Comparison of the sensitivity of primary isolation of *Chlamydia*

In irradiated McCoy cells or HeLa 229 cells treated with diethylaminoethyl-dextran (DEAE-D)

S. DAROUGAR, D. KUNDU, AND B. R. JONES

Institute of Ophthalmology, University of London

The technique of culture in irradiated McCoy cells for the isolation of *Chlamydia* was developed by Gordon, Magruder, Quan, and Arm (1963), Magruder, Gordon, Quan, and Dressler (1963), and Gordon, Dressler, and Quan (1967), and subsequently modified and simplified by Darougar, Kinnison, and Jones (1971a), Darougar, Jones, Kinnison, Vaughan-Jackson, and Dunlop (1972), and Darougar, Cubitt, and Jones (1974). The technique has been shown to be three to four times more sensitive than the detection of inclusions in smears and the isolation of agent in yolk-sac (Gordon, Harper, Quan, Treharne, Dwyer, and Garland, 1969; Darougar, Treharne, Dwyer, Kinnison, and Jones, 1971b). This technique has been extensively used for the study of chlamydial infection of the eye, genital tract, and rectum and for the routine isolation of *Chlamydia* in different laboratories (Gordon and others, 1969; Dunlop, Hare, Darougar, Jones, and Rice, 1969; Darougar and others, 1971b; Dunlop, Vaughan-Jackson, Darougar, and Jones, 1972; Oriel, Reeves, Powis, Miller, and Nicol, 1972; Richmond, Hilton, and Clarke 1972).

The enhancing effect of diethylaminoethyl-dextran (DEAE-D) has been reported for the culture of viruses in cell-culture (Pagano and Vaheri, 1965; Kaplan, Wiktor, Maes, Campbell, and Koprowski, 1965; Vaheri, Sedwick, and Plotkin, 1967; Vogt, 1967; Duc-Nguyen, 1968), as well as for *Chlamydia* (Harrison, 1970; Rota and Nichols, 1971). Recent studies by Kuo and Wang (1972) have indicated that culture in HeLa 229 cells treated with DEAE-D is as sensitive as culture in irradiated McCoy cells for the primary isolation of *Chlamydia* from the genital tract.

The purposes of this study were:

- (a) To investigate the enhancing effect of DEAE-D on the primary isolation of *Chlamydia* in irradiated McCoy cell-culture.
- (b) To compare the sensitivity of culture in irradiated McCoy cells (IMcCoy cells) with that of culture in HeLa 229 cells (HeLa 229) treated with DEAE-D for the primary isolation of *Chlamydia*.
- (c) To compare the increase in numbers of inclusions during serial passage in the two systems of cell-culture.

Methods and material

SOURCE OF SPECIMENS

Clinical specimens used in this study consisted of conjunctival and genital material from patients in London who were referred to the External Diseases Clinic, Moorfields Eye Hospital, and who were suffering from sexually-transmitted chlamydial infections of the eye and genital tract. Other specimens consisted of conjunctival material from persons in Iran suffering from hyperendemic trachoma.

COLLECTION OF SPECIMENS

Specimens were collected by swabbing different areas of the conjunctiva, and were placed in plastic capsules containing 2SPS transport medium (2SP with additional serum: Darougar and others, 1972). Specimens from the genital tract were collected by swabbing or scraping the urethra of the male or cervix of the female and placed in plastic capsules containing 2SPS (Dunlop and others, 1972).

All specimens collected in London or Iran were stored in a liquid nitrogen refrigerator for transportation to the laboratory and were stored there in a -70°C . refrigerator until the time of inoculation.

TREATMENT OF SPECIMENS

McCoy cells

Methods used for growth and irradiation of McCoy cells and for preparation of monolayers of these cells in flat-bottomed tubes ("bijou" bottles, Sterilin Limited, Richmond, Surrey), containing coverslips are reported by Darougar and others (1971a).

HeLa 229 cells

Methods for growth of HeLa 229 cells and the preparation of monolayers in flat-bottomed tubes containing coverslips are reported by Kuo and Wang (1972).

