

## *Chlamydia trachomatis* in Cell Culture

### I. Comparison of Efficiencies of Infection in Several Chemically Defined Media, at Various pH and Temperature Values, and After Exposure to Diethylaminoethyl-Dextran

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Three chemically defined cell culture media, Eagle minimum essential medium (MEM) with Earle basal salt solution, Eagle MEM with Hanks basal salt solution, and a modified Eagle MEM, were tested and found capable of supporting the development of *Chlamydia trachomatis* in <sup>60</sup>Co-treated McCoy cells. The enhancement of trachoma infection by diethylaminoethyl-dextran (DEAE-D) was greater at pH values closer to neutrality than at any other pH values measured at the start of the experiments. Centrifugation of the trachoma inoculum onto cell monolayers at 33 C increased the number of inclusions when compared to centrifugation at 20 C. When the inoculum was centrifuged onto cell monolayers and subsequent incubation was at temperatures ranging from 34 to 39 C, the greatest number of inclusions was observed after incubation from 35 through 37 C. Enhancement of the trachoma infection by DEAE-D was tested at temperatures ranging from 35 to 37 C. These cultures had three- to fivefold increases in inclusions when compared to previously reported experiments in which DEAE-D-treated cultures were incubated at 34 C.

Infection of several chlamydia organisms in cell culture in chemically defined medium has been reported (13, 19, 22). Cultivation of meningopneumonitis in mammalian cells in serum-free medium in suspension cultures has recently been achieved (17); trachoma elementary bodies have been propagated serially in monolayer cultures in media devoid of serum (T. R. Rota, unpublished results); however, there have been no quantitative, comparative studies on the ability of commercially available synthetic culture media to support trachoma infection in cells in vitro.

Studies on the effect of temperature on the growth of some chlamydia have been carried out in embryonated eggs (20). Chlamydia which propagate serially in cell culture are usually maintained at 35 to 36 C (4) or at 37 C (5, 21). Slow-growing chlamydial strains, and especially trachoma, have been incubated at 35 C or lower, both in embryonated eggs (15, 18) and in cell culture (10, 23). There are no reports on the effect and the range of temperatures at which trachoma or inclusion conjunctivitis (TRIC) agents may infect and replicate in cell culture.

The present studies are components of a research project designed to grow trachoma

elementary bodies in cells in suspensions to produce batch quantities of antigen useful in trachoma vaccine studies. The following environmental conditions were studied in an effort to increase the efficiency of trachoma infectivity for cell culture. (i) Three chemically defined media available commercially were tested in the absence of serum for their effect on the trachoma infection in cells in vitro. (ii) When possible, the media were used at different pH values to determine whether these differences influenced the yield of inclusions in the cultures. (iii) A range of temperatures during centrifugation of the inoculum onto the cells and subsequent incubation of the cultures was used in an attempt to increase the yield of trachoma infected cells. (iv) The enhancing effect of diethylaminoethyl-dextran (DEAE-D) (14, 23) on the trachoma infection was investigated by using one synthetic medium at several pH values, and several temperatures of incubation.

#### MATERIALS AND METHODS

**Chlamydia strain.** *Chlamydia trachomatis* strain Har-36 (TRIC/2/SAU/HAR-36/OT) (9) was cloned in eggs by the method of Bell et al. (2), and propagated

serially in irradiated McCoy cells (23). The inoculum used in these experiments was the 28th cell culture passage. Irradiated McCoy cells were infected for 48 h, disrupted in a blender (Omnimix, Ivan Sorvall) in an ice bath, and centrifuged at  $250 \times g$  for 10 min at 5 C. The supernatant fluid was stored in sealed ampoules and frozen under dry ice. The inoculum containing  $2 \times 10^8$  inclusion-forming units per ml was diluted  $10^{-2}$  in the cell culture media tested. The concentration of the inoculum was the same in all experiments reported here, except where noted. A fresh ampoule was used for each experiment.

