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Early Detection of Chlamydial Inclusions Combining the Use of Cycloheximide-Treated McCoy Cells and Immunofluorescence Staining

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Detection of *Chlamydia trachomatis* inclusions only 21 h after a specimen reaches the laboratory has been achieved by the combined use of cycloheximide-treated McCoy cells and immunofluorescence staining. Moreover, cells exposed to cycloheximide were more sensitive for detecting chlamydial inclusions than those pretreated by irradiation, since larger numbers of inclusions were found in the former cells. The application of this rapid and sensitive method allows a diagnosis of chlamydial infection to be made before antibiotic therapy is started. In this way, it should enable the treatment of nonspecific genital infections to be placed on a more rational basis.

The recent implication of *Chlamydia trachomatis* in nonspecific genital infections has resulted in a number of publications describing the most efficient method for isolating and identifying this organism in tissue culture. Several different cell lines and various experimental conditions have been tried, and varying success has been claimed for each method (1-3, 6, 9, 11, 14, 15, 17). The McCoy cell line has become widely used for chlamydial isolation, but the necessity of pretreating the cells with metabolic inhibitors to increase their sensitivity to chlamydiae remains controversial (5, 8, 10, 15). It must be concluded from the published evidence that the success of a single isolation method varies in different laboratories, and that the most sensitive method of one group of workers may prove less useful in the hands of another.

We have found that the most sensitive culture system involves the use of nonreplicating McCoy cells. These cells have been produced in the past by irradiation with 4,500 rads from a cobalt source at least 4 days before being used for chlamydial isolation. More recently, chemical inhibitors of cells, such as 5-iodo-2-deoxyuridine and cytochalasin B, have been described as alternatives to irradiation for the pretreatment of McCoy cells (18, 20). Cycloheximide, which is a specific inhibitor of eukaryotic cell protein synthesis (4), has also proved useful in recent isolation studies (7, 16). It is added to 24-h cell monolayers after specimen inoculation and centrifugation, thereby eliminating the delay between cell treatment and inoculation that is essential with irradiation, 5-iodo-2-deoxyuridine, or cytochalasin B treatment. We report here our use of cycloheximide-treated McCoy cells, which we have found to have increased sensitivity for chlamydial isolation. In addition, we have combined this method with immunofluorescence staining of chlamydial inclusions, which not only increases the sensitivity of the isolation technique but also allows a more rapid diagnosis of chlamydial infection. The clinical implications of such a rapid diagnostic method are discussed.

MATERIALS AND METHODS

McCoy cells. McCoy cells were obtained from J. H. Pearce (Microbiology Department, Birmingham University) and have been maintained for 3 years in this laboratory.

Media. Complete medium with antibiotics (CMA), for cell growth and maintenance, consisted of Eagle minimal essential medium containing 5% heat-inactivated fetal calf serum, 1% vitamins (Flow Laboratories), 1% 200 mM glutamine, 100 μg of vancomycin per ml, 50 μg of streptomycin per ml, and 4.4 mM bicarbonate to pH 7.0. Complete medium containing glucose and antibiotics (CMGA), used as chlamydial growth medium, was CMA containing 5% extra fetal calf serum and 0.5% glucose.

Transport medium for clinical samples consisted of 0.2 M sucrose in 0.02 M phosphate buffer, pH 7.0 (2SP), containing 10% heat-inactivated fetal calf serum, the antibiotics mentioned above, and 25 U of nystatin per ml.

Cycloheximide. Cycloheximide was obtained from Sigma and was added to cells as a 1 μg/ml solution in CMGA (CY).

Laboratory strains. A urethral isolate from a pa-

tient who had nongonococcal urethritis was used in most experiments. It was designated 78α and had been passed four times in irradiated McCoy cells. Two different batches of this material were used (P4A and P4B). *C. trachomatis* serotypes A to I were originally obtained from J. Treharne, Institute of Ophthalmology, London. *Chlamydia psittaci* strains (bovine abortion and sheep abortion) were obtained from Wellcome Laboratories, Beckenham.