DEAE-D treatment

Irradiated McCoy cell monolayers were either rinsed twice with 1 ml. CMGA containing $30\text{ }\mu\text{g./ml.}$ DEAE-D or were treated with 1 ml. of this suspension for 30 min. before inoculation of specimens.

HeLa cell monolayers were rinsed twice with 1 ml. HBSS containing $30\text{ }\mu\text{g./ml.}$ DEAE-D as described by Kuo and Wang (1972).

INOCULATION OF CLINICAL SPECIMENS

Aliquots of clinical specimens from patients in London were inoculated in parallel into two tubes of IMcCoy cells and two tubes of HeLa 229 cells using the simplified one-passage technique of culture in irradiated McCoy cells (Darougar and others, 1971a). In the case of Iranian specimens, the technique of inoculation was modified in that the speed of centrifugation used was at 15,000 G instead of 2,500 G as in the standard technique (Darougar and others, 1974).

INFECTIVITY ASSAY IN CELL-CULTURE

After incubation for 72 hrs, the inoculated monolayers were fixed with methanol for 10 min. and stained with iodine (Gordon and others, 1969). The iodine-stained coverslips were examined for inclusions using a $\times 40$ oil-immersion objective and $\times 10$ eye piece, and the number of inclusions in the whole area of each monolayer was recorded. Monolayers with degenerated cells or defective areas were excluded from this study.

One of two tubes of IMcCoy cells and one of two tubes of HeLa 229 cells inoculated with aliquots of each specimen, were stained and examined for the presence of inclusions. If inclusions were found in the first tube, the cells in the second tube were harvested and stored for further studies. If examination of the first tube was negative, the second tube was stained with iodine not later than 72 hrs after inoculation and examined for the presence of inclusions.

SERIAL PASSAGE

Aliquots of clinical specimens were inoculated in parallel into two tubes of IMcCoy cells and two tubes of HeLa 229 cells treated with DEAE-D (first passage). After 60 hrs of incubation, the monolayer of each cell-culture system was fixed and stained with iodine and examined for inclusions. If inclusions were found in the first tube, the cells in the second tube of each system were harvested no later than 72 hrs after inoculation by adding a few glass beads and shaking on a "Whirlimixer" for 30 sec. The harvested suspensions were diluted from 10^{-1} to 10^{-3} after which 1 ml. of each dilution was inoculated into each of two tubes for each type of cell monolayer for a second passage.

The subsequent third and fourth passages were carried out in the same manner as the second passage.

The number of inclusions in each monolayer at each passage was recorded.

Results

Effect of DEAE-D on the sensitivity of culture in irradiated McCoy cells

In a preliminary study, monolayers of IMcCoy cells were treated with different dilutions of 10–50 $\mu\text{g./ml.}$ DEAE-D (M.W. 2×10^6 Pharmacia, Uppsala, Sweden), in Hanks's balanced salt solution (HBSS), or in Eagle's complete medium with glutamine and antibiotics (CMGA, Gordon and others, 1969). Using iodine staining to detect inclusions, there was no significant difference in the sensitivity of cell-cultures treated with different concentrations of DEAE-D whether prepared in HBSS or CMGA, for the primary isolation of *Chlamydia*. Using Giemsa's stain, particles of DEAE-D were seen attached to the surface of cells which caused difficulties in the detection of inclusions by either dark-field or bright-field illumination. In this initial study, there was no difference in the activity between DEAE-D solutions sterilized by autoclaving or by filtration.

Therefore, in further studies, the IMcCoy cell monolayers were either rinsed twice with 1 ml. CMGA containing 30 $\mu\text{g./ml.}$ DEAE-D or were treated with 1 ml. of this solution for 30 min. before the inoculation of specimens.