**Cells.** McCoy cells (10, 23) were maintained in monolayers in saniglass bottles in Eagle minimum essential medium (MEM) (7) with Earle basal salt solution (8) (MEM-Earle; Microbiological Associates), with 10% horse serum (Microbiological Associates) supplemented with L-glutamine (200 mmol/ml) and streptomycin (50  $\mu$ g/ml). The cultures were gassed with 5%  $\text{CO}_2$  in air to lower the pH to 7.4. The cell line was kept at 34 to 35 C. Cells used for experiments were irradiated with 5,000 roentgens in a  $^{60}\text{Co}$  source, and the medium was changed. The monolayers were trypsinized 4 or 5 days after irradiation, and 1 ml of cell suspension ( $2.3 \times 10^8$  cells/ml) was dispensed into flat-bottomed tubes containing a 12-mm diameter cover slip (10, 23). The cover slip cultures were used 7 to 9 days after irradiation.

**Cell culture media.** Three commercially available synthetic cell culture media were used. They were prepared as follows. (i) MEM-Earle contained 2,200 mg of  $\text{NaHCO}_3$  per liter and required gassing with 5%  $\text{CO}_2$  in air, since it tends to become alkaline once opened. It was tested at a pH value of 7.0, 7.4, and 7.7. The latter values were attained in two 100-ml vials of MEM-Earle which had previously been opened and exposed to air. MEM-Earle is dispensed by the manufacturers at pH 7.0 ( $\pm 0.1$ ). (ii) A slightly modified Eagle MEM medium (modified MEM) (25) was obtained in a powdered, autoclavable form (Auto-Pow) from Flow Laboratories. The ingredients were reconstituted in water and sterilized by autoclaving at 121 C for 15 min.  $\text{NaHCO}_3$  at 7.5% in distilled water was sterilized by filtration and was added in measured amounts to the rest of the medium, prior to use, to obtain pH values of 7.0, 7.2, 7.5, 7.6, 7.8, and 8.0. Modified MEM medium offered greater versatility in the manipulation of the pH. It was gassed with 5%  $\text{CO}_2$  in the centrifuged cultures. (iii) MEM with Hanks basal salt solution (7, 12) (Microbiological Associates) dispensed at pH 7.2 ( $\pm 0.1$ ) contained 350 mg of  $\text{NaHCO}_3$  per liter and did not require gassing. It was used at pH 7.4 (pH was measured in one vial of 100 ml of this medium).

All media received 30 mmol of extra glucose per ml and phenol red as a pH indicator. No serum was added. The pH of a sample of medium was measured in a pH meter at the start of the experiment and in the control cell cultures at the end of the experiment. The pH of the trachoma inoculated cultures was recorded at the end of the experiment by visual comparison with phenol red standards.

**Inoculation of cultures.** Cover slip cultures of irradiated McCoy cells received 1.0 ml of the trachoma elementary bodies suspension diluted in the

medium used at a specific pH value. They were gassed, if required to maintain a low pH, centrifuged (International PR-1 model) at  $800 \times g$  for 1 h at either 20 or 33 C, and incubated at the temperature specified for each experiment.

**DEAE-D treatment.** DEAE-D (Pharmacia; molecular weight  $2 \times 10^6$ ) was used at a concentration of 20  $\mu$ g/ml in combination with modified MEM medium at the following pH values: 7.0, 7.2, 7.5, 7.6, 7.8, and 8.0. Cultures exposed to DEAE-D were treated according to the method developed by Harrison (13) for an ovine chlamydial agent and reported previously for trachoma in cell cultures (23). Monolayers of irradiated McCoy cells were exposed to 1.0 ml of a solution containing 20  $\mu$ g of DEAE-D per ml in the culture medium. The cultures were allowed to stand at room temperature for 30 min and were washed twice with Hanks solution before receiving the trachoma inoculum which had been diluted in the modified MEM medium at a given pH. Cultures not treated with DEAE-D and not centrifuged were also washed twice with Hanks solution prior to inoculation with trachoma organisms. Control cultures of uninoculated cells treated like the experimental cultures were also prepared, for the study of the effect of pH, temperature, and DEAE-D treatment on the cells.