Clinical specimens. Clinical specimens were obtained from patients attending venereal disease clinics. Urethral swabs were taken from men as previously described (13), and cervical material was obtained by rotating a cotton-wool swab in the cervix. Swabs were vigorously agitated in 2SP in a plastic vial, and the samples were immediately placed in liquid nitrogen for storage.

Antisera. Lymphogranuloma venereum antiserum, which had a microimmunofluorescence titer of 1/$\geq$256, was obtained from a convalescent patient. For use, it was diluted to give a titer of 1/64. Antisera to sheep and cattle abortion agents, each of which had a titer of 1/256, were obtained from animals experimentally infected with the sheep or cattle agents.

Conjugated serum. Sheep anti-human, rabbit anti-sheep, and rabbit anti-bovine immunoglobulin, all fluorescein labeled (Wellcome Reagents Ltd.), were used at a dilution of 1/20.

Growth and preparation of McCoy cells. Cells were grown in CMA in plastic tissue culture flasks (A/S NUNC). Confluent monolayers were either passaged or irradiated (IR), or used to seed cover slips. Irradiation was carried out in a ^{60}Co source, using a total dose of 4,500 rads. After exposure of the cells, the CMA was replaced with fresh medium, and the flasks were incubated at 35°C for a further 3 to 4 days. The cells were then seeded onto 13-mm-diameter cover slips in flat-bottomed tubes (Turner-Stayne Laboratories) at a concentration of 1.25×10^5 cells in CMA per cover slip. After 18 to 24 h of incubation confluent monolayers of IR cells were used for inoculation. Cells to be CY treated after inoculation (see below) were seeded from monolayers in plastic flasks onto cover slips at 2×10^5 cells/tube and used 18 to 24 h later.

Inoculation of cell monolayers. All specimens to be tested were stored in 2SP in liquid nitrogen. They were rapidly thawed in a 37°C water bath and thoroughly agitated on a Vortex mixer. Each clinical specimen was diluted in a sufficient volume of CMGA to inoculate two tubes of cells, and the laboratory-passaged isolate (78α) was diluted as required for each experiment. The CMA was removed from each tube of cells immediately before inoculation. After thorough mixing, 0.5 ml of diluted sample was added to each tube with an automatic syringe to ensure equal distribution of the material. The tubes were centrifuged at $2,800 \times g$ for 1 h in an MSE Super Medium centrifuge and then incubated for 2 h at 35°C. The fluid was replaced with 2 ml of CMGA (for IR cells) or 2 ml of CY, and the cells were incubated for at least another 16 h at 35°C.

Immunofluorescence (IF) staining. The medium was removed from the tubes, and the cells were fixed with methanol for 10 min at 35°C. The cover slips were washed with pH 6.8 buffer, rinsed in methanol to aid drying, and mounted on microscope slides, monolayer uppermost, with Diatex (Raymond Lamb). Lymphogranuloma venereum antiserum was applied to each cover slip, and the slides were incubated for 10 min at 35°C in a moist chamber. They were then washed twice for 10 min in phosphate-buffered saline (Dulbecco A), rinsed in distilled water, and dried. Fluorescein-conjugated serum was spread over each cover slip, and the slides were incubated for 10 min at 35°C. The excess serum was then removed by two 10-min washes in phosphate-buffered saline (Dulbecco A), the second wash containing a few drops of 0.25% trypan blue as counter stain. The slides were finally rinsed in distilled water and dried, and the monolayers were mounted with Diatex under cover slips. Cells were examined with a Nikon S-Ke microscope fitted with an epifluorescence attachment (Projectina Co., Ayrshire). Known coded chlamydia-positive and -negative specimens were tested against the lymphogranuloma venereum antiserum whenever clinical or other specimens were examined by IF staining.

