

The use of cycloheximide-treated cells for isolating trachoma agents under field conditions*

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Standard procedures for the isolation of Chlamydia trachomatis require pretreatment of the tissue culture cells. We evaluated the use of cycloheximide, an antimetabolite that can be added to the cells with the inoculum. Cycloheximide-treated cells provided a sensitive system for isolating trachoma agents. This system was applicable to field studies as requirements for equipment were minimal and the cells were 2 weeks old when inoculated.

Chlamydia trachomatis is a significant pathogen of man, being the agent of trachoma, which is still a major cause of blindness in some parts of the world (6, 7). Chlamydiae are also recognized as common agents of sexually transmitted disease (6, 9). Much of the recent information on human chlamydial infections has been due to the development of a tissue culture technique for isolating these organisms (5). The commonly used isolation procedures require pretreatment of the host cells with radiation or an antimetabolite (2, 11). This pretreatment requirement poses potential difficulties to diagnostic studies and large-scale surveys. Cycloheximide suppresses the metabolism and reproduction of eukaryotic cells while not affecting prokaryotic cells (1), and this substance therefore provides an alternative method of treating cells for chlamydial isolation because it can be added to host cells after infection (8).

In previous studies on trachoma in Tunisia, we used Giemsa- and immunofluorescent-stained conjunctival smears to determine the prevalence of chlamydial infections (3, 4, 12). *C. trachomatis* was isolated from only 5% of specimens from selected patients with active trachoma, although up to 60% of matched conjunctival smears had inclusions. It appeared that technical problems were preventing chlamydial recovery in the cell culture systems. In previous studies, frozen clinical specimens were shipped in liquid nitrogen and took 3-5 weeks in transit from Tunisia to San Francisco. When Ripa & Mårdh (8) described the use of cycloheximide in the treatment of cells for *C. trachomatis* isolation, we decided to employ this technique in our studies. An attractive feature of this method is that the cells need no pretreatment. Because untreated cell cultures are stable for 2-3 weeks at moderate temperatures (20°C), it would be feasible to send normal cells from San Francisco to Tunisia and process the specimens there.

* This work was supported in part by National Institutes of Health grants EY 00427 and CA 14427; by PL 83-480 grant 07-075 from the Health Services Administration, US Public Health Service, and by the World Health Organization.

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MATERIALS AND METHODS

The collection medium consisted of Eagle's minimal essential medium with 10% fetal calf serum and contained 1 µg cycloheximide, 10 µg gentamicin, 100 µg vancomycin, and 4 µg amphotericin B per ml. Approximately 75 000 McCoy cells were planted on 12-mm cover slips in 15-mm-diameter shell vials (10), sealed with silicone rubber stoppers, and incubated at 37°C for 48 h. The vials and other tissue culture supplies were then sent by air freight from San Francisco to Tunis in a suitcase measuring

approximately 760×460×230 mm.^a Before inoculation, the cells were incubated in a standard bacteriological incubator at 37°C for about 36 h. Clinical specimens were collected from the conjunctiva of the lower lid of patients with active trachoma by means of a wire stem calginate nasopharyngeal swab and placed into 3 ml of collection medium in 15-ml screw-cap tubes. The specimens were held slightly below ambient temperature for 10 h after collection and then refrigerated overnight (another 12 h) prior to inoculation into the cells. One ml of each specimen was placed on each of three cover slips and centrifuged for 1 h at 1850 *g*. The vials were then incubated at 37°C for 48 h without changing the medium. The cell monolayers from one vial/specimen were then fixed with absolute methanol, stained with 5% iodine, and examined for the presence of inclusions. After 72 h of incubation, the second inoculated tissue culture vial was frozen in liquid nitrogen and the third was taken by hand back to San Francisco (about a further 40 h) at ambient temperature. The material was passed into new cycloheximide-treated cells immediately on arrival at the Collaborating Centre. A duplicate clinical specimen had been frozen in liquid nitrogen in the field and both the frozen cell passage material and duplicate clinical material were sent by air freight to San Francisco, arriving approximately 5 weeks after collection. These frozen specimens were thawed and spun into cycloheximide-treated cells using the same media, the only modification in the procedure being that they were centrifuged at 2700 *g*.

For cytological studies, scrapings collected from the tarsal conjunctiva were spread on glass slides, air dried, and fixed in methanol. They were then stained by Giemsa's technique.

RESULTS

Of 31 specimens collected in the field and directly inoculated into the cells, 24 were examined at 48 h (7 tubes were lost owing to various handling mishaps). Twelve of these 24 (50%) were inclusion-positive. All 31 inoculated cell monolayers were carried back to San Francisco and 19 (62%) yielded iodine-staining inclusions on subsequent passage there. Two of the positive specimens had been inclusion-

negative in the first passage. Of the 31 matched Giemsa-stained scrapings, 12 (39%) were inclusion-positive. The isolation attempts on all 12 of these patients were positive.

Only 2 (6%) of the 31 frozen duplicate clinical specimens yielded isolates in San Francisco. The cell cultures that had been inoculated in Tunis and then frozen in liquid nitrogen were also thawed and passaged in San Francisco and 3 of the 31 (10%) yielded chlamydial isolates.