**Assessment of infectivity.** Cultures were fixed after 48 or 72 h of incubation, stained with iodine (11), and examined at  $\times 320$ . The number of infected cells was counted in 18 or in 100 fields in the centrifuged cultures. In one experiment, cells having multiple inclusions were counted and were recorded separately. The infected cells were counted over the entire cover slip area in the DEAE-D treated cultures, and in the trachoma-inoculated cultures which had neither been centrifuged nor exposed to DEAE-D. The results were expressed as geometric means of the number of cells with trachoma inclusions per group (usually three, five, or six cultures per group). One-way or two-way analysis of variance and a non-parametric ranking test were performed where indicated. All microscope readings were coded to eliminate bias. Control cell cultures stained with iodine and by Giemsa's method were used to examine cell morphology at  $\times 800$ .

## RESULTS

**Trachoma infection in chemically defined media.** The efficiency of trachoma infection for irradiated McCoy cells was tested in three chemically defined media after centrifugation of the inoculum at 20 C onto cell monolayers and incubation at 34 C for 72 h. The efficiency of infection of trachoma elementary bodies for cell culture in MEM-Earle varied inversely with the pH of the medium. Geometric means of 70, 47, and 32 cells (counted in 18 fields, 6 cultures/group) were infected at pH values of 7.0, 7.4, and 7.7, respectively. These differences are significant ( $P < 0.01$ ).

The highest efficiency of MEM-Earle at pH 7.0 was shown in a second experiment with triplicate cultures. Trachoma elementary

bodies in modified MEM medium at pH 7.0 and 7.2 produced fewer infected cells (geometric means of 46 and 24, respectively) than MEM-Earle at pH 7.0 and at the routine pH of 7.4. Trachoma elementary bodies in MEM-Hanks at pH 7.4 yielded about the same number of infected cells as in MEM-Earle at the same pH (geometric means of 53 and 47, respectively). All the above results have been confirmed in additional experiments testing the media with inocula derived from two other passages of HAR-36 in irradiated McCoy cells.

**DEAE-D-treated cultures.** DEAE-D enhancement of trachoma infectivity for irradiated McCoy cells was dependent on the starting pH of the medium in two experiments with modified MEM medium (Table 1). In DEAE-D-treated cultures, there were significantly ( $P < 0.01$ ) more infected cells at pH 7.0 and 7.2 than at other pH values tested with this medium (7.5, 7.6, 7.8, and 8.0). These cultures were neither centrifuged nor gassed with 5% CO<sub>2</sub>. Control cultures which were inoculated, but neither centrifuged nor treated with DEAE-D, contained very few inclusions. At pH 7.0, DEAE-D treatment gave a 22-fold increase over untreated cultures at the same pH. In a second experiment, with modified MEM at pH 7.2, DEAE-D-treated cultures showed a 73-fold increase over comparable untreated cultures. The cultures infected by centrifugation of the inoculum onto the cells produced, by far, the highest

number of infected cells, as previously reported (23).

Morphological studies of the Giemsa-stained uninfected control cells indicated that neither the pH of the medium nor the DEAE-D treatment caused visible deleterious effects on the host cells, which might account for the lower number of inclusions observed as the pH of the medium was increased towards alkalinity. During the course of these experiments, the pH of the medium did not change more than 0.4 units from the starting value in the inoculated cultures. The experiments were concerned with the starting pH of the medium at the time of the trachoma inoculation, rather than with the maintenance or regulation of the pH during the 72-h incubation period. No attempts were made to supplement the synthetic media with additional buffers other than the salts and the sodium bicarbonate and by gassing in the centrifuged cultures of MEM-Earle and modified MEM. In general, the association holds for all groups of cultures—those started at lower pH had the lowest pH after 72 h and had also the highest number of inclusions when compared to cultures started with media at pH values ranging from 7.6 to 8.0.

