corp., Carle Place, N.Y.) was dissolved in RPMI medium (GIBCO) while it was still warm to make 35%, pH was adjusted to 7.2, and it was filter sterilized, distributed in small portions, and stored at 37°C. PEG treatment was done by adding 0.1 ml of PEG along with 0.1 ml of organism suspension at the time of inoculation. The concentrations of these chemicals tested were 0, 10, 20, 30, and 40 μg/ml for DEAE-dextran; 0, 2.5, 5, 10, and 15 μg/ml for poly-L-lysine; 0, 2, 4, 6, 8, and 10 for Polybrene; and 35 and 17.5% for PEG. The reported optimal concentrations of these chemicals for *C. trachomatis* were 30 μg/ml for DEAE-dextran (7, 8), 10 μg/ml for poly-L-lysine (7), 4 μg/ml for Polybrene (14), and 35% for PEG (11).

Storage temperature and viability. The effect of storage temperature on viability of *Chlamydia* sp. strain TWAR and the kinetics of inactivation were studied as follows. HeLa cells were infected with strain AR-39. Three days after inoculation, infected cells were harvested. The organisms were suspended in SPG chlamydia transport medium (sucrose, 75 g; KH_2PO_4, 0.52 g; Na_2HPO_4, 1.22 g; glutamic acid, 0.72 g; H_2O to 1 liter; pH 7.4 to 7.6) and distributed into small vials for storage. The infectivity titers were assayed by titration in HeLa cells immediately after harvesting (zero-time titers). After storage at different temperatures for the indicated times, the viable TWAR organisms were assayed by titration in HeLa cells (*x*-time titers) to obtain the percentage of residual titers: (inclusion count at *x*-time/inclusion count at zero time) × 100. The temperatures studied were 22°C (room temperature), 4°C (refrigerator temperature), and −75°C (deep freezer temperature).

RESULTS

35°C versus 37°C incubation temperature. Two incubation temperatures have traditionally been used for culturing chlamydiae: 35°C for trachoma biovar of *C. trachomatis* and 37°C for LGV and mouse biovars of *C. trachomatis* and for *C. psittaci* (12). Therefore, we compared these two incubation temperatures to see which temperature would be optimal for *Chlamydia* sp. strain TWAR. It was found that 35°C is better than 37°C for both inclusion formation and burst size (Table 1). The first passage inclusion counts were, on average, 1.5 and 2.5 times greater for the two isolates at 35 than at 37°C. The burst size was 1.2 and 4.6 times greater at 35 than at 37°C. Even at 35°C the burst sizes were small, averaging 13 and 52.

Centrifugation speeds. Gordon and Quan (3) originally described a centrifugal force of 900 × *g* for cell culture of *C. trachomatis*. We have used that speed routinely and found that centrifugation of *Chlamydia* sp. strain TWAR inocula onto cell monolayers increased inclusion counts 2,000 to 4,000 times compared with stationary absorption (6). Since a greater centrifugal force has been shown to enhance cell culture infectivity of *C. trachomatis* (5), we experimented with a centrifugal force of 1,700 × *g* to see whether further enhancement could be obtained. Table 2 shows that the inclusion counts with centrifugation at 1,700 × *g* averaged 2.2 and 3.6 times greater for the two isolates than centrifugation at 900 × *g*.

Chemical treatments of cell monolayers. We have reported that pretreatment of HeLa cell monolayers with DEAE-dextran before inoculation of *Chlamydia* sp. strain TWAR enhanced inclusion counts 1 to 3 times with stationary absorption and 1.5 to 2.5 times in combination with centrifugation at 900 × *g* as compared to controls (6). We studied enhancement by pretreatment of cells with other polycations, poly-L-lysine and Polybrene, in addition to DEAE-dextran, and with PEG when centrifugal force was increased to 1,700 × *g*. The results showed that the infectivity of *Chlamydia* sp. strain TWAR (first-passage inclusion count) was enhanced by DEAE-dextran and poly-L-lysine but not by Polybrene and PEG. The best enhancement was obtained with DEAE-dextran; 2.4 times enhancement at the optimal concentration of 20 μg/ml (Table 3). Poly-L-lysine gave 1.6-fold enhancement with the optimal concentration of 5 μg/ml. PEG was toxic to HeLa cells at a 35% concentration.

