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Factors affecting the sensitivity of replicating McCoy cells in the isolation and growth of chlamydia A (TRIC agents)

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SUMMARY

Normal non-irradiated McCoy cell cultures provide a sensitive and reproducible method for the isolation of oculo-genital strains of chlamydia A directly from human secretions and for laboratory studies with these agents. Since September 1973, chlamydia have been isolated from 175 of 562 women (32·1 %) attending venereal disease clinics. Freshly isolated and low passage strains have been used to determine the importance of centrifugation, constitution and pH of the tissue culture medium, and the temperature of incubation in controlling the efficiency of plating in the method.

INTRODUCTION

Non-gonococcal urethritis in the male, and cervicitis in the female, are now the commonest form of venereal disease in Britain, and over one-third of these conditions may be associated with chlamydia group A organisms (TRIC agents) related to *C. trachomatis*. Ocular infections, spread from the genital tract to the eye of the patient or to the eye of infants born of infected women, have many of the clinical and pathological features associated with early trachoma infection in the tropics. Prompt diagnosis is important (*a*) to differentiate chlamydial infections from gonorrhoea, (*b*) for socio-epidemiological reasons, (*c*) to indicate the need for specific and prolonged therapy with tetracyclines, and (*d*) to investigate the extent to which sequelae of venereal diseases, such as salpingitis and sterility in the female, and Reiter's syndrome in the male may be the result of untreated chlamydial infection. The development of simple methods for isolating chlamydia A directly from the human genital tract or eye has been remarkably slow, although it has been known for many years (Furness, Graham & Reeve, 1960) that certain 'fast' laboratory-adapted strains, isolated and serially passaged in the yolk sac of chick embryos, could be grown in tissue culture by methods in general use in virology laboratories, and that a wide variety of cell lines could be used for this purpose (Blyth & Taverne, 1974). Adsorption of chlamydia to tissue culture cells is inefficient and slow, but this can be overcome to some extent by centrifuging the infected inoculum onto the monolayer, as with rickettsias (Weiss & Dressler, 1960) or by pretreatment of tissue cultures with DEAE-dextran (Rota & Nichols, 1973). The main problem, however, has been the lack of precise information on the optimum conditions for the growth of genital strains of chlamydia A in tissue culture. In recent years, many *ad hoc* isolation methods have been described, and

as Blyth & Taverne (1974) have noted, '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 agents in the genital tract'. Specialized tissue culture methods involving irradiation of each batch of cells with 4000–6000 rad (Gordon *et al.* 1972) before inoculation of clinical specimens are beyond the facilities of most diagnostic laboratories, and in our experience are not necessary. For the past two years we have used coverslip monolayers of non-irradiated cells grown and maintained by conventional methods (Hobson, Johnson, Rees & Tait, 1974). These monolayers are inoculated with clinical specimens under centrifugation, incubated at 37° C. for 48–72 hr. in 5 % CO_2 in air and then stained by a modified Giemsa technique (Johnson, 1975). Positive cultures show large well-defined intracytoplasmic inclusions, packed with deeply basophilic elementary bodies. Under dark ground microscopy the stained inclusions show an intense yellow fluorescence, as has long been described for free elementary bodies.

The object of the present paper is to re-examine critically various steps in our empirical method to determine some of the optimum cultural conditions for the growth of genital strains of chlamydia, not only with the aim of improving the precision of diagnostic work, but as a preliminary to more fundamental laboratory studies on this group of organisms.

MATERIALS AND METHODS

The source and methods of subculture of McCoy cells, methods of preparation of McCoy monolayer coverslip cultures (MCC) and the collection and inoculation of clinical specimens from patients with suspected chlamydial infection have already been described in detail (Hobson *et al.* 1974).

The results of inoculating MCC with known laboratory strains of chlamydia or with infected clinical material were assessed quantitatively. After 48 hr. incubation, MCC were Giemsa-stained and examined under dark ground microscopy at 400 magnification. The total number of inclusions in the whole coverslip were counted, except where preliminary inspection suggested the count would be in excess of 5000–6000, in which case the inclusions in 60 microscope fields (approx. 1/15th of the coverslip area) were counted, and the number per whole coverslip was derived by multiplication. The accuracy and reproducibility of this procedure were determined as described in the text, and the results indicated that 95 % confidence limits of $\pm 23{\cdot}5$ % could be assigned to the counts shown in the various tables which describe the separate investigation of individual factors in the isolation procedure. Each count in the tables represents the average of 2–4 replicate MCC examined.