**Trachoma infection at varying temperatures.** Cultures were centrifuged with the inoculum at 20 and at 33 C and incubated at temperatures ranging from 34 to 37 C (Table 2). When the cultures were centrifuged at 20 C and stained at 48 h (Table 2, column A), there was an increase in infected cells as the temperature of incubation increased from 34 to 37 C. Infection at 36 C was lower than at 35 and 37 C; this finding was noted in some, but not all, of the experiments. The results obtained from increasing the temperature of centrifugation to 33 C (the maximal temperature reached by the centrifuge available) and incubating from 34 to 37 C are shown in Table 2, columns C and D. The levels of infection at 48 h in cultures centrifuged at 33 C and incubated at 34, 35, 36 and 37 C (Table 2, column C) represented 1.2- to 1.7-fold increase over those centrifuged at 20 C and incubated at comparable temperatures (Table 2, column A). More cells were infected at 48 h than at 72 h for all temperatures tested (except in the cultures centrifuged at 20 C and incubated at 37 C, where the infection increased by 72 h). The number of infected cells decreased by 72 h because of the rupture of mature inclusions and falling off the glass of infected cells between 48 and 72 h.

One-way analysis of variance ( $\log_{10} [X]$ ) on the levels of infection obtained at each incubation time showed that inclusion counts after incubation at each temperature were signifi-

TABLE 1. Effect of DEAE-dextran on the number of inclusions observed in McCoy cells in modified MEM medium at several pH values

Cells treated with DEAE-D	Exp 1			Exp 2	
	Inoculum centrifuged	pH of medium	No. of infected cells <sup>a</sup>	pH of medium	No. of infected cells <sup>a</sup>
Yes	No	7.0	66	7.2	146
		7.6	6	7.5	49
		8.0	1	7.8	33
		7.0	3	7.2	2
ANOVA <sup>c</sup> No	No	7.0	( $P < 0.01$ ) 3	7.5	2
		7.6	3	7.8	2
		8.0	1	7.2	2
		7.0	1,837	7.2	925

<sup>a</sup> Geometric mean number of infected cells counted on the entire cover slip monolayer at  $\times 320$ . Five or six cultures per group. All cultures were incubated at 34 C for 72 h.

<sup>b</sup> Centrifuged at 20 C,  $800 \times g$ , 1 h.

<sup>c</sup> Analysis of variation.

cant, and particularly so after 48 h ( $P < 0.01$ ). Because there was indication that the variances within the groups were unequal, a non-parametric test was applied with essentially the same results, except at 72-h incubation (Table 2, column D). Two-way analysis of variance applied to the data of temperature of centrifugation versus incubation time showed the temperature effect to be significant ( $P = 0.03$ ), with the incubation time showing less effect ( $P = 0.07$ ). The results indicated that, by increasing both the temperature of centrifugation (above 20 C) and the temperature of incubation (up to 37 C), there was a significant increase in the number of infected cells produced in the cultures. These experiments have been repeated three times with similar results.

**Effect of temperature on the number of multiply infected cells.** Table 3 shows the percentage of infected cells with multiple inclu-

sions in cultures centrifuged at 33 C and incubated from 35 to 39 C. At all temperatures tested, the highest number of total inclusions was present at 48 h; at this time multiply infected cells were more numerous in cultures incubated from 35 to 37 C than in those incubated at 38 and 39 C. At all temperatures except 35 C, multiply infected cells were more numerous at 72 than at 48 h. The smaller number of cells with multiple inclusions after 72 h at 35 C could possibly be due to a faster rate of rupturing of mature inclusions and/or faster rate of fusion of multiple inclusions within one cell (3). The decrease in the total number of cells with inclusions at 38 and 39 C by 72 h is partly associated with degeneration of inclusions, as indicated by the appearance of some inclusions which were a faded gray color, instead of brown, after staining with iodine. These inclusions appeared to be arrested, without morphological differentiation, and occasionally extruded from the cells in toto.

