J. Hyg., Camb. (1974), **72**, 121
Printed in Great Britain

Cultivation of TRIC agents: a comparison between the use of BHK-21 and irradiated McCoy cells

By W. A. BLYTH* and JANICE TAVERNE†
MRC Trachoma Unit, Lister Institute of Preventive Medicine, Chelsea Bridge Road, London, SW1W 8RH

(*Received* 26 *June* 1973)

SUMMARY

BHK-21 cells and HeLa cells were as sensitive as irradiated McCoy cells for titrating both fast- and slow-growing strains of TRIC agents. BHK-21 cells were as efficient as irradiated McCoy cells for isolating TRIC agents from trachoma and from the genital tract of patients with non-gonococcal urethritis. More inclusions were formed in BHK-21 than in irradiated McCoy cells.

INTRODUCTION

Like some other *Chlamydia*, the agents of trachoma and inclusion conjunctivitis (TRIC agents) were first isolated in the chick embryo yolk sac, and this host is still used both for isolation and for growing the organisms in the laboratory. Many of the disadvantages of chick embryos can be avoided by using cell cultures. For isolation work, Gordon, Magruder, Quan & Arm (1963) pioneered the use of cell cultures by demonstrating that trachoma organisms from the conjunctiva of experimentally infected monkeys formed inclusions in McCoy cell cultures. Later the agent was isolated from patients with trachoma in McCoy cells previously exposed to gamma irradiation (Gordon & Quan, 1965). Irradiated McCoy cells were shown to be as sensitive as chick embryos to several TRIC agents that had been grown in yolk sacs or McCoy cell cultures (Gordon, Dressler & Quan, 1967); subsequently these irradiated cultures were reported to be more sensitive than eggs for the isolation of TRIC organisms from both human conjunctival and genital specimens (Gordon *et al.* 1969; Darougar *et al.* 1971; Gordon *et al.* 1972*b*), although Ford and McCandlish (1971) found that the isolation rates from urethral specimens were similar in eggs and in irradiated McCoy cells.

In the first direct comparison of unirradiated and irradiated cultures, Gordon *et al.* (1972*a*) showed that, from a given yolk sac suspension of a TRIC agent, more and bigger inclusions developed in irradiated than in unirradiated cultures of McCoy cells. By contrast, in the only reported comparison with a different cell line, unirradiated cultures of HeLa-229 cells proved to be as efficient as irradiated

* Present address: Department of Bacteriology, The Medical School, University Walk, Bristol, BS8 1TD.
† Present address: Department of Pathology, Royal College of Surgeons of England, London, WC2A 3PN.

McCoy cells for isolation of TRIC agents (Kuo, Wang, Wentworth & Grayston, 1972); both sorts of culture were, however, treated with DEAE-dextran. We report here a comparison of the sensitivity of BHK-21 cells and McCoy cells, both irradiated and unirradiated, for isolating TRIC agents from clinical specimens and for titrating laboratory strains.

METHODS

TRIC agents

The following strains were used; the suffix *f* denotes a fast-killing variant (Taverne, Blyth & Reeve, 1964): yolk-sac (YS) pools of MRC-4 (Jones, 1961) and MRC-4*f* purified with M-KCl (Taverne & Blyth, 1971), and a 20% YS suspension from the 25th egg passage of TW 3 (Wang & Grayston, 1963) kindly provided by Dr F. B. Gordon, Naval Medical Research Institute, Bethesda, Maryland.

Conjunctival specimens

Specimens were collected from Gambian children by Dr S. Sowa of this Unit, who also provided the relevant clinical and laboratory information. Scrapings, suspended in 1 ml. of phosphate-buffered saline (PBS), pH 7·4, containing 0·25 M sucrose, 10% calf serum and 1000 μg. of streptomycin, were stored at $-70°$ C. Before inoculation of cell cultures the suspensions were treated in an ultrasonic bath at 0° C. for 30 sec. on stage 8 (Soniprobe Cleaning Tank, Type 1130/2A, Dawe Instruments Ltd.) and diluted with 3 ml. of the maintenance medium used for BHK-21 cells.

Urethral specimens

Specimens were taken from men with non-gonococcal urethritis attending St Thomas's Hospital, London, S.E.1. They were stored at 4° C., each in 1 ml. of PBS containing 0·25 M sucrose, 1% bovine serum albumen, 500 μg. streptomycin and 25 units neomycin; if not used within 24 hr. they were stored at $-70°$ C. Before inoculation they were shaken vigorously for 30 sec. with glass beads in a mechanical shaker and then diluted with 3 ml. of McCoy cell maintenance medium.