The SAL strain of chlamydia A was isolated in Liverpool from a post-partum cervical swab of an 18 year old woman whose baby developed purulent conjunctivitis 9 days after birth; chlamydia A were also isolated from the baby's eye swabs. Primary MCC infected with the cervical swab material from patient SAL were incubated at 37° C. for 72 hr. and then disrupted by exposure to a 9 mm. sonic

Table 1. *The effect of different tissue culture media on the growth of chlamydia (SAL strain) in McCoy cells*

Medium	Final concentration (%) of additional: Fetal calf serum	Glucose	Inclusion count per coverslip*
199	10	0·5	3180
	10	0	2625
	2	0	1500
Eagles' MEM	10	0	1695
	2	0	1275
Eagles' BME	2	0	210

All media were obtained from Wellcome Laboratories, Beckenham, Kent.
Fetal calf serum was obtained from Flow Laboratories, Irvine, Scotland.
* In this and subsequent tables, the inclusion count is on coverslip monolayers stained after 48 hr. incubation under the specified conditions.

probe (Mullard MSE. 60W). for 2 min.; 0·4 ml. samples of this suspension were inoculated into 4 further MCC. After 72 hr. incubation, one coverslip was removed and stained; about 3000 mature inclusions were present. The remaining MCC were harvested as described above, and the suspension stored at $-70°$ as a master stock. To obtain the large amount of chlamydial culture required for serial laboratory experiments, 0·2 ml. of a 10^{-2} dilution of this stock in SPG buffer (Bovarnick, Miller & Snyder, 1950) was inoculated into the yolk sac of 7-day-old chick embryos and incubated at 37° C. Embryos began to die 9–10 days after inoculation; yolk sac membranes were immediately removed from dead or dying embryos, and were homogenized by shaking in 10 ml. SPG in bottles containing glass beads. Smears of each suspension were stained by Giemsa and examined microscopically; those containing high concentrations of elementary bodies were pooled and clarified by low speed centrifugation to remove cell debris and yolk. The final yolk sac emulsion was stored in 1 ml. amounts at $-70°$ C. and formed the source of all experiments with SAL strain described below.

The STU strain was isolated here from a 17-year-old girl with cervicitis. Primary MCC inoculated with swabs from this patient were harvested as for SAL strain, and a working pool (the K pool) was made in chick embryos inoculated directly from this primary tissue culture material.

RESULTS

Tissue culture media

The objective was to compare the sensitivity to infection by chlamydia of McCoy cells grown and maintained in the various media listed in Table 1. All media contained a constant concentration (20 mM) of $NaHCO_3$ and were incubated at 37° C. in 5 % CO_2 in air to maintain the pH between 7·0–7·2.

Suspensions of McCoy cells from cultures grown in 12 oz. bottles as previously described (Hobson *et al.* 1974) were seeded into each medium at a concentration of 2×10^5 cells per ml., and MCC were prepared. After 24 hr. growth, each medium

Table 2. *The effect of temperature of incubation on the growth of chlamydia (SAL strain) in McCoy cells*

Temp. °C.	Inclusion count per coverslip
30	0
33	0
35	2200
37	3100
39	1590

was replaced by an equal volume of the same medium. Each MCC was infected with 0·4 ml. of a 10^{-3} dilution of SAL strain (hereafter referred to as 'the standard inoculum') and centrifuged for 1 hr. at 2400*g*. Inclusion counts after 48 hr. incubation (Table 1) suggested that medium 199 was better than either Eagles' MEM or BME media; 10 % calf serum (FCS) gave somewhat better results than 2 %, and there was a small further increase in sensitivity with extra glucose. The difference in inclusion counts from the best to the worst medium was from 3180 to 210 inclusions per coverslip. In all subsequent experiments the standard medium consisted of medium 199 + 10 % FCS + 20 mM-$NaHCO_3$ + 0·5 % glucose.