Effect of storage temperatures on viability. To simulate the clinical situation, 10,000 to 20,000 IFU of AR-39 organisms contained in 1 ml of SPG medium were stored at different temperatures for different durations and assayed to determine the titer of remaining viable organisms (Table 4). The organisms were inactivated rapidly at room temperature. About 40% of the organisms remained viable after 4 and 6 h, 6% remained viable after 10 h, and 1% remained viable after

TABLE 2. Comparison of two centrifugal forces on cell culture infection of two TWAR isolates

Strain and expt	Inclusion count (IFU/ml)[a]		Ratio, B/A
	900 × *g* (A)	1,700 × *g* (B)	
TW-183			
1	110	270	2.5
2	80	150	1.9
Avg			2.2
AR-39			
1	80	350	4.4
2	20	50	2.5
3	120	480	4.0
Avg			3.6

[a] Average of triplicate cover slips.

TABLE 3. Enhancement of infectivity of *Chlamydia* sp. strain TWAR AR-39 by pretreatment of HeLa cells with DEAE-dextran

Concn of DEAE-dextran (μg/ml)[a]	IFU (10)/ml in given expt (fold increase over control)[b]			Avg
	1	2	3	
0	137 (1.0)	32 (1.0)	65 (1.0)	1.0
10	199 (1.3)	51 (1.6)	134 (2.1)	1.7
20	243 (1.6)	58 (1.8)	249 (3.8)	2.4
30	291 (1.9)	58 (1.8)	140 (2.2)	2.0
40	129 (0.9)	18 (0.6)	124 (1.9)	1.4

[a] HeLa cells were incubated with DEAE-dextran at indicated concentrations for 15 min at room temperature before inoculation with organisms.
[b] Average counts from triplicate cultures in each experiment.

1 day. Inactivation was slow at 4°C. There was little titer loss within 4 h, 88 to 92% remained viable; 75% remained viable at 10 h; and 70 and 53% viability was found after 1 and 3 days, respectively. One percent of the organisms was still found viable for 7 days. Two conditions of freeze and thaw were studied; quick and slow freezing and quick and slow thawing. In quick freezing, the organisms were put in a −75°C freezer immediately after harvesting. In slow freezing, the organisms were stored in a 4°C refrigerator for 0.5, 2, and 4 h after harvesting and then transferred to the freezer. Following 3 days of storage in the freezer, the titer was assayed after the vials were either thawed quickly by submerging the bottom half of the vial in lukewarm water or slowly by leaving the vials on a test tube rack at room temperature. Slow freezing was less damaging to the organisms than quick freezing. An average of 77% of the viable organisms were recovered following slow freezing versus an average of 39% with quick freezing. The methods of thawing caused no significant difference in the viability; the titers with slow thawing were 90 to 92% those with the quick thawing (data not shown).

DISCUSSION

Our previous (6) and current studies show that *Chlamydia* sp. strain TWAR behaves more like the trachoma biovar of *C. trachomatis* than the other two biovars of *C. trachomatis* and *C. psittaci* in that cell culture growth is enhanced by DEAE-dextran treatment, centrifugation, use of cycloheximide, and incubation at 35°C versus 37°C. However, even with the combination of these enhancing factors, *Chlamydia* sp. strain TWAR is the most difficult organism among the chlamydiae to grow in either cell cultures or chicken embryo yolk sacs. It is possible that a cell line more sensitive to *Chlamydia* sp. strain TWAR than HeLa 229 may be found in the future. However, among other cell lines that we have tested, including L and McCoy cells, none was as good as HeLa 229 (6).