Cell cultures

BHK-21 cells (Stoker & Macpherson, 1964) and HeLa cells (bought from Flow Laboratories Ltd.) were grown in 20 oz. glass bottles from Friday to Monday, when they were stripped off the glass, suspended in fresh growth medium and stored at 4° C. for the rest of the week. For use, cells were suspended in warm growth medium and seeded, 2×10^5 cells per tube, in flat-bottomed plastic tubes containing glass cover-slips 12 mm. in diameter. The tubes were incubated at 35° C. overnight in air with a 5% CO_2 concentration maintained by Pardee's buffer (Bellett, 1960).

McCoy cells were obtained through the courtesy of Dr J. Treharne (Institute of Ophthalmology, London) and Mrs Phillipa Powis (St Thomas's Hospital, London, S.E.1.). They were shown to be mouse cells by chromosome analysis, for which we are grateful to Dr A. Whitaker, Wellcome Laboratories, Beckenham, Kent; this confirms the finding of Gordon *et al.* (1972*a*). Confluent cell layers in plastic

bottles (Falcon Plastics) were subjected to 4000 R. from a ^{60}Co source at the Imperial College of Science and Technology; after irradiation the medium was changed. Next day 2×10^5 cells were seeded onto cover-slips in flat-bottomed plastic tubes. These were then incubated at 35° C. in 5% CO_2 in air for 3–5 days. Cultures of unirradiated McCoy cells were used after incubation overnight.

Growth medium for BHK-21, HeLa and unirradiated McCoy cells used in titrations was Eagle's tissue culture medium (BHK) (Wellcome Reagents Ltd.) supplemented with 0·035% $NaHCO_3$, 10% tryptose phosphate broth (Difco Laboratories) and 10% calf serum (Flow Laboratories Ltd.).

Maintenance medium (MM) was growth medium without serum; the pH was adjusted to 7·5 with tris and HCl to a final concentration of 0·05 M tris. It contained streptomycin 200 μg./ml. and, when used with human specimens, neomycin 25 units/ml.

Growth and maintenance medium for irradiated McCoy cells used for isolation of TRIC agents was Eagle's MEM tissue culture medium (Wellcome Reagents Ltd.) supplemented with 0·15% $NaHCO_3$, 10% foetal bovine serum, 1% vitamins (MEM vitamins, Flow Laboratories Ltd.), 0·03% *l*-glutamine and 200 μg./ml. streptomycin. Neomycin 25 units/ml. was added after inoculation of clinical material.

Infectivity titrations in cell cultures

Yolk sac suspensions were diluted in the appropriate maintenance medium (MM) and 1 ml. volumes were centrifuged onto the cells in tubes in a swing-out head at 600 ***g*** for 30 min. at 35° C. Concentrations of organisms, when known, were adjusted to infect no more than 10% of the cells (less than 5 inclusions per high power field of the microscope) (Blyth & Taverne, 1972). For isolation from clinical specimens three cultures of BHK-21 and three of irradiated McCoy cells were inoculated each with 0·4 ml. volumes and centrifuged at 3500 ***g*** for 1 hr. at 35° C. After centrifugation, the medium was replaced by fresh MM and the cultures were incubated in 5% CO_2 in air at 35° C. for 3 days, or for 2 days when *f* strains were titrated. Cultures were then fixed and stained with iodine and the inclusions were counted; titres are expressed as the number of inclusion-forming units (IFU) per ml. of original suspension. With clinical specimens, if no inclusions were found in 100 microscope fields chosen at random, the whole cover-slip was examined before being recorded as negative.

RESULTS

Our preliminary experiments with McCoy cell cultures confirmed the observations of Gordon *et al.* (1972*a*) on the effects of irradiation; by the 4th or 5th day after irradiation many giant cells, often with bizarre nuclei, had formed and the cultures slowly degenerated by the 8th or 9th day. The greatest numbers of inclusions and the most reproducible titrations were obtained with cultures which had been irradiated with 4000 R. and infected 4–5 days later; this procedure was therefore used as a routine. The numbers of inclusions and their morphology were about the same whether we grew and maintained the McCoy cells in BHK-21

Table 1. *Infectivity titrations of MRC-4 and MRC-4f strains of TRIC agent in unirradiated and irradiated McCoy cell cultures: duplicate titrations of two yolk sac suspensions*