Giemsa staining. Cell monolayers were fixed in methanol for 10 min as described above, followed by a wash with pH 6.8 buffer. Freshly prepared 10% Giemsa stain (Gurr Improved) was added, and the tubes were incubated for 30 min at 35°C. The stain was removed and the cover slips washed with 30% methanol in buffer, followed by two washes in buffer. The cover slips were removed from the tubes, dried, and mounted, monolayer downwards, on microscope slides with either Gurr Neutral Mounting Medium or Gurr Clearmount. Cells were examined under dark-field illumination with a Nikon S-Ke microscope. Positive and negative controls for staining specificity were included as above.

Inclusion counts. Inclusion counts were carried out independently by at least two experienced observers, each examining either the whole cover slip three times or making counts of three sets of 30 fields chosen at random at $\times$200 magnification. An average count was calculated for the readings observed by either method. Specimens were coded, and the results obtained by one technique were not revealed until those of the other were available.

Experiments. Experiments were designed (i) to determine the relative sensitivity of CY-treated and IR McCoy cells for the isolation of chlamydiae, (ii) to compare the efficiency of Giemsa and IF staining for demonstrating the inclusions at various stages in the chlamydial growth cycle, and (iii) to determine the earliest time at which a diagnosis of chlamydial infection could be made by combining these two techniques.

Abbreviations. Various combinations of cell treatments, staining procedures, and incubation times have been abbreviated. For example, cycloheximide treatment of McCoy cells followed by immunofluorescence staining 19 h after inoculation (i.e., incubation for 18 h) is designated CY:IF:19 h.

RESULTS

Comparison of CY-treated and IR McCoy cells for the isolation of strain 78α. The labo-

ratory-passaged chlamydial isolate 78α (batches P4A and P4B) produced at least as many inclusions in CY-treated McCoy cells as in IR McCoy cells (Table 1). This suggested that cells exposed to cycloheximide were at least as sensitive for chlamydial isolation as those pretreated by irradiation.

After Giemsa staining, the inclusions seen in CY-treated cells were smaller than those in IR cells, probably because chemical inhibition of cell multiplication does not produce such extensive areas of cytoplasm as does irradiation. The inclusions were, however, more easily identifiable than those seen in untreated McCoy cells. In this laboratory, we have found the inclusions in the latter cells to be very small, difficult to identify, and not countable with any degree of accuracy.

Comparison of IF and Giemsa staining for identification of chlamydial inclusions in IR cells. Twelve urethral specimens were each divided into two equal portions, which were inoculated onto IR McCoy cells. After incubation at 35°C for 23 h, one cover slip of each specimen was examined by IF, and after 47 h the second cover slip was stained with Giemsa. Inclusions were detected in 4 of the 12 specimens by both staining methods (Table 2). However, the inclusions counted in the samples incubated for 23 h and stained by IF were much more numerous than those in the 47-h samples stained by Giemsa. In one sample (10A) one inclusion was detected only by IF, and in another sample (12A) one inclusion was detected only by Giemsa staining.

The inclusions stained by the IF method were bright green, discrete, and clearly identifiable in both the positive control and the positive clinical specimens. The trypan blue counterstain greatly reduced background and nonspecific fluorescence, although the latter was not usually a problem when dealing with urethral specimens.

TABLE 1. *Number of inclusions produced by strain 78α in CY-treated and IR McCoy cells*

Expt	Inoculum		Avg no. of inclusions[a]/cover slip in cells treated with:		CY:IR ratio
	Batch	Dilution	CY	IR	
1	78α P4A	1/20	2,179[b]	1,955	1.1:1
		1/40	1,010	732	1.4:1
		1/80	421	438	1.0:1
		1/160	173	179	1.0:1
2		1/40	665	441	1.6:1
3	78α P4B	1/40	7,060	3,963	1.8:1

[a] Giemsa stained after 47 h of incubation.
[b] Each count derived from examining two cover slips.