Temperature of incubation

MCC were grown and maintained in standard medium; 24 hr. after seeding they were all infected with the standard inoculum of SAL strain, centrifuged for 1 hr. at 2400*g*, and incubated in 5 % CO_2 in air at 37° C. for 2 hr. to bring the initial pH to about 7·2. Bottles were then tightly sealed and incubated for 48 hr. in separate water baths at temperatures between 30–39° C. The growth and condition of the McCoy cells were similar at each temperature, but the plating efficiency of chlamydia (Table 2) was better at 37° C. than at the lower temperatures used in most diagnostic techniques (e.g. Croy, Kuo & Wang, 1975); even at 39° C., 50 % of the inclusion forming units (i.f.u.) detectable at 37° C. were still able to establish growth, although the inclusions were smaller. Further attempts to determine the ceiling temperature of growth were unsuccessful since McCoy cells survived poorly after 48 hr. incubation at 40° C. or more.

The effect of antibiotics

Vancomycin and streptomycin were routinely incorporated at 50 μg./ml. final concentration in the MCC medium to prevent bacterial contamination. In MCC inoculated with *ca* 3000 i.f.u. of SAL strain the concentrations of each antibiotic could be increased to 500 μg./ml. without reducing the final inclusion count below that in control cultures incubated without antibiotics. At concentrations of 1000 or 1500 μg./ml. of vancomycin or streptomycin there was a progressive reduction in the size and number of inclusions, but the McCoy cells showed obvious signs of direct toxicity at these high doses. In contrast, the standard inoculum of SAL strain was completely incapable of forming inclusions in monolayers incubated throughout in penicillin at > 0·01 μg./ml., in chloramphenicol at > 2·5 μg./ml. or

Table 3. *The effect of the pH of tissue culture medium on the growth of chlamydia (SAL strain) in McCoy cells*

Buffer	Condition of incubation	pH	Inclusion count per coverslip
HEPES 14 mM	Sealed bottles in 37° water bath	6·6	870
		7·0	3000
		7·4	660
		7·8	540
		8·2	20
$NaHCO_3$ 20 mM	Sealed bottles in 37° water bath	7·0	3440
	Unsealed in 5 % CO_2 in air incubator at 37°	7·0	3600

in tetracyclines at $>0{\cdot}01$ μg./ml. Further quantitative studies of the antibiotic sensitivity of genital strains of chlamydia are in progress.

The effect of pH

MCC prepared and grown under standard conditions were washed and changed to standard medium, but without $NaHCO_3$. Buffering activity was provided solely by the addition of HEPES buffer at a final concentration of 14 mM, adjusted to the required pH in the medium by the addition of 0·04 M-NAOH. In a variety of tissue culture systems inoculated with various viruses this material has been found to be a satisfactory substitute for $NaHCO_3$ (e.g. Williamson & Cox, 1968).

MCC with HEPES-buffered media at initial pH ranging from 6·6–8·2 were inoculated with SAL strain. Control MCC in normal medium containing $NaHCO_3$ were similarly inoculated. After centrifugation at 2400*g* for 1 hr., all the HEPES cultures and half the $NaHCO_3$ MCC were sealed and incubated at 37° C. for 48 hr. in a water bath; the remaining infected cultures were incubated at 37° C. in 5 % CO_2 in air. The pH of the cultures was monitored throughout incubation by comparison with prepared pH colour standards. There were no significant changes from the initial pH values. MCC in medium with $NaHCO_3$ gave closely similar counts (Table 3) whether sealed or continuously exposed to CO_2 in air. At pH 7·0 HEPES-buffered MCC showed no significant difference from these controls in number or quality of inclusions, but the efficiency of infection fell with rising pH, and at pH 8·2 only 0·7 % of the i.f.u. detected at pH 7·0 produced inclusions and these were much smaller than usual. At pH 6·6 there was again a reduction in efficiency.

In a subsequent experiment, MCC in HEPES-buffered media at pH 6·0, 7·0 or 8·2 were inoculated with SAL strain and centrifuged. After 48 hr. incubation in sealed bottles, half the MCC at each pH were removed, stained and examined for inclusions, and the remainder were changed to standard growth medium with $NaHCO_3$ and incubated for a further 48 hr. in CO_2 in air. After the initial 48 hr. incubation there were 3300 inclusions at pH 7·0 but at pH 6·0 and pH 8·2 no inclusions were visible. After a further 48 hr. incubation at pH 7·2–7·4 MCC previously incubated at pH 6·0 showed no inclusions; MCC previously incubated

Table 4. *The effect of the force and time of centrifugation on the efficiency of infection of chlamydia (SAL strain) for McCoy cells*