DISCUSSION

The conditions and logistic problems in this study were not unique. Relatively sophisticated laboratory procedures are often not available in a trachoma-endemic area. Indeed, the transportation problems, lack of dry ice, and the high ambient temperatures (these specimens were taken in the northern Sahara, where daytime temperatures are frequently above 30°C, even in November) are common in trachoma-endemic areas. Although our previous studies had employed a variety of different holding media for freezing specimens, none had successfully preserved the trachoma agents during transit. We do not know why specimens lose infectivity during their 3- to 5-week shipment period from North Africa to San Francisco, since specimens stored in liquid nitrogen in our laboratory lose very little infectivity during this time. However, the tissue culture procedure we used in this study proved to be a sensitive method for isolating trachoma agents. It was approximately 60% more sensitive than the Giemsa stain (62% positive versus 39% positive).

We used the iodine stain to detect inclusions in the infected tissue culture monolayers after 48 h of incubation. If there is urgency in establishing a diagnosis, or there are technical restrictions, the monolayers can be incubated for 24 h and examined for inclusions by immunofluorescent techniques. Our goal was not early diagnosis but rather the establishment of isolates for further laboratory studies.

The use of cycloheximide-treated cells for the isolation of trachoma agents requires only a centrifuge and a bacteriological incubator as permanent laboratory equipment. We have often transported this equipment and an electrical generator to the sites of our field studies. The rest of the supplies needed for the isolation attempts are easily carried and may be disposable. The major advantage of cycloheximide is that it can be added with the inoculum, eliminating the need to pretreat cells with radiation or 5-iodo-2-deoxyuridine, the previously

^a Details of this portable tissue culture system are available on request from the WHO Collaborating Centre for Reference and Research on Trachoma and Other Chlamydial Infections, University of California, San Francisco, CA 94143, USA.

employed techniques (2, 11). Untreated, light monolayers of McCoy cells maintain viability for several weeks at moderate temperatures. The cells used in this study had been planted 17 days before the time of infection; it is therefore possible to send material from a tissue culture laboratory for use in the field 2 weeks later. The 48 h in transit from Tunis to

San Francisco resulted in no loss of isolates, although infectivity (titre) may have decreased. Thus, the use of McCoy cell monolayers prepared in the base laboratory, shipped with the materials necessary to process the clinical specimens and the subsequent cycloheximide cell treatment, provides a useful technique for the isolation of trachoma agents in the field.

RÉSUMÉ

EMPLOI DE CELLULES TRAITÉES PAR LE CYCLOHEXIMIDE POUR ISOLER LES AGENTS DU TRACHOME DANS LES SPÉCIMENS RECUEILLIS SUR LE TERRAIN

Dans les études menées précédemment sur le trachome en Tunisie, on a eu recours aux méthodes cytologiques pour déterminer la prévalence des infections à *Chlamydiae*. Mais seules 5% des tentatives faites pour isoler *Chlamydia trachomatis* dans des prélèvements conjonctivaux ont abouti, alors qu'un examen sur lame avait permis de déceler des inclusions dans 60% des échantillons appariés. Les tentatives d'isolement avaient été effectuées sur des spécimens cliniques congelés dans l'azote liquide et expédiés à San Francisco.

La communication de Ripa & March au sujet de l'emploi du cycloheximide pour traiter les cellules en vue de l'isolement de *C. trachomatis* a déterminé les auteurs de la présente étude à utiliser cette technique pour leurs travaux. Le cycloheximide stoppe le métabolisme et la reproduction des cellules eucaryotes sans affecter les cellules acaryotes, et l'avantage de cette substance est de pouvoir être ajoutée au milieu de culture tissulaire après l'inoculation. Ce sont donc des couches monocellulaires non traitées de cellules McCoy (dont la viabilité est toujours d'au moins 2 semaines) qui ont été expédiées de San Francisco en Tunisie.

La collecte de matériel conjonctival a été faite chez des enfants atteints de trachome. Les échantillons ont été placés dans un milieu approprié et centrifugés dans les 24 heures sur des couches monocellulaires de cellules

McCoy. Après une incubation à 37°C pendant 48 heures sans changer le milieu, les cellules avec l'inoculum ont été transportées directement à San Francisco (comme bagage à main).

L'examen des cellules, traitées par le cycloheximide pour un second passage dès l'arrivée à San Francisco, a permis d'isoler des *Chlamydiae* sur 19 des 31 échantillons (soit 62%). On avait décelé des inclusions dans 12 (39%) des prélèvements conjonctivaux appariés colorés au Giemsa, et les tentatives d'isolement intéressant ces 12 sujets ont toutes été positives. En revanche, les difficultés rencontrées précédemment avec les échantillons expédiés dans l'azote liquide se sont répétées, puisque seuls 2 des 31 spécimens cliniques correspondant aux inoculums (soit 6%) qui ont été congelés pour leur expédition ont permis d'isoler des *Chlamydiae*.

Les cellules traitées par le cycloheximide constituent par conséquent un système favorable à l'isolement de l'agent du trachome, et leur emploi ne nécessite qu'un incubateur et une centrifugeuse comme équipement de laboratoire permanent. Quant aux couches monocellulaires de cellules McCoy initiales, celles-ci peuvent être envoyées sur le terrain par le laboratoire de base, puisque leur viabilité est de plusieurs semaines à température modérée.

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