TABLE 2. *Inclusions detected in IR cells after inoculation of urethral samples*[a]

Urethral sample[b]	Avg no. of inclusions/cover slip after staining by:		IF:Giemsa ratio
	IF	Giemsa	
3A	190[c]	43	4.4:1
10A	1	0	
12A	0	1	
15A	223	79	2.8:1
24A	17	6	2.8:1
30A	2,214	92	24.0:1

[a] Cells were incubated for 23 h and stained by IF and for 47 h followed by Giemsa staining.
[b] Six samples were negative by both staining methods.
[c] Each count derived from examining one cover slip.

Determination of the times at which inclusions may be demonstrated by IF or Giemsa staining. It is technically not possible to detect inclusions within the same working day as a specimen is inoculated, and so no attempt was made to seek inclusions before the next day, by which time at least 19 h had elapsed. Strain 78α (P4A) was diluted 1/40 and centrifuged onto IR and CY-treated McCoy cells. After 18, 19, 21, 23, and 47 h of incubation (i.e. 19, 20 h, etc., after inoculation), duplicate CY-treated cover slips were examined after IF and Giemsa staining, and IR cells were examined after Giemsa staining only.

Chlamydial inclusions could be easily identified in CY-treated cells by IF 19, 24, and 48 h after inoculation (Fig. 1 a, b, and c), and there was no increase in the number of inclusions during the 47-h incubation period. In contrast, inclusions could not be detected in either CY-treated or IR McCoy cells by Giemsa staining after 19 to 24 h. Furthermore, a greater number of inclusions (between 2,147 and 2,780/cover slip) were detected in CY-treated McCoy cells stained by IF at all the times tested than in either CY-treated (1,660/cover slip) or IR cells (1,310/cover slip) stained with Giemsa at 48 h. These results suggested that both CY treatment of cells and IF staining of inclusions contributed to a method that appeared to be more sensitive for the isolation of chlamydiae.

Comparison of CY-treated and IR McCoy cells and IF and Giemsa staining for chlamydial isolation. In light of the previous result, an experiment was designed to determine further the relative importance of CY treatment of cells and of IF staining for the improved sensitivity of the isolation method.