**Enhancement of infection by DEAE-D.** The enhancing effect of the trachoma infection by DEAE-D was previously demonstrated when cultures were incubated at 34 C (23). Table 4 combines those results with the results obtained after incubating the treated cultures at 35, 36, and 37 C. The cultures exposed to the polycation (column A) were compared to uncentrifuged controls which were not treated with DEAE-D (column B). Concomitantly, the inoculum was centrifuged onto monolayers at 20 C and incubated at 35 to 37 C (column C). At all temperatures tested, DEAE-D-treated cultures (column A) showed between 30- and 80-fold increases in infected cells when compared to the uncentrifuged, non-DEAE-D-treated trachoma-inoculated controls (column B). As in our previous studies, centrifuged cultures showed a much greater number of infected cells when compared to DEAE-D-treated monolayers.

Increasing the temperature of incubation in-

TABLE 2. Effect of the temperature of centrifugation and the subsequent temperature of incubation on the number of inclusions observed in MEM-Earle medium

Temp of incubation (C)	Centrifugation at 20 C		Centrifugation at 33 C	
	(A) 48 h	(B) 72 h	(C) 48 h	(D) 72 h
34	146 <sup>a</sup>	131	196	190
35	208	139	359	168
36	165	144	230	179
37	231	314	264	236
ANOVA	$P < 0.01$ Prob. (H) 0.025 <sup>b</sup>	$P < 0.01$ Prob. (H) 0.056	$P < 0.01$ Prob. (H) 0.040	$P < 0.01$ Prob. (H) 0.264

<sup>a</sup> Geometric means of number of cells with inclusions in 100 fields at  $\times 320$ , three cultures per group. Inoculum used at 1:25 dilution.

<sup>b</sup> Non-parametric ranking test for differences between treatment means were performed with  $\log_{10}(X)$ . Two-way analysis of variance: temperature of centrifugation  $P = 0.03$ ; incubation time,  $P = 0.07$ .

TABLE 3. Effect of temperature on number of multiple inclusions per cell in MEM-Earle medium

Temp of incubation (C)	48 h			72 h		
	Total inclusions <sup>a</sup>	Cells with multiple inclusions	Infected cells with more than one inclusion (%)	Total inclusions	Cells with multiple inclusions	Infected cells with more than one inclusion (%)
35	1,181	22	1.90	554	6	1.10
36	1,008	10	1.00	612	33	5.80
37	1,451	20	1.40	867	25	3.00
38	718	7	0.98	271	9	3.43
39	616	4	0.65	228	19	9.10

<sup>a</sup> All cultures were centrifuged at 33 C,  $800 \times g$  for 1 h; six cover slips per group. Inclusions in 18 fields at  $\times 320$ .

TABLE 4. *Effect of temperature of incubation and DEAE-D treatment on the number of inclusions<sup>a</sup> observed in irradiated McCoy cells in MEM-Earle's medium*

Temp of incubation (C)	(A) Noncentrifuged, DEAE-D treated		(B) Noncentrifuged, non- DEAE-D treated		(C) Centrifuged, <sup>b</sup> non- DEAE-D treated	
	48 h	72 h	48 h	72 h	48 h	72 h
34 <sup>c</sup>	92	121	2	3	1,130	1,953
35	304	329	4	4	4,308	2,905
36	360	533	11	7	3,907	3,486
37	ND <sup>d</sup>	411	ND	ND	3,011	3,205

<sup>a</sup> Geometric mean number of cells with inclusions on the entire cover slip monolayer. Five or six cover slips per group; fixed at 48 and 72 h after inoculation.  $\times 320$ .