	Infectivity titre (inclusion-forming units/ml.)	
TRIC agent	Unirradiated	Irradiated
MRC-4	$5{\cdot}5 \times 10^6$	$3{\cdot}7 \times 10^6$
	$1{\cdot}2 \times 10^7$	$7{\cdot}4 \times 10^6$
	Mean $8{\cdot}8 \times 10^6$	$5{\cdot}5 \times 10^6$
MRC-4*f*	$5{\cdot}8 \times 10^7$	$6{\cdot}0 \times 10^7$
	$1{\cdot}2 \times 10^8$	$5{\cdot}4 \times 10^7$
	Mean $8{\cdot}9 \times 10^7$	$5{\cdot}7 \times 10^7$

medium or in the medium provided by Dr Gordon (Gordon *et al.* 1972*a*). Likewise, there was no obvious difference whether we used 0·22 % $NaHCO_3$ in the medium (as have others) or 0·035 %. When irradiated McCoy cells were used for isolation of TRIC agents, we grew and maintained them on Eagle's MEM – rather than the BHK medium – since this has generally been used by others for isolation work.

Infectivity titrations in irradiated and unirradiated McCoy cells

Yolk sac suspensions of MRC-4 and MRC-4*f* were titrated simultaneously in unirradiated and irradiated McCoy cells; Table 1 shows the results of two representative tests. Although the infectivity titres obtained in the two types of culture were similar the morphology of the inclusions was very different. In unirradiated cells, they were often smaller than the cell nuclei, stained almost black with iodine and contained few elementary bodies. In irradiated cells, the inclusions were 5- to 10-fold greater in diameter; they stained reddish brown with iodine and contained many elementary bodies.

Infectivity titrations in BHK-21 cells, HeLa cells and irradiated McCoy cells

Yolk sac suspensions of MRC-4, MRC-4*f* and TW3 were titrated in parallel in BHK-21 cells and irradiated McCoy cells or in BHK-21 cells and HeLa cells. No significant differences were found between the three types of cell culture; the minor variations reflected the state of health of a particular series of cultures rather than any inherent differences in susceptibility to TRIC agents. (Table 2).

Some investigations were also made of BHK-21 cells irradiated and treated like McCoy cells. The cells did not enlarge after irradiation but, instead, gradually degenerated. After infection with TRIC agents, fewer inclusions developed in them than in control unirradiated cultures.

Isolation of TRIC agents from conjunctival specimens using BHK-21 cells and irradiated McCoy cells.

Conjunctival scrapings were taken from children with active trachoma (stage II or III) from whom TRIC agents had previously been isolated in eggs. The specimens were inoculated in parallel into cultures of BHK-21 cells and irradiated

Table 2. *Infectivity titrations of MRC-4, MRC-4f, and TW3 strains of TRIC agents in irradiated McCoy, BHK-21 and HeLa cells*

TRIC agent	Pool	Infectivity titres (inclusion-forming units/ml.) Irradiated McCoy	BHK-21	HeLa
MRC-4*f*	a	$1{\cdot}6 \times 10^8$	$2{\cdot}1 \times 10^8$	ND
	b	ND	$1{\cdot}4 \times 10^9$	$1{\cdot}5 \times 10^9$
MRC-4	a	$5{\cdot}4 \times 10^7$	$1{\cdot}0 \times 10^8$	ND
	b	ND	$1{\cdot}9 \times 10^7$	$2{\cdot}7 \times 10^7$
TW3		$7{\cdot}3 \times 10^4$	$1{\cdot}1 \times 10^5$	ND

ND = not done.

McCoy cells. Both here and with urethral specimens, a control titration of a yolk sac suspension of MRC-4 of known infectivity titre was always included as a check on the health of the cultures and their sensitivity to infection.

None of the specimens tested gave sufficient numbers of inclusions for valid comparisons to be made between the cell systems on this basis. However, if the observation of at least one iodine-staining inclusion per cover-slip is accepted as evidence of isolation, there were no significant differences; of the seven specimens examined, six were positive in BHK-21 cells, five of which were also positive in irradiated McCoy cells.

Isolation of TRIC agents from urethral scrapings using BHK-21 cells and irradiated McCoy cells

For preliminary work, Mrs Phillipa Powis provided material from three specimens which had already been shown to contain TRIC agents and, in our comparative tests, inclusions developed in both culture systems from all of them. A further 74 specimens (not previously screened) were subsequently tested in parallel in BHK-21 cells and irradiated McCoy cells. Inclusions developed in both cell types from six specimens; a further two were positive only in BHK-21 cells. In eight of the total of nine specimens positive in both cell types more inclusions developed in BHK-21 cells (a mean of 23 per 100 microscope fields, with a range of 0·8–91) than in irradiated McCoy cells (a mean of nine per 100 fields, with a range of 0·01–30).