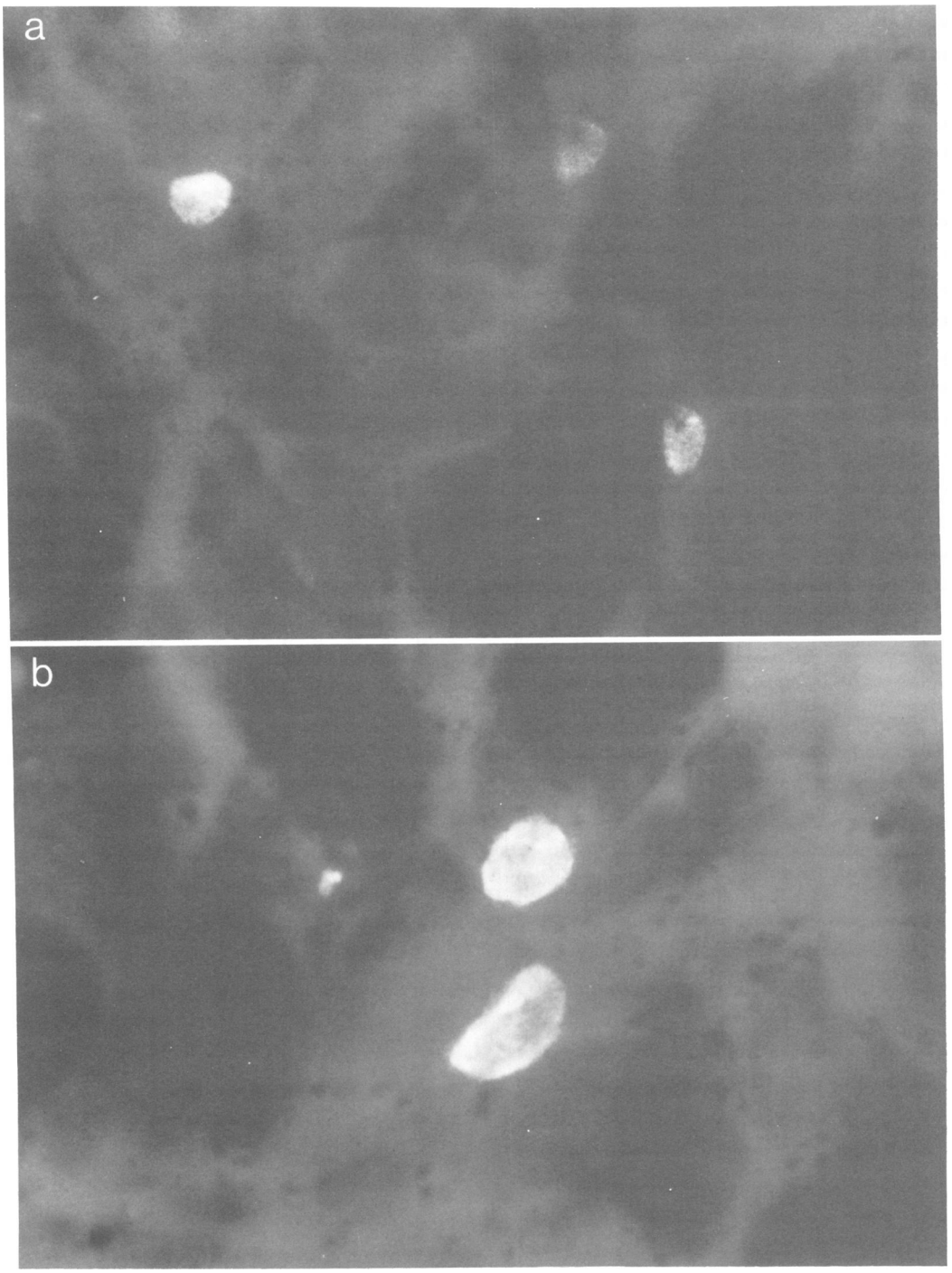
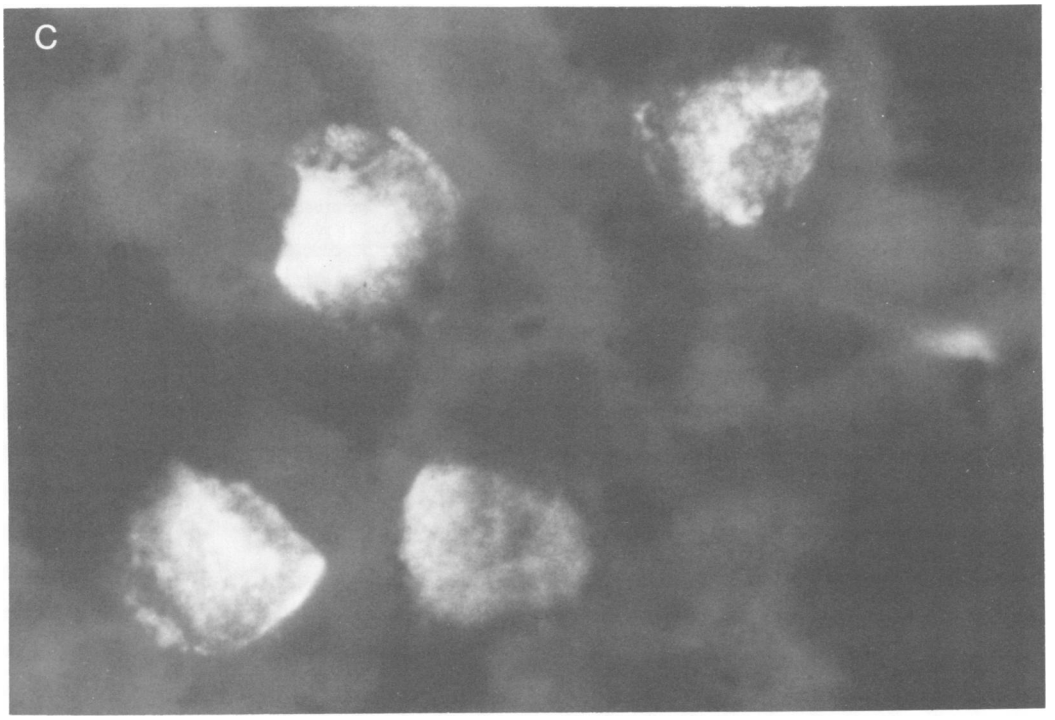