The growth of *Chlamydia* sp. strain TWAR was enhanced by increasing the centrifugal force for inoculating the cell culture from 900 × *g* to 1,700 × *g*. DEAE-dextran treatment of cells increased infectivity at a centrifugal force of 1,700 × *g*. Among three polycations tested, DEAE-dextran gave the best enhancement. Based on these studies and until new enhancing methods or sensitive cell lines can be found, the optimal conditions for culturing *Chlamydia* sp. strain TWAR is to use HeLa 229 cells, pretreat cells with DEAE-dextran, centrifuge (1,700 × *g*) the inoculum, incorporate cycloheximide in the culture medium, and incubate inoculated cells at 35°C for 3 days.

Chlamydia sp. strain TWAR has a unique ultrastructure of the elementary body (2). It is pear shaped, with a large periplasmic space. Whether this unique structure affects the attachment and internalization needs further study. Although there was a continuous increase in elementary bodies through 4 days of culture, suggesting that passage at 4-day intervals might be more effective, experiments showed that the yields of infectious organisms were greater when the cultures were harvested at 3 rather than 4 days due to toxicity of *Chlamydia* sp. strain TWAR to host cells (6).

The ultrastructural observation of intracellular development of *Chlamydia* sp. strain TWAR in HeLa cells showed that maturation of reticulate bodies to elementary bodies was retarded (2). This finding is supported by the data in this study showing small burst size. The burst size of a trachoma serovar of *C. trachomatis* in HeLa cells under similar culture condition has been estimated to be about 500 (13). The small burst size of *Chlamydia* sp. strain TWAR may explain the difficulty of passage. It is possible that the nutritional requirement of *Chlamydia* sp. strain TWAR is different from that of other chlamydiae. If so, the culture medium adequate for growing *C. trachomatis* and *C. psittaci* may not support the full development of *Chlamydia* sp. strain TWAR.

Chlamydia sp. strain TWAR seems to be more labile than *C. trachomatis* to storage at room temperature and freezing, although the viability was relatively stable at 4°C. Tjiam et al. (15) were able to isolate *C. trachomatis* from 86% of positive clinical specimens stored at room temperature for 48 h, from 89% of specimens stored at 4°C for 48 h, and from 89% of specimens stored at −70°C for 1 week. Similarly, Mahoney and Cherensky (9) reported isolation of *C. trachomatis* from one (14%) of seven clinical specimens stored at 4°C for 7 days but not from any of three specimens stored for 8 days. Their isolation rates were 10 (34%) of 29 with specimens stored at room temperature (23°C) for 1 day, but none (of 29) after room temperature storage for 2 and 3 days. The recovery rates with freezing (−70°C) for 3 days were 86 and 92% by snap and slow freezing, respectively. We did not test clinical specimens. However, until such studies are done, it seems reasonable, based on this study, to recommend storage of clinical specimens at 4°C if isolation can be done within 24 h and at <−70°C after 1 to 4 h at 4°C if isolation cannot be done within 24 h. Freezing specimens has the advantage of preventing bacterial and fungal growth which may occur at either room temperature or 4°C.

TABLE 4. Effect of storage temperature and duration on viability of *Chlamydia* sp. strain TWAR AR-39

Time	% Viability remaining (no. of expts)			
	22°C	4°C	4°C/−75°C[a]	−75°C[b]
0	100[c]	100	100	100
2 h	70 (3)	92 (4)		
4 h	39 (3)	88 (4)		
6 h	40 (3)	79 (4)		
10 h	6 (3)	75 (3)		
1 day	1 (3)	70 (3)		
2 days	0 (3)			
3 days		53 (3)	77 (9)	39 (4)
7 days		1 (2)		
10 days		0 (2)		

[a] Storage at 4°C for 0.5, 2, or 4 h before freezing for 3 days; three experiments at each time period.
[b] Freezing immediately after harvesting.
[c] Number of organisms at time zero = 1×10^4 to 2×10^4 IFU in 1 ml of SPG medium.

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