<sup>b</sup> Centrifuged at 20 C,  $800 \times g$  for 1 h.

<sup>c</sup> Reference (23).

<sup>d</sup> Not done.

creased the yield of infected cells in DEAE-D-treated cultures. After 72 h of incubation, the greatest number of inclusions was observed at 36 C, representing 4.6-fold increase over that seen at 34 C. Uncentrifuged, non-DEAE-D-treated, trachoma-inoculated cultures showed an increase in the number of infected cells as the temperature of incubation increased from 34 to 36 C (column B).

## DISCUSSION

This is the first report in the literature of a quantitative study on the ability of several commercially available synthetic media to support trachoma infections in cells in culture.

Trachoma has been propagated in irradiated McCoy cells in MEM-Earle with 10% horse serum at pH 7.4 (23). This medium requires gassing, and when serum was omitted we observed more inclusions when the medium was pH 7.0 than when it was pH 7.4. Experiments using modified MEM medium indicated that a pH close to neutrality was more favorable for the development of trachoma inclusions. The latter medium needs addition of  $\text{NaHCO}_3$  to produce the desired pH and may require gassing also. In contrast to the two other media tested, MEM-Hanks, which was almost as effective as MEM-Earle, does not need gassing and can be used successfully at the pH supplied by the manufacturers. For simplicity, MEM-Hanks is highly recommended for work with irradiated McCoy cells. A suitable combination of media would be 50% MEM-Earle with 50% MEM-Hanks. This medium would be sufficiently buffered and would require neither gassing nor additional  $\text{NaHCO}_3$  (T. R. Rota, unpublished results).

Large inclusions with mature elementary bodies were produced in all media tested de-

spite the absence of serum. The possibility of using a chemically defined medium for growth of trachoma in cell cultures will facilitate biochemical studies related to the metabolism of the agent and its interaction with the host cells during the developmental stages of the parasites' infectious cycle.

DEAE-D-treated cultures, like untreated cultures, contained more inclusions when the pH of the medium was close to neutrality. Strain Har-36 can develop inclusions when centrifuged onto irradiated McCoy cells and incubated at temperatures ranging from 34 to 39 C. We have not been able to propagate this strain serially in McCoy cells without centrifugation. Har-36 grown in cell cultures has been shown to be pathogenic for owl monkey eyes (MacDonald, McComb, and Rota, unpublished results). The observation that centrifugation at 33 C was more favorable than centrifugation at 20 C is in agreement with other workers (6; W. A. Blyth, personal communication).

The net effect of increasing temperature was an increased number of infected cells and an acceleration of the infectious cycle. When centrifugation was at 33 C, inclusions were demonstrable with iodine stain after 24 h. Most of the inclusions contained visible elementary bodies by 48 h, and by 72 h the number of elementary bodies and the size of the inclusions had increased. In cultures centrifuged at 33 C, more inclusions were observed by 48 h when incubated at 35 and 37 C than at other temperatures tested.

TRIC strains that propagate indefinitely in cell cultures have often been maintained at 37 C (5, 21). It is possible that adaptation of a strain to grow at 37 C would select elementary bodies increasingly more effective in initiating a successful cell infection. This might lead to variants which would grow without need for

centrifugation onto cells. Whether this adaptation, if attained, would involve antigenic changes is a matter for speculation. The nature of the trachoma strains that propagate easily in cell cultures has been the subject of long-standing controversy and study (16, 24). The study of the temperature range of TRIC agents for taxonomic purposes deserves exploration.

Sensitivity of the trachoma infection to the temperature of centrifugation and to the subsequent temperature of incubation indicates that penetration of the elementary bodies into the cells and the development of the inclusion are temperature-dependent processes. Temperature might have an effect on the activity of enzymes (1) involved in the development of inclusions. Temperature may also have an effect on host-cell functions thereby influencing the uptake of elementary bodies or the supply of energy available to the parasite.

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