FIG. 1. *Chlamydial inclusions in CY-treated cells stained by the IF technique after (a) 18 h of incubation, (b) 23 h of incubation, and (c) 47 h of incubation.* ×1,500.



CY-treated and IR McCoy cells were inoculated with a 1/40 dilution of strain 78α P4B and stained by IF after 19 h and by Giemsa after 48 h.

The sensitivity of the combinations of cell treatments, staining methods, and times after inoculation, as judged by the number of inclusions detected, was in the following decreasing order: CY:IF:19 h; CY:Giemsa:48 h; IR:IF:19 h; and IR:Giemsa:48 h (Table 3). Thus, as in previous experiments, CY-treated cells were more sensitive than IR cells for detection of chlamydial inclusions, irrespective of incubation time and the staining method used. In addition, significantly more inclusions were seen after IF staining of either IR or CY-treated cells at 19 h than after Giemsa staining of IR cells at 48 h. However, fewer inclusions were seen by IF staining of IR cells than by Giemsa staining of CY-treated cells, suggesting that CY treatment is more important in the increased sensitivity of the method than is IF staining.

The sensitivity of the technique that appeared to have the greatest value, namely, CY:IF:19 h, was further compared with that of the more conventional method (IR:Giemsa: 48 h) by testing increasing dilutions of strain 78α P4B. Thus, a 1/40 dilution of strain 78α was further diluted (10^{-1} to 10^{-3}) in CMGA, and 0.5 ml of each dilution was centrifuged onto IR and CY-treated cells. Cells of both sorts were stained by IF after 19 h, and IR cells only were stained by Giemsa after 48 h. The numbers of inclusions counted are recorded in Table 4. At each dilution of the inoculum, the greatest number of inclusions was detected in CY-treated cells stained by IF after 19 h. The results indicated that by this technique there would be a greater likelihood of detecting chlamydiae in clinical specimens containing few organisms than by the use of IR cells stained by Giemsa at 48 h.

TABLE 3. *Number of inclusions produced by strain 78α in CY-treated or IR cells stained by IF and Giemsa at 19 h and by Giemsa at 48 h*

Treatment of cells	Avg no. of inclusions/cover slip in cells stained by:		
	IF at 19 h	Giemsa at 19 h	Giemsa at 48 h
CY	30,414[a]	NV[b]	24,940
IR	21,125	NV	15,907

[a] Each figure derived from the counts of three observers on three sets of 30 ×200-magnification fields on each of three cover slips.
[b] NV, Not visible.

Inclusions produced by *C. trachomatis* serotypes and *C. psittaci* strains detected by the CY:IF:19 h method. The most sensitive and

rapid technique, namely CY-treated cells followed by IF staining at 19 h, was tested for its ability to detect inclusions of several *C. trachomatis* serotypes and two *C. psittaci* strains. These were laboratory stocks of *C. trachomatis* serotypes A, B, C, D, E, F, G, H, and I and of *C. psittaci* strains of sheep and cattle abortion.

Inclusions were clearly visible 19 h after inoculation with all these strains, although the sensitivity of the rapid diagnostic method for each strain was not assessed by comparison with the standard isolation procedure (IR:Giemsa:48 h).

