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Comparison of Methods for Cultivation and Isolation of *Chlamydia trachomatis*

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McCoy cells treated with cycloheximide, iododeoxyuridine, and DEAE-dextran and untreated McCoy cells were inoculated with two stock strains of *Chlamydia trachomatis* and with 231 urethral specimens from men, 53 (23%) of which contained *C. trachomatis*. Isolation rates, number and quality of inclusions, and quality of the cell monolayers were compared. There were no significant differences between the isolation rates in the four systems, although the most isolations were made in the untreated and cycloheximide-treated cells. Cycloheximide-treated cells produced, from both the clinical specimens and the two stock strains, significantly more inclusions than any of the other systems. The monolayer of the cycloheximide-treated cells and the inclusions that grew in these cells were optimal for examination and detection of *C. trachomatis*.

The diagnosis of infection due to *Chlamydia trachomatis* is based primarily on the isolation of the agent in cell culture. It is widely believed that chlamydiae grow better in nonreplicating cells. To prevent their replication, cells that are to be used for chlamydial cultivation or isolation are either irradiated (8) or treated with antimetabolites such as 5-iodo-2'-deoxyuridine (IUdR) (20), cycloheximide (16), cytochalasin B (19), emetine (6), or hydrocortisone (3). Replicating cell systems have also been used, including untreated McCoy cells (5, 9) and HeLa 229 cells pretreated with DEAE-dextran, which enhances chlamydial infectivity (12). DEAE-dextran treatment of HeLa 229 cells is also used to differentiate strains that cause lymphogranuloma venereum from other strains of *C. trachomatis*, since the infectivity of the former for these cells is not enhanced by DEAE-dextran (13).

Although cycloheximide and IUdR are the most common agents used to treat cells that are to be used for chlamydial cultivation, there have been few comparisons of the relative efficiency of cells treated with these drugs and that of untreated cells (6, 10, 14, 16). To our knowledge, no comparison has been made between normal McCoy cells treated with DEAE-dextran and those treated with IUdR or cycloheximide or untreated. This investigation was undertaken to assess and quantitate the relative efficiency, in our laboratory, of McCoy cells treated with cycloheximide, IUdR, and DEAE-dextran and of untreated McCoy cells for the isolation and cultivation of *C. trachomatis*.

MATERIALS AND METHODS

Cells. McCoy cells (line A9) were originally obtained from the World Health Organization Reference Center, G. W. Hooper Foundation, University of California, San Francisco, Calif., and have been maintained in our laboratory in 75-cm^2 Falcon plastic flasks.

Media. Cells were grown in Eagle minimum essential medium with Hanks salts (M. A. Bioproducts, Walkersville, Md.), supplemented with 2 mM L-glutamine (M. A. Bioproducts), 10% heat-inactivated fetal bovine serum (Sterile Systems, Logan, Utah), which was pretested for chlamydial antibodies, 50 μg of vancomycin (Eli Lilly & Co., Indianapolis, Ind.) per ml, 10 μg of gentamicin (Lilly) per ml, 2 μg of amphotericin B (E. R. Squibb & Sons, Princeton, N.J.) per ml and 2 to 4 ml of 7.5% sodium bicarbonate to pH 8.0 to 8.2. This was called growth medium. For inoculation of clinical specimens and stock chlamydial cultures, growth medium supplemented with 0.06% glucose was used. This was called inoculation medium.

Reagents for cell treatment. Cycloheximide (Sigma Chemical Co., St. Louis, Mo.), IUdR (Sigma), and DEAE-dextran (molecular weight, 2 × 10^6; Pharmacia, Uppsala, Sweden) were used.