Centrifugal force (*g*)	Duration of centrifugation (mins)	Inclusion count per coverslip
1*	—*	30
1	—	60
2500	30	2600
2500	60	3100
2500	180	19830
2000	60	2700
2500	60	3000
2000	60	5520
3500	60	7500
4000	60	24000
4500	60	23400

* Control MCC were inoculated together with the MCC to be centrifuged, but placed in the 5% CO_2 air incubator at 37° C. without centrifugation, either immediately* or after 60 min. stationary incubation at 33° C.

at pH 7·0 showed a considerable increase in size of inclusions, many of which were bursting, but no extra new inclusions were seen; MCC previously incubated at pH 8·2 now contained approximately 2000 inclusions of the size normally seen after 48 hr. It would appear that chlamydia can establish delayed or latent infections in MCC under excessively alkaline conditions, but can revert to normal behaviour on re-establishing normal pH, whereas there was no such reversion after excessively acid conditions of incubation. In all the above experiments, the pH variations and change from $NaHCO_3$ to HEPES buffer had no apparent effect on McCoy cell morphology or growth.

The effect of centrifugation

Replicate MCC were inoculated with SAL strain and either left uncentrifuged, or immediately centrifuged at a constant 2500*g* for 30, 60 or 180 min. with the centrifuge bowl temperature maintained at 33° C., before incubating at 37° C. for 48 hr. in 5% CO_2 in air. The effect of centrifugation for a constant time but with varying *g* force was similarly estimated. Replicate MCC were infected as above and immediately centrifuged for 1 hr. at 2000–4500*g* at 33° C. After 48 hr. incubation, inclusion counts were as shown in Table 4. Centrifugation at high speeds for a long time obviously increases the efficiency of infection, but would be impracticable in a diagnostic procedure. Rises in temperature and breakage of glassware were difficult to control, and atmospheric effects tended to leach out CO_2 progressively from the screw-capped bottles, with a rise of pH to $>8{\cdot}0$.

It seemed of practical importance to determine whether penetration of the cell was completed within the period of centrifugation. Accordingly, replicate MCC were inoculated with SAL strain as before, and centrifuged for 1 hr. at 2500*g*. In one set of MCC the medium was removed immediately after centrifugation and the

Table 5. *The effect of washing McCoy cell monolayers at various times after centrifugation of chlamydia (SAL strain) onto the coverslip*

Interval between centrifugation and washing (hr)	Inclusion count per coverslip
0	1500
1	2040
2	2400
3	2700
4	2850
Unwashed infected control	3150

coverslip was washed 3 times; 2 ml. of fresh medium was added before incubation. Other sets of MCC were incubated immediately after centrifugation, but were removed for washing and replacement of medium 1, 2, 3 or 4 hr. later, then immediately returned to the incubator. A final set of MCC were incubated immediately after centrifugation and left untouched in the original medium for the whole incubation period. After 48 hr. incubation, inclusion counts were as shown in Table 5. It was apparent that the attachment of chlamydia particles to the McCoy cell surface brought about by centrifugation could be reversed by washing the monolayer and replacement of the medium, unless a long further period of undisturbed incubation is allowed. In a further experiment, even simple replacement of the medium, without washing, immediately after centrifuging SAL strain onto MCC reduced the inclusion count to 60 % of that found in cultures where the medium was not changed.

The sensitivity and reproducibility of the diagnostic method

In the light of the experiments described above, the diagnostic method of Hobson *et al.* (1974) was slightly modified, although still representing to some extent a compromise between the ideal circumstances and those feasible in large-scale diagnostic work.

McCoy cells were dispensed into coverslip cultures (MCC) in the standard medium described above and incubated for 24 hr. in 5 % CO_2 in air with the bottle tops loosened. To minimize pH changes, clinical specimens were inoculated immediately after removing MCC from the incubator. The MCC medium was not changed, and bottle tops were tightly sealed during centrifugation at 2500*g* for 1 hr. at 33° C. Bottles were then immediately replaced in the incubator at 37° C. for 48 hr. in a constant atmosphere of 5 % CO_2 in air with bottle tops loosened. The staining procedure was modified (Johnson, 1975) to give better resolution of chlamydial inclusions, and dark ground microscopy was used routinely for examining all MCC.