Comparison of CY:IF:19 h and IR:Giemsa:48 h methods for the isolation of chlamydiae from clinical specimens. Most of the previous results were obtained by using a laboratory-passed chlamydial strain. The sensitivity of the improved technique (CY:IF:19 h) was, therefore, compared with that of the conventional method (IF:Giemsa:48 h) by examining fresh clinical specimens. Seventy-three urethral and two cervical specimens were each inoculated onto both CY-treated and IR McCoy cells, which were incubated and stained as appropriate for each method. Chlamydiae were demonstrated in 16 of the 73 urethral and 1 of the cervical specimens by both IF and Giemsa staining (Table 5). No specimen was found to be positive by IF that was negative by Giemsa staining. However, the number of inclusions detected by IF was always more than by Giemsa, again indicating the greater sensitivity of the CY:IF:19 h technique. The intense fluorescence, generalized distribution, and intracellular location of inclusions clearly differentiated them from the small amount of nonspecifically fluorescing cell debris occasionally seen in clinical specimens.

TABLE 4. *Number of inclusions produced by various dilutions of strain 78α in CY-treated or IR cells stained by IF at 19 h and in IR cells stained by Giemsa at 48 h*

Dilution of inoculum	Treatment of cells	Avg no. of inclusions/cover slip in cells stained by:	
		IF at 19 h	Giemsa at 48 h
10^{-1}	CY	7,395[a]	
	IR	4,471	1,866
10^{-2}	CY	826	
	IR	494	307
10^{-3}	CY	100	
	IR	48	9

[a] Each count derived from examining five cover slips.

TABLE 5. *Comparison of CY-treated cells stained by IF at 19 h and IR cells stained by Giemsa at 48 h for the isolation of chlamydiae from clinical specimens*

Source of specimen	Avg no. of inclusions/cover slip in cells treated with:		Ratio of inclusions detected by IF and Giemsa
	CY and stained by IF at 19 h	IR and stained by Giemsa at 48 h	
Ur[a]	171[b]	12	14.4:1
Cervix	3,096	1,161	2.7:1
Ur	159	60	2.7:1
Ur	3,248	1,840	1.8:1
Ur	63	43	1.5:1
Ur	180	27	6.6:1
Ur	206	7	19.5:1
Ur	974	134	7.3:1
Ur	5,087	1,198	4.2:1
Ur	928	123	7.5:1
Ur	1,414	177	8.0:1
Ur	74	13	6.0:1
Ur	38,968	7,963	4.9:1
Ur	9,659	2,304	4.2:1
Ur	101	5	20.0:1
Ur	744	91	8.2:1
Ur	1,349	112	12.0:1

[a] Ur, Urethra.

[b] Each count derived from examining one cover slip.

DISCUSSION

In the past, we have used irradiated McCoy cells stained with Giemsa after 48 h of incubation for chlamydial isolation. Wang and colleagues (19) used IF staining but considered it more complicated than Giemsa staining and apparently have not used it routinely. However, we have found that CY treatment of McCoy cells in combination with IF staining of inclusions provides increased sensitivity and the ability to obtain results the day after a clinical specimen has been taken. Furthermore, the technique is simple. These criteria for an acceptable new isolation method are discussed further.

Sensitivity. The results of initial experiments indicated that both CY treatment of McCoy cells and IF staining contributed to increased sensitivity. It seems, however, that CY treatment plays the major role, since more inclusions were detected by Giemsa staining of CY-treated cells than by IF staining of IR cells. This suggests that the increase in sensitivity conferred by the IF staining does not compensate for that lost by using IR rather than CY-treated cells.