Of the aliquots of eight clinical specimens inoculated equally into IMcCoy cells either treated or untreated with DEAE-D, six were positive in each system (Table I). There was

Table I *Effect of DEAE-Dextran on the sensitivity of irradiated McCoy-cell culture for the primary isolation of Chlamydia*

Serial number of specimens	No. of inclusions		
	Cells not treated	Cells treated with DEAE-Dextran	
		Rinsed twice	Treated for 30 min.
8167	4	2	2
8334	1	1	2
8090	22	20	33
8089	64	140	174
8125	172	195	130
8164	112	63	71
8050	0	0	0
8090	0	0	0

no significant difference between the numbers of inclusions formed in the DEAE-D treated cells and the untreated cells, nor was there a significant difference in the numbers of inclusions formed in the cells rinsed twice with DEAE-D suspension and in the cells which were treated with DEAE-D for 30 min before inoculation.

Comparison of irradiated McCoy cell culture with HeLa 229 cell culture treated with DEAE-D

Of a total of 111 clinical specimens, 64 were positive in IMcCoy cells and 57 were positive in HeLa 229 cells (Table II). Although the difference in the positivity of the two systems of cell-culture did not reach statistical significance, the mean number of inclusions formed in IMcCoy cells was seven times higher than the number of inclusions formed in the HeLa 229 cells (Table III). The inclusions formed in IMcCoy cells were two or three times larger than those formed in the HeLa cells: there being many more elementary bodies in inclusions stained with Giemsa in IMcCoy cells than those formed in HeLa 229 cells.

Table II *Comparison of sensitivity, for primary isolation of Chlamydia, of irradiated McCoy-cell cultures and of DEAE-D treated HeLa 229 cell cultures*

Source of specimens	No. of specimens	No. of isolations	
		McCoy cells	HeLa 229 treated with DEAE
Conjunctiva (London)	30	30	28
Genitalia (London)	18	18	17
Conjunctiva (Iran)	63	16	12
Total	111	64	57

Table III *Ratio of inclusions obtained by inoculation of clinical specimens onto irradiated McCoy cells and HeLa 229 cells treated with DEAE-Dextran*

Source of specimens	No. of specimens	Mean ratio of inclusions
		McCoy cells : HeLa 229 cells
Conjunctiva (London)	30	8 : 1
Genitalia (London)	18	9 : 1
Conjunctiva (Iran)	63	5 : 1
Total	111	7 : 1

It was noteworthy that, for those clinical specimens that gave rise to many inclusions in the first passage, there was no difference in the rate of isolation between the two systems of cell-culture, but in the case of fifteen specimens which were positive in the IMcCoy cells with only a few inclusions, only eight were positive in HeLa 229 cells.

Serial passage

The result of serial passage of seven chlamydial isolates obtained in McCoy cells has shown that *Chlamydia* can be propagated continuously in this system of cell-culture (Table IV).

The number of inclusions obtained in IMcCoy cells after each passage was increased by 10^2 to 10^3 . Of the seven chlamydial isolates which were obtained in HeLa 229 cells and passaged into the same cell-culture system, five were positive in the second passage, only one was positive in the third passage, and none in the fourth passage (Table IV).

Table IV Results of serial passage of different chlamydial isolates obtained and passed in either irradiated McCoy cell-culture or HeLa 229 cells treated with DEAE-Dextran

Dilutions of inoculum	No. of positive cultures							
	Passage 1		Passage 2		Passage 3		Passage 4	
	McCoy	HeLa	McCoy	HeLa	McCoy	HeLa	McCoy	HeLa
10^0	7	7						
10^{-1}			7	5	7	1	7	0
10^{-2}			7	4	7	1	7	0
10^{-3}			7	3	7	0	7	0

The number of inclusions in the positive HeLa cell-cultures increased slightly (approximately 5 times) in the second passage; thereafter they declined. It was noted that those harvests from infected HeLa 229 cells that contained less than ten inclusions per ml failed to produce a positive culture in subsequent passage. In contrast, a fraction of an inclusion per ml. was sufficient to maintain serial passage in IMcCoy cells.

Discussion

The improved technique of culture in irradiated McCoy cells (Darougar and others, 1972, 1974) has provided a high level of sensitivity for the isolation of *Chlamydia*, approximating to 80 per cent. isolations for material from the infected eye, 90 per cent. for the genital material from women suffering from TRIC infection of the eye, and 70 per cent. for such material from mothers of babies with TRIC ophthalmia neonatorum (Darougar and others, 1972). The simplicity, speed, and sensitivity of this technique has made it the method of choice for routine diagnostic isolation services for chlamydial infections, for epidemiological studies, and for the microbiological assessment of the results of treatment of trachoma in hyper-endemic areas.