Treatment of cells. Confluent monolayers of cells were prepared on 12-mm-diameter no. 1 thickness glass cover slips (Bellco Glass, Inc., Vineland, N.J.) in 15 by 45-mm 1-dram glass "shell" vials (VWR Scientific, San Francisco, Calif.) provided with loose caps. Three-day-old cells were suspended in either plain growth medium or growth medium containing 50 μg of IUdR per ml. The following concentrations of cells were dispensed to produce optimal monolayers: 5 ×

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10^4/ml for those treated with IUdR, 2.5×10^4/ml for those to be treated with cycloheximide, and 1.8×10^4/ml for those to be treated with DEAE-dextran and for those that were to be untreated. Cells were dispensed in a volume of 1 ml per vial and incubated at 37°C in 5% CO_2 for 72 h, at which time the quality of the monolayer was assessed. Before inoculation of clinical specimens, the medium was discarded; the monolayer was washed with phosphate-buffered saline; and either 0.5 ml of inoculation medium (for untreated and IUdR-treated cells), 0.5 ml of inoculation medium containing 0.6 μg of cycloheximide per ml, or 1.0 ml of Hanks solution containing 30 μg of DEAE-dextran per ml was added, the latter being left on the monolayer for 30 min and then replaced with 0.5 ml of inoculation medium. The same procedure was performed before the inoculation of stock cultures, except that the inoculation medium contained the inocula.

Clinical specimens. Urethral specimens were collected from 231 men attending a clinic for sexually transmitted diseases. The specimens were collected with type 1 calcium alginate swabs (Inolex, Glenwood, Ill.). The ends of the swabs were cut into 1-dram screw-cap glass vials (Wheaton Scientific, Millville, N.J.) which contained 0.4 to 0.5 ml of precooled sucrose-phosphate-glutamate medium (2) supplemented with 50 μg of streptomycin (Lilly) per ml, 10 μg of gentamicin (Lilly) per ml, and 25 μg of nystatin (Squibb) per ml. After collection, the vials were kept at 4°C and, within 1 to 3 h, sealed, quick-frozen in dry ice and alcohol, and stored at −85°C.

Stock cultures. We used two strains of *C. trachomatis*: (i) an immunotype D conjunctival isolate (World Health Organization Reference Center), which we passed at a concentration, as tested on cycloheximide-treated McCoy cells, of approximately 10^5 inclusion-forming units per ml and termed strain 1C-3; and (ii) a strain which we isolated from the lymph node of a patient who had lymphogranuloma venereum. We passed this isolate (called strain 1L-1) once at a concentration of approximately 3.5×10^6 inclusion-forming units per ml. Samples in inoculation medium supplemented with fetal bovine serum (1:1) were stored at −85°C.

Inoculation, identification, and quantitation of chlamydiae. The frozen clinical specimens were thawed in a 37°C water bath and shaken with glass beads for 2 min on a Vortex vibrator. A 0.1-ml sample of each specimen was inoculated into the medium of a vial containing monolayers of cycloheximide-, IUdR-, or DEAE-dextran-treated cells or untreated cells.

Before inoculation of the stock cultures, four 10-fold dilutions (2.0×10^{-1} to 2.0×10^{-4} for strain 1C-3 and 1.25×10^{-2} to 1.25×10^{-5} for strain 1L-1) were performed in inoculation medium. After the removal of phosphate-buffered saline and DEAE-dextran from the monolayers, 0.5 ml of each dilution of each strain was added to each of three vials containing cycloheximide-, IUdR-, or DEAE-dextran-treated cells or untreated cells.

After inoculation of either the stock cultures or the clinical specimens, the vials were centrifuged for 1 h at $3,200 \times g$ at 18 to 22°C in a Sorvall centrifuge equipped with an HG-4L rotor (Dupont, Newton, Conn.) and then incubated for 2 h at 37°C in 5% CO_2. The medium was then replaced with 1 ml of inoculation medium or inoculation medium containing 0.6 μg of cycloheximide per ml. The vials containing the clinical specimens and the 1C-3 strain were incubated for an additional 63 h; those containing the 1L-1 strain were incubated for an additional 46 h. After incubation, the medium was discarded. The monolayers were washed with phosphate-buffered saline, fixed with absolute methanol for 10 min, and stained with Lugol solution (0.33% I, 0.66% KI) for 20 min. Cover slips were examined at 100× and, when necessary, at 250× or 400×. Inclusions were counted with the help of a 1-cm^2 ocular micrometer. All of the inclusions were counted on cover slips containing less than approximately 10^4 inclusions. When there were more inclusions, they were counted on 24 randomly assigned micrometer surfaces, and the number of inclusions on the entire cover slip was estimated. The same 24 micrometer surfaces were examined on each cover slip that contained approximately 10^4 or more inclusions.