The results obtained with this procedure are detailed below:

The sensitivity of the standard method to infection by chlamydia

Clinical specimens. Chlamydia have been isolated on one or more occasions from cervical swabs of 175 of 562 women (32·1 %) with various genital complaints seen

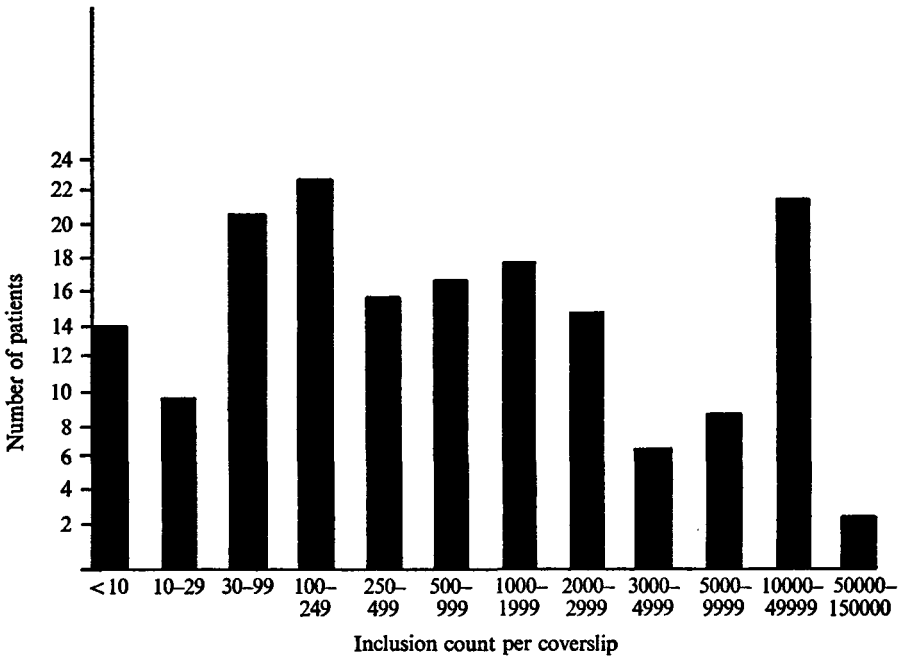

Fig. 1. The distribution of inclusion counts per coverslip from primary cervical swabs of 175 chlamydia-positive patients.

in venereal disease clinics in Liverpool. The chlamydial inclusions in the McCoy coverslip monolayer culture (MCC) 48–72 hr. after inoculation represent a single cycle of development, and their number thus reflects the number of infective particles in the original swab. The inclusion counts in the primary swabs of the 175 positive women ranged from 1 to 159,000 (median count = 900). The distribution of counts is shown in Fig. 1. Only 8·0 % of specimens yielded 10 or fewer inclusions. A repeat cervical swab was taken 5–10 days later from 106 women; 98 (92·5 %) were again positive, with counts similar to the first result. In two patients who refused treatment, chlamydia were re-isolated from swabs taken on several occasions, over a period of 14 weeks in one case and over one year in the other. After treatment with tetracyclines or erythromycin, chlamydia have not been re-isolated on repeated swabs for up to 1 year after therapy from any of the other 173 cases.

Laboratory strains of chlamydia. To determine the accuracy of the procedure, 14 replicate MCC prepared from a single batch of McCoy cells were simultaneously centrifuged immediately after infection with a constant inoculum of the K pool of STU strain. After 48 hr. incubation under standard conditions all the MCC were stained and the inclusions counted. The mean count was 455 inclusions per MCC (standard deviation = 55); the 95 % confidence limits of the coverslip count was thus 455 ± 107 inclusions, and in all 14 instances the counts were within ± 0·75 times the standard deviation.

The importance of strict control of centrifugation was shown by centrifuging a second set of 14 infected monolayers, prepared at the same time as the set described

above, using the same batch of cells and the same chlamydial dilution, but centrifuging in an older general purpose laboratory centrifuge which only gradually and erratically reached the required speed. The mean inclusion count was 300 and the standard deviation was 147 after identical incubation with the first batch of MCC. This confirmed and extends the previous findings on centrifugation.

Over sixty 1 ml. samples of the K pool of STU strain have now been used in experimental work over the past 18 months. The variation in inclusion counts obtained by infecting MCC with a constant dilution of this pool, in the standard procedure described above, has been comparatively small, when it is considered that ampoule to ampoule variation, the possible deleterious effects of long storage and subsequent thawing together with dilution errors must be added to the standard error of the method as described above. The highest count recorded over this long period was 4300 i.f.u. and the lowest 2800 i.f.u. at the constant dilution chosen.