Treatment of irradiated McCoy cells with DEAE-D in this study failed further to increase the sensitivity of cell-culture for the primary isolation of *Chlamydia* from specimens centrifuged onto the monolayers. The mechanism of action of DEAE-D is not well understood, but it has been suggested that this poly-cation works on the interaction between cells and infective agents in the early stages of penetration of the cell. The particles of DEAE-D may attach to the surface of cells, leaving electro-positive charges available for combination with electro-negative particles, thus facilitating the uptake of agent by cells (Harrison, 1970; Rota and Nichols, 1971).

In this study the iodine-staining technique was used for the detection of inclusions in irradiated McCoy cells and HeLa 229 cells treated with DEAE-D. Using a Giemsa stain, particles of DEAE-D attached to the surface of cells caused difficulties in the detection of inclusions using dark-field or bright-field illumination. The fluorescent antibody staining technique, which provides easier and more rapid reading of the monolayers in comparison to the Giemsa stain (Kuo and Wang, 1972), was not used in this study.

Studies by Darougar and others (1974) have shown that the use of the higher temperature of 35°C. during the period of centrifugation and a higher force of centrifugation up to 15,000 G will significantly increase the rate of isolation of *Chlamydia* in irradiated McCoy cells. Therefore, in this study, the higher temperature of 35°C. during the period of centrifugation and the force of 2,500 G or 15,000 G were used in place of the 20°C. and 900 G used by Kuo and Wang (1972) for the inoculation of HeLa cells.

From 111 specimens inoculated, the overall rate of isolation in McCoy cells was slightly higher (65) than that in HeLa 229 cells (57). However, in the case of specimens containing few infective particles, the sensitivity of McCoy cells was almost twice that of HeLa 229 cells. Therefore, in instances of mild or late stages of chlamydial disease of the eye (especially in hyperendemic trachoma) or in genital material in which only small quantities of viable *Chlamydia* are to be found, it can be expected that cultivation in DEAE-D treated HeLa 229 cells would give significantly fewer positives than the present method of cultivation in irradiated McCoy cells.

The successful serial passage of *Chlamydia* in irradiated McCoy cell-culture in this study was consistent with previous observations (Darougar and others, 1971). Failure of serial passage in HeLa 229 cells treated with DEAE-D was in keeping with the fact that the inclusions that developed in this system after primary inoculation were smaller and contained fewer of the mature elementary bodies that are necessary for the successful passage of this agent.

Thus, the technique of culture in HeLa 229 cells treated with DEAE-D is not as sensitive as the technique of culture in irradiated McCoy cells, particularly in the case of specimens with a lower number of infective particles, nor is it as suitable for serial cultivation of *Chlamydia*. It does, however, provide a reasonably sensitive and practical alternative to the irradiated McCoy cell-culture for the primary detection of *Chlamydia* in laboratories where facilities for the irradiation of cells are not available.

Summary

The treatment of irradiated McCoy cells with diethylaminoethyl-dextran (DEAE-D) failed to increase the sensitivity of this cell-culture system for the primary isolation of *Chlamydia*.

Comparison of the sensitivity of the technique of culture in irradiated McCoy cells with culture in HeLa 229 cells treated with DEAE-D for the primary isolation of *Chlamydia* from conjunctival and genital material has shown that the irradiated McCoy cell-culture was slightly more sensitive than the HeLa 229 cells. In the case of specimens with lower numbers of infective particles, the McCoy cells were significantly more sensitive. The mean number of inclusions developed in the irradiated McCoy cell-culture was, on average, seven times higher than the mean number of inclusions found in the HeLa 229 cells treated with DEAE-D. Furthermore, the inclusions were larger and contained more elementary bodies.

Serial passage of *Chlamydia* in irradiated McCoy cell-culture was successfully continued with an increase in the number of inclusions of between 10^2 to 10^3 in each passage. The HeLa cells treated with DEAE-D failed to support the propagation of *Chlamydia* in serial passage.