DISCUSSION

A virtually unsubstantiated belief that irradiated McCoy cells have some special advantage for the isolation of TRIC agents has become widespread among workers investigating the incidence of these organisms in the genital tract. Indeed, a considerable number of isolations were made in various laboratories with this culture system before any evidence was published to suggest that irradiation increased the susceptibility of McCoy cells to infection by TRIC agents, or that such irradiated cells were more efficient than other cell lines used unirradiated. Although there is no doubt that irradiation of McCoy cells has a remarkable effect

on the morphology of inclusions that form in them, inclusions of similar morphology form in other cells that have not been irradiated; it thus seems that the choice of McCoy cells for work with TRIC agents was unfortunate. Our work with other cell lines shows that irradiation of the host cell is unnecessary for efficient multiplication of TRIC agents, whether they are laboratory strains grown in the chick embryo yolk sac or wild-type strains obtained directly from their human host. Indeed, a consideration of the particle/infectivity ratios of TRIC agents makes such a finding likely. When fast-growing strains of TRIC agents (*f* variants) are titrated by centrifugation onto BHK-21 cells, the number of infective organisms measured as inclusion-forming units is almost as great as the total number of organisms in the inoculum, counted by dark-ground illumination (Taverne & Blyth, 1971). It is thus impossible for another cell culture system to yield substantially more inclusions when inoculated with the same suspension. By contrast, the ratio of particles to infectivity for BHK-21 cells of yolk sac suspensions of slow-growing strains is usually between 100:1 and 1000:1. These organisms may be potentially infective, and their viability might be demonstrated by adaptation to another host cell, but so far no other system has been reported that supports the multiplication of the apparently uninfective organisms. Our comparative titrations were done with organisms passaged in the chick embryo; strains that had been selectively passaged in particular cell lines were deliberately avoided.

In our hands, no more inclusions were formed from a given inoculum of slow-growing TRIC agents titrated in irradiated McCoy cells than in BHK-21 cells. Similarly the yields of new infective elementary bodies from such inclusions were at best approximately the same: 52 hr. after infection with strain MRC-4 in conditions designed to avoid multiple infection, cultures of BHK-21 cells yielded 1 IFU per inclusion; irradiated McCoy cells maintained in medium containing 0·035 % $NaHCO_3$ yielded 0·05 IFU per inclusion, which was increased to 1 IFU per inclusion in medium containing 0·22 % $NaHCO_3$ (unpublished results).

When unirradiated and irradiated cultures of the same cell line – McCoy cells – are compared, our results conflict with those of Gordon and his co-workers (1972*a*), who found 4–8 times more inclusions in irradiated cultures. However, we used different criteria to identify inclusions in the two sorts of culture. In irradiated cells inclusions were large and resembled those commonly obtained in BHK-21 cells, but in unirradiated cells they were often extremely small and stained black with iodine, an appearance sometimes seen in conditions unfavourable to normal development of TRIC agents. Thus, although the numbers counted were similar in both instances, the cycle of development in terms of morphology was complete only in irradiated cells. Moreover, not only the morphology but the number of inclusions obtained from a given inoculum is well known to vary with the condition of the cultures, including, among other factors, their age and the pH of the medium. The higher yields of infective organisms from irradiated cultures reported by Gordon *et al.* (1972*a*) were expressed not per inclusion but per volume of culture harvest. This result would be expected, irrespective of whether development was complete in the unirradiated cells, since more inclusions were present in each irradiated culture.

Apart from results obtained with irradiated McCoy cells, there is evidence that TRIC agents grow best in cells that are not multiplying. The conditions used in our routine work with BHK-21 cells produce cultures that contain very few dividing cells and are parasynchronous (Newton & Wildy, 1959). Suspended cells taken from storage at 4° C. were cultured overnight, during which time their number increased but failed to double. The cultures were then maintained in medium without serum, which prevented further multiplication. By contrast, a transformed line of BHK-21 cells that continued to multiply even after the cultures were infected, yielded at least ten times fewer inclusions than cultures of the same line in which cell multiplication was inhibited, either by exposing the cultures to γ-irradiation or to medium containing a low concentration of glutamine. (In cultures deprived of glutamine the number of inclusions were similar to those in irradiated cultures but the growth cycle of the agent was not complete.) (T. Rota & W. A. Blyth, unpublished results.)