Reports from some laboratories indicate that inhibition of cell metabolism is unnecessary for efficient chlamydial isolation (8, 12, 15). We have found that 19 to 48 h after inoculation, inclusions in untreated McCoy cells are readily visible by IF staining. They are, however, very difficult to detect by Giemsa staining after 48 h because the metabolizing cells overgrow during this time. Giemsa-stained inclusions in CY-treated cells after 48 h were sometimes less distinct than in IR cells, but they were much more readily detected than in untreated cells. Thus, because of the comparative ease of demonstrating inclusions, as well as the increase in sensitivity, we prefer to use CY-treated cells rather than untreated cells for chlamydial isolation, irrespective of the incubation time and staining method used.

Splitting of any specimen for comparative purposes always entails a risk of uneven distribution of material. In tests on halved clinical specimens, greater numbers of inclusions were consistently detected by the rapid CY:IF:19 h method than by the IR:Giemsa:48 h method. Such consistency would be most unlikely if there were unequal distribution of organisms, and it seems that the most reasonable explanation for the results is the enhanced sensitivity of the rapid technique. Although increased sensitivity of the CY:IF:19 h method was again indicated by the results of the dilution experiment with strain 78α, there was no evidence that the enhanced sensitivity resulted in a greater rate of chlamydial isolation from clinical specimens. This is, perhaps not unexpected, since many specimens contain large numbers of chlamydiae. An increased isolation rate may be revealed only by testing many more specimens containing few organisms.

Specificity. After IF staining of cells inoculated with strain 78α or urethral isolates, there was no difficulty in distinguishing between fluorescing inclusions and nonspecific fluorescence. The inclusions were readily recognizable by their intense staining, color, shape, and intracytoplasmic location against the red background produced by the trypan blue counterstain. However, the presence of endocervical cells and mucus in some cervical specimens partially obscured the monolayer, but an extra, thorough wash with phosphate-buffered saline before fixing in methanol solved this in most instances. There was consistent agreement among observers on the nature of the fluorescence that was seen in clinical specimens, and the results of coded positive and negative controls were always unequivocal.

Simplicity and rapidity. CY treatment of cells avoids the need for a cobalt source of irradiation, so the method is readily applicable in routine microbiology laboratories. In addition, tubes containing cover slips of untreated cells may be prepared daily, if necessary even from nonconfluent cell cultures, thus making cells available for inoculation and CY treatment at any time. Thus, the problems of calculating when to irradiate cells and prepare them on cover slips, several days in advance of receipt of samples, are overcome.

The short time required for the detection of chlamydiae by the CY:IF:19 h technique is due principally to our ability to detect inclusions after 18 h of incubation. This short incubation period also helps to reduce the damaging effect on the cell monolayers of bacteria and fungi which occur particularly in cervical specimens. The time required for chlamydial detection was also reduced by shortening the IF staining method as much as possible. Methanol fixation, incubation with antiserum, and incubation with fluorescein-conjugated antiserum, each for 10 min, produced results equally as good as fixing and incubating for longer periods. Speed and efficiency were further increased by using a nonfluorescing, fast-drying mountant for attaching cover slips to microscope slides so that they did not detach during the washing procedures.

Apart from the increased sensitivity, the potentially most useful aspect of the modified isolation method is its ability to provide a rapid diagnosis of chlamydial infection. This depends on the combined use of both innovations, CY treatment and IF staining. McCoy cells are treated with cycloheximide after a specimen has been inoculated so that such treatment does not cause a delay in processing it. Inclusions are detectable by IF staining after 18 h of incubation; Giemsa-stained inclusions are not detectable in this time. IF staining may be completed in 90 min, so a diagnosis of chlamydial infection becomes available within 21 h of a sample reaching the laboratory. The implications of this are important. It is now possible to see patients with nongonococcal urethritis and ask them to return the following day, when the results of chlamydial isolation will be available. Patients from whom chlamydiae have been isolated may be treated with tetracyclines, and those who are chlamydia negative may be further investigated to determine the cause of their disease and whether it is worth treating them with tetracyclines. In this way, it may be possible to place the treatment of nongonococcal urethritis, at least in our hands, on a more rational basis.

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