Although the technique of culture in HeLa 229 cells treated with DEAE-D is not as sensitive as that of irradiated McCoy cells, it provides a reasonably sensitive and practical alternative for the primary isolation of *Chlamydia* in laboratories where facilities for the irradiation of cells are not available.

The authors are grateful to the staff of Moorfields and other hospitals for referring their patients, to our colleagues at The External Eye Diseases Clinic, Moorfields Eye Hospital, and the Diagnostic Clinic, Institute of Ophthalmology, for the collection of clinical specimens, to Prof. M. A. Faghih, Prof. H. Mohsenine, and Dr. J. Allami, School of Public Health, Teheran, for the collection of specimens from patients with hyper-endemic trachoma in Iran, to Dr. San-Ping Wang for sending us the HeLa 229 cells, and to Miss J. Johnstone for secretarial assistance.

We are indebted to the Research and Development Division of the Department of Health and Social Security and to the World Health Organisation for financial support for this work.

References

- DAROUGAR, S., CUBITT, S., and JONES, B. R. (1974) *Brit. J. vener. Dis.*, **50**, 308
- , JONES, B. R., KINNISON, J. R., VAUGHAN-JACKSON, J. D., and DUNLOP, E. M. C. (1972) *Ibid.*, **48**, 416
- , KINNISON, J. R., and JONES, B. R. (1971a) "Simplified irradiated McCoy cell-culture for the isolation of *Chlamydia*", in "Trachoma and Related Disorders", ed. R. L. Nichols, p. 63. Excerpta Medica, Amsterdam and New York
- , TREHARNE, J. D., DWYER, R. ST. C., KINNISON, J. R., and JONES, B. R. (1971b) *Brit. J. Ophthalm.*, **55**, 591
- DUC-NGUYEN, H. (1968) *J. Virol.*, **2**, 643
- DUNLOP, E. M. C., HARE, M. J., DAROUGAR, S., JONES, B. R., and RICE, N. S. C. (1969) *J. infect. Dis.*, **120**, 463
- , VAUGHAN-JACKSON, J. D., and DAROUGAR, S. (1972) *Brit. J. vener. Dis.*, **48**, 421
- GORDON, F. B., DRESSLER, H. R., and QUAN, A. L. (1967) *Amer. J. Ophthalm.*, **63**, 1044
- , HARPER, I. A., QUAN, A. L., TREHARNE, J. D., DWYER, R. ST. C., and GARLAND, J. A. (1969) *J. infect. Dis.*, **120**, 451
- , MAGRUDER, G. B., QUAN, A. L., and ARM, H. G. (1963) *Proc. Soc. exp. Biol. (N.Y.)*, **112**, 236
- HARRISON, M. J. (1970) *Aust. J. exp. Biol. med. Sci.*, **48**, 207
- KAPLAN, M. M., WIKTOR, T. J., MAES, R. F., CAMPBELL, J. B., and KOPROWSKI, H. (1967) *J. Virol.*, **1**, 145
- KUO, C. C., and WANG, S. P. (1972) *J. infect. Dis.*, **125**, 665
- MAGRUDER, G. B., GORDON, F. B., QUAN, A. L., and DRESSLER, H. R. (1963) *Arch. Ophthalm. (Chicago)*, **69**, 300
- ORIEL, J. D., REEVE, P., POWIS, P., MILLER, A., and NICOL, C. S. (1972) *Brit. J. vener. Dis.*, **48**, 429
- PAGANO, J. S., and VAHERI, A. (1965) *Arch. ges. Virusforsch.*, **17**, 456
- RICHMOND, S. J., HILTON, A. L., and CLARKE, S. K. R. (1972) *Brit. J. vener. Dis.*, **48**, 437
- ROTA, T. R., and NICHOLS, R. L. (1971) *J. infect. Dis.*, **124**, 419
- VAHERI, A., SEDWICK, W. D., and PLOTKIN, S. A. (1967) *Proc. Soc. exp. Biol. (N.Y.)*, **125**, 1092
- VOGT, P. K. (1967) *Virology*, **33**, 175