That irradiation does not confer a unique advantage is plain from our results both with TRIC agents cultivated in the chick embryo yolk sac and with organisms from human tissues. We emphasize that our work with material from men with non-gonococcal urethritis does not give a measure of the rate of isolation of TRIC organisms; it merely demonstrates that isolations from human tissues can be made equally well in BHK-21 or in irradiated McCoy cells.

It is a pleasure to thank Mrs Anne Fitzpatrick and Mr R. C. Ballard for their skilled technical assistance. We should also like to thank Dr P. Clay for his great help with the irradiation of our cultures. We are grateful to Dr F. B. Gordon for his kind co-operation and for giving us samples of cells, media and TRIC organisms. Without the generous help of our colleagues at St Thomas's Hospital, Drs P. Reeve, J. D. Oriel and Ann Miller and Mrs Phillipa Powis, the work on urethral specimens would not have been possible.

REFERENCES

Bellett, A. J. D. (1960). Use of Pardee's CO_2 buffer in plaque titration of EMC virus. *Virology* **10**, 285.

Blyth, W. A. & Taverne, J. (1972). Some consequences of the multiple infection of cell cultures by TRIC organisms. *Journal of Hygiene* **70**, 33.

Darougar, S., Treharne, J. D., Dwyer, R. St C., Kinnison, J. R. & Jones, B. R. (1971). Isolation of TRIC agent (Chlamydia) in irradiated McCoy cell culture from endemic trachoma in field studies in Iran. Comparison with other laboratory tests for detection of Chlamydia. *British Journal of Ophthalmology* **55**, 591.

Ford, D. K. & McCandlish, L. (1971). Isolation of human genital TRIC agents in nongonococcal urethritis and Reiter's disease. *British Journal of Venereal Diseases* **47**, 196.

Gordon, F. B., Dressler, H. R. & Quan, A. L. (1967). Relative sensitivity of cell culture and yolk sac for detection of TRIC infection. *American Journal of Ophthalmology* **63**, 1044.

Gordon, F. B., Dressler, H. R., Quan, A. L., McQuilkin, W. T. & Thomas, J. I. (1972*a*). Effect of ionising irradiation on susceptibility of McCoy cell cultures to *Chlamydia trachomatis*. *Applied Microbiology* **23**, 123.

Gordon, F. B., Harper, I. A., Quan, A. L., Treharne, J. D., Dwyer, R. St C., & Garland, J. A. (1969). Detection of Chlamydia (Bedsonia) in certain infections of man. I. Laboratory procedures: comparison of yolk sac and cell culture for detection and isolation. *Journal of Infectious Diseases* **120**, 451.

Gordon, F. B., Magruder, G. B., Quan, A. L. & Arm, H. G. (1963). Cell cultures for detection of trachoma virus from experimental simian infections. *Proceedings of the Society for Experimental Biology and Medicine* **112**, 236.

Gordon, F. B. & Quan, A. L. (1965). Isolation of the trachoma agent in cell culture. *Proceedings of the Society for Experimental Biology and Medicine* **118**, 354.

Gordon, F. B., Woolridge, R. L., Quan, A. L., Gillmore, J. B., Arm, H. G., Yang, Y.-F. & Magruder, G. B. (1972*b*). Field studies on McCoy cell cultures for detection of *Chlamydia trachomatis*. *Southeast Asian Journal of Tropical Medicine and Public Health* **3**, 69.

Jones, B. R. (1961). TRIC virus infections in London. *Transactions of the Ophthalmological Society of the United Kingdom* **81**, 367.

Kuo, C.-C., Wang, S.-P., Wentworth, B. B. & Grayston, J. T. (1972). Primary isolation of TRIC organisms in HeLa 229 cells treated with DEAE-dextran. *Journal of Infectious Diseases* **125**, 665.

Newton, A. A. & Wildy, P. (1959). Parasynchronous division of HeLa cells. *Experimental Cell Research* **16**, 624.

Stoker, M. & Macpherson, I. (1964). Syrian hamster fibroblast cells, line BHK-21 and its derivatives. *Nature, London* **203**, 1355.

Taverne, J., Blyth, W. A. & Reeve, P. (1964). Toxicity of the agents of trachoma and inclusion conjunctivitis. *Journal of General Microbiology* **37**, 271.

Taverne, J. & Blyth, W. A. (1971). Interactions between trachoma organisms and macrophages. In *Trachoma and Related Disorders* (ed. R. L. Nichols), pp. 88–107. Proceedings of a symposium held in Boston, Mass., August 1970. Amsterdam: Excerpta Medica.

Wang, S. P. & Grayston, J. T. (1963). Classification of trachoma virus strains by protection of mice from toxic death. *Journal of Immunology* **90**, 849.