Statistics. McNemar's test (1) and either the paired-data or the two-group Student's two-tailed *t* test were used.

RESULTS

Stock strains 1C-3 and 1L-1. The results for strains 1C-3 and 1L-1 are shown in Table 1. With both strains, the cycloheximide-treated cells contained significantly more inclusions than either the untreated cells or those that underwent the other treatments. There were significantly more inclusions of the 1C-3 strain in untreated cells than in cells treated with either IUdR or DEAE-dextran. No difference was noted between the latter two. More inclusions of the 1L-1 strain were noted in untreated and IUdR-treated cells than in cells treated with DEAE-dextran.

Clinical specimens. Of the 231 specimens, 53 (23%) contained *C. trachomatis* (1 to 1,239 inclusions per specimen) (Table 2). No significant differences were found between the isolation rates in the treated and untreated cells, even when the analysis was limited to specimens that contained 1 to 20 inclusions. There were 40 such specimens, 7 of which formed inclusions in cells in all four cell treatment groups and 33 of which formed inclusions in cells in only one to three of the cell treatment groups. The other 13 positive specimens contained 35 to 1,239 inclusions per specimen and were all identified in each of the cell treatment groups. All 53 specimens that contained *C. trachomatis* were considered in the assessment of number of inclusions (Table 3). Significantly more inclusions were formed in the cycloheximide-treated cells. Virtually the same number of inclusions were seen in the untreated and in the DEAE-dextran-treated cells, and there was no statistically significant difference between the number of inclusions formed in these cells and in the IUdR-treated cells.

Appearance of cells and inclusions. Inclusions were easier to see in the cycloheximide-treated cells. Most of the dividing cells completed divi-

TABLE 1. Number of inclusions in treated and untreated McCoy cells inoculated with *C. trachomatis* 1C-3 and 1L-1

Cell treatment	No. of inclusions of 1C-3[a]	*P* value[b] as compared with the following treatment:			No. of inclusions of 1L-1[a]	*P* value[b] as compared with the following treatment:		
		None	IUdR	DEAE-dextran		None	IUdR	DEAE-dextran
Cycloheximide	9,597 ± 1,432	<0.01	<0.001	<0.001	24,418 ± 1,901	<0.01	<0.001	<0.001
No treatment	4,969 ± 396		<0.001	<0.01	17,117 ± 1,737		≈0.1	<0.001
IUdR	2,885 ± 272			>0.2	13,145 ± 1,492			<0.005
DEAE-dextran	3,409 ± 350				7,020 ± 769			

[a] Each value represents the mean inclusion count on 12 cover slips ± standard error after converting all counts to the lowest dilution.

[b] Determined with use of the two-group, two-tailed *t* test.

sion after the addition of cycloheximide. The cells were elongated and separated. There was no overcrowding. The monolayer was light and translucent, allowing inclusions to contrast sharply with the background. The cells had an adequate volume of cytoplasm. The inclusions were bright and full and easy to detect. The IUdR-treated monolayers had many of the same qualities, but the cells had less cytoplasm, and the inclusions were smaller and sometimes stained darkly, making it difficult to distinguish them from artifacts. The same difficulties were encountered to a greater extent with untreated and DEAE-dextran-treated cells. The problem was compounded in these cells by overcrowding of the monolayer due to uninhibited growth. In addition, the DEAE-dextran-treated cells showed a tendency to cluster in some areas, leaving other areas devoid of cells, making examination more difficult. DEAE-dextran-treated cells were also more sensitive to cytotoxic effects of the specimens.