DISCUSSION

Attempts to isolate and propagate genital strains of chlamydia group A in conventional tissue culture systems have given discrepant results in different laboratories (see Blyth & Taverne, 1974). This is not surprising, since a wide variety of cell lines and cultural conditions have been used. Moreover, the strains of chlamydia employed have had differing histories of laboratory passage, and certain of the 'fast' strains previously used widely have recently been shown by serological examination to resemble the organism of lymphogranuloma venereum rather than *C. trachomatis* (Becker, 1974). Although McCoy cells have been the most frequently used tissue culture over recent years, it must be borne in mind that at some unknown time this cell line, at first believed to originate from human synovial tissue, became indistinguishable in karyotype and antigenic constitution from the mouse L929 tumour cell line (Gordon *et al.* 1972), which has long been known to be highly sensitive to infection by chlamydia group B organisms, but to be considerably affected by changes in the tissue culture medium. In fact, mouse L929 cells can be infected by chlamydia A as readily as present day sources of 'mouse' McCoy cells (F. W. A. Johnson & D. Hobson, unpublished). Recently, there has been a growing tendency to interfere with the replication of McCoy cells by physical or chemical trauma to cellular DNA, presumably as a method of stabilizing the cultural conditions over the 48–72 hr. period required for chlamydial inclusions to form, and thus to increase the apparent sensitivity to infection. In our experience, there has been no advantage of non-replicating cells over normal cells in tissue culture, provided that optimum conditions for each type of culture were closely maintained. As Rota & Nichols (1973) found with irradiated McCoy cultures, the efficiency of plating of chlamydia can be affected considerably by changes in cultural conditions. In the present experiments Eagles' medium, although widely used in work with chlamydia, appears inferior to medium 199 and there is a small added advantage in higher concentrations of calf serum than customarily used. The nutritional demands of chlamydia in tissue culture are known to be complex (Bader & Morgan, 1961; Becker, 1974) and there is a specific

demand upon the host cell for sources of energy such as adenosine triphosphate (ATP). Changes in pH and temperature of incubation can obviously affect the result of infecting McCoy cells with chlamydia either directly, by reducing the viability of the elementary body itself, or indirectly by altering the metabolism of the host cell, or by a combination of both factors. Once the infecting particle has penetrated within the McCoy cell, the second of these factors seems more likely to operate.

The main limiting factor in the growth of chlamydia A in tissue culture appears to be the adsorption to and penetration of the host cell by elementary bodies. In the present experiments, the infectivity of a standard inoculum of chlamydia A increased significantly with both the time and speed of centrifugation; in our routine diagnostic procedure, clinical specimens have been centrifuged onto McCoy cell cultures for 1 hr. at the maximum speed available on conventional laboratory centrifuges (approx. 2500*g*) although it is apparent that even this procedure gives far from the maximum result. Indeed Darougar, Cubitt & Jones (1974) have found a progressive increase in inclusion counts when inoculated cultures were centrifuged at forces ranging from 800 to 15,000*g*. However, in 86·3% of positive clinical specimens examined in our laboratory in the last two years, the inclusion count has been greater than 30. In practice the method is thus more likely to underestimate the degree of infection of a patient rather than to give false negative results. The results obtained with laboratory strains established from clinical isolates suggest that the sensitivity of the method does not vary widely from one monolayer to another or from time to time.

Although centrifugation improves the rate or degree of contact between the elementary bodies and the surface of McCoy cells, several hours of incubation after centrifugation seem to be required for penetration to be completed, presumably by active pinocytosis. During this time, the attachment of elementary bodies to the cell is readily reversible by washing or changing the medium; as with myxoviruses, it seems probable that the infectivity of the particle after attachment to, but before penetration of, the host cell could be greatly affected by exposure to unfavourable pH, temperature or to toxic agents present in the specimen or its transport medium.

The establishment of a simple, reproducible and sensitive tissue culture system for the isolation of chlamydia A is now of obvious importance for the diagnosis of ophthalmia neonatorum and genital infections (Dunlop, 1975). The present results suggest that there is no inherent difficulty in achieving this objective with conventional replicating cell cultures, and that the method described above could usefully offer a working standard procedure against which more academic studies on the exact cultural requirements of chlamydia A can be assessed.

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