DISCUSSION

Most methods of chlamydial isolation and cultivation in cell culture involve treatment of the host cells with agents that prevent their multiplication (16). Replicating cells have been favored by some because of potential risks of handling some of the agents used to inhibit replication and for reasons of technical convenience (5, 9, 15). Many comparative investigations have shown that treated cells contain more inclusions than untreated cells (3, 6, 7, 14, 16, 19). This finding was confirmed in the present study.

McCoy cells treated with cycloheximide emerged as the current system of choice (6, 10, 14, 16). We also found that cycloheximide-treated McCoy cells are better than the other systems we tested. Cycloheximide-treated McCoy cells contained the most inclusions, and the inclusions produced in these cells were the easiest to detect.

We found that inclusion counts in untreated cells were either higher than, or not significantly different from, those in cells treated with IUdR, results which agree with those of other investigators (14). DEAE-dextran has been used mostly with the HeLa 229 cell line (12). It has also been tried with IUdR-treated (4, 17), cycloheximide-treated (17, 21), and irradiated (18) McCoy cells. In the latter case, the inoculum was not centrifuged onto the cells. The results were poorer than those with centrifugation in the absence of treatment with DEAE-dextran. In our hands, in conjunction with centrifugation, normal McCoy cells treated with DEAE-dextran had inclusion counts that were either lower than, or not significantly different from, those in untreated or IUdR-treated cells. However, the

TABLE 2. Isolation of *C. trachomatis* from 53 urethral specimens from men with chlamydial urethritis in treated and untreated McCoy cells

Specimen	No. of isolates					
	Any treatment	All treatments	Cycloheximide treatment	No treatment	IUdR treatment	DEAE-dextran treatment
Specimens containing 1 to 20 inclusions	40	7	27	27	24	20
Specimens containing more than 20 inclusions	13	13	13	13	13	13
All specimens	53	20	40	40	37	33

TABLE 3. Number of inclusions in treated and untreated McCoy cells from urethral specimens of 53 men with chlamydial urethritis

Cell treatment	Total no. of inclusions[a]	*P* value[b] as compared with the following treatment:		
		None	DEAE-dextran	IUdR
Cycloheximide	1,685	<0.05	<0.025	<0.025
No treatment	981		⩾0.2	>0.05
DEAE-dextran	938			>0.05
IUdR	459			

[a] Total number of inclusions formed by the 53 clinical specimens.

[b] Determined with use of the paired-data, two-tailed *t* test.

monolayer of the DEAE-dextran-treated cells was of poor quality, and undercounting cannot be ruled out.

None of the systems we used isolated more than 75% of the total number of positive specimens. This was due to the large proportion of poor-yield specimens and clearly indicates that the sensitivity of the culture technique in chlamydial diagnosis leaves something to be desired. In earlier studies in which systems for the isolation of *C. trachomatis* were compared, specimens that did not produce inclusions in all of the systems produced fewer than 5 to 10 inclusions in the systems in which inclusions were found (7, 10, 16). It seems reasonable to assume that specimens that contain many inclusion-forming units will be found to produce inclusions with the use of any of the available systems. This was the case in our study. We believe that comparative isolation studies should focus on low-yield specimens. The limit we used was 20 inclusions, the highest number seen in any specimen that was negative in at least one of the systems we tested.

The choice of a cell culture system may be influenced by the source of the specimens. Bacterial overgrowth and toxicity occur more often with cervical than with urethral specimens.

In conclusion, in our hands, cycloheximide-treated McCoy cells were better than untreated, IUdR-treated, or DEAE-dextran-treated McCoy cells. More inclusions were noted, and the qualities of the cell monolayer and chlamydial inclusions were optimal for examination and diagnosis.

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