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Detection of Experimental *Chlamydia trachomatis* Eye Infection in Conjunctival Smears and in Tissue Culture by Use of Fluorescein-Conjugated Monoclonal Antibody

HUGH R. TAYLOR,* NISHEETA AGARWALA,† AND SHIRLEY L. JOHNSON

The Dana Center for Preventive Ophthalmology, The Wilmer Ophthalmological Institute, The Johns Hopkins University, Baltimore, Maryland 21205

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The immunofluorescent staining of conjunctival cytology smears with an antichlamydial monoclonal antibody is a new method for the detection of chlamydial eye infection. Using a monkey model of chlamydial eye infection, we compared the monoclonal antibody staining cytology with the established methods of Giemsa-stained cytology and chlamydial culture with cycloheximide-treated McCoy cells. Compared with either culture or monoclonal antibody-stained cytology, Giemsa cytology had a low sensitivity (30 and 24%, respectively) but was highly specific (100% in each case). Compared with culture, monoclonal antibody-stained cytology was highly sensitive (94%) but appeared to lack specificity (70%). However, examination of the changes in identification rates with time suggested that monoclonal antibody-stained cytology may be even more sensitive than culture and detect lower levels of infection. This study showed that monoclonal antibody-stained cytology of conjunctival smears might be a rapid, efficient, and inexpensive method of diagnosing ocular chlamydial infection.

Chlamydia were first identified by Halberstaedter and von Prowazek in Giemsa-stained conjunctival scrapings; these authors demonstrated the pathogenicity of chlamydia by experimentally infecting the eyes of an orangutan (6). Giemsa cytology was the only laboratory method available to diagnose chlamydial infection with any assurance until the embryonated hens egg culture of chlamydia was introduced by T'ang and his co-workers in 1957 (19). Egg yolk culture, using newly developed antibiotics and a blind second passage, soon became the standard for diagnosing chlamydial infection. The availability of large quantities of cultured antigen made it possible to serotype chlamydia (25) and to measure specific chlamydial antibodies in serum and other body fluids (24). Despite its widespread use in seroepidemiological studies, serodiagnosis has not been particularly useful in the diagnosis of active infection because of the high prevalence and longevity of the immunoglobulin G response, although the presence of serum immunoglobulin M titers may indicate more serious systemic infections, especially in neonates and infants (14, 23).

The introduction of tissue culture systems during the 1970s made a significant advance in the culture of chlamydia (5, 12, 27). More recently, tissue culture systems have been miniaturized (30) and further enhanced by the use of monoclonal antibodies against species-specific chlamydial antigens (15, 16). Today the use of cycloheximide-treated McCoy cells or DEAE-dextran-treated HeLa cells cultured in microtiter plates and stained with a fluorescein-labeled monoclonal antibody is widely, although not universally, accepted as the most reliable method of diagnosing chlamydial infection. This method, however, is still slow, taking 3 to 6 days, it is relatively expensive, and it requires a well-equipped tissue culture laboratory.

In this study, in a controlled, experimental infection, the direct staining of conjuctival cytology smears with a recently developed antichlamydial monoclonal antibody was compared with the standard microtiter culture technique. The monoclonal antibody-stained cytology technique gave far more positive results than did Giemsa cytology and may even have been more sensitive than culture.

MATERIALS AND METHODS

Specimens. Conjunctival specimens were collected from 10 young adult cynomolgus monkeys. Five monkeys received a single inoculation of 20 μl of a $10^{3.2}$ 50% egg lethal dose suspension of *Chlamydia trachomatis* strain HAR36 (B serotype) in both eyes. The other five monkeys received similar inoculations in both eyes initially and repeated weekly for 18 weeks. The group that received the single inoculations developed acute inclusion conjunctivitis which resolved over 8 weeks, and the reinoculated group developed the typical monkey model of chronic trachoma as previously described (20).

Ocular specimens for this study were collected from the left eye of each animal of both groups at weeks 0, 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, and 18. For those animals being reinoculated, specimens were collected immediately before reinoculation. The inferior fornix was scraped with a sterile platinum spatula, and duplicate smears were made on alcohol-precleaned glass slides. Then the conjunctiva was swabbed with a calcium alginate swab (Calgiswab, type 1; INOLEX Division, American Can Co.) which swept the inferior tarsus and fornix, the lateral fornix, the superior tarsus and fornix, and the medial fornix.

Chlamydial culture. Immediately after collection, the conjunctival swab was placed in 2 ml of collection medium (24) with vancomycin substituted for amphotericin B. Specimens were kept on wet ice for 5 to 60 min until taken to the laboratory. Specimens were then disrupted by 2 min of vortexing. Samples of 100 μl of that medium were then placed on 3-day-old confluent McCoy cell monolayers in 96-well microtiter plates (Costar, no. 3596). One specimen was inoculated onto eight wells of a primary culture plate and also onto another plate to be used for a second passage. The

* Corresponding author.
† Present address: 11, Saras Bang, Deonar, Bombay 400088, India.

inoculated plates were centrifuged at 1,100 × *g* for 60 min at 25°C. The medium was replaced with isolation medium containing 1 μg of cycloheximide (Sigma Chemical Co.) per ml (24), again with vancomycin substituted for amphotericin B. The plates were cultured for 3 days at 37°C in a humidified incubator with 5% CO_2.

At the end of 3 days, culture medium was aspirated from the primary culture plates, and the adherent cells were washed once with phosphate-buffered saline and fixed by the addition of ethanol for 5 min. Plates were washed twice with distilled water. Then 25 μl of fluorescein-labeled antichlamydial monoclonal antibody with Evans blue counterstain (MicroTrak Chlamydia Culture Confirmation Reagent; Syva Co.) was added to appropriate wells and incubated at 37°C in a humidified incubator for 30 min. The stain was aspirated and the wells were rinsed twice with deionized water. After aspiration of the water, a drop of mounting fluid (Syva Co.) was placed in each well, and a 5-mm circular cover slip (Bellco Glass, Inc.) was placed on top. Plates were inverted and read under a transmitted fluorescence microscope at ×500. Chlamydial inclusions were recognized by their characteristic apple green staining (10). The first well of all cultures was stained with the monoclonal antibody; if this was not positive, wells 3 and 5 were also stained with monoclonal antibody. The remaining five wells were stained with the iodine stain.

At the end of 3 days of culture, the cells in the plates set up for secondary passage were dislodged by pipetting. The cells and medium were transferred directly to a corresponding well in a new plate of McCoy cells. After a further 3 days of incubation, the second-passage plate was fixed, stained, and read by using the techniques described above.

Giemsa cytology. Immediately on collection, slides for Giemsa stain were air dried, fixed for 5 min in methanol, and stored at room temperature until stained with Giemsa stain (Eastman Kodak, no. C8685) (29). These slides were examined at ×1,000 and were graded positive only when characteristic Halberstaedter-Prowazek intracytoplasmic inclusions were seen.

Monoclonal antibody-stained cytology. Slides for monoclonal antibody staining were air dried, fixed in cold acetone for 5 min, and stored at −20°C until stained. Slides were allowed to come to room temperature, and a few drops of acetone were placed on scrapings to get rid of any condensation. The slides were then dried and overlaid with 30 μl of fluorescein-labeled monoclonal antibody reagent with Evans blue counterstain (MicroTrak Chlamydia Direct Reagent; Syva Co.) for 15 min in a covered, moist chamber. Slides were turned on edge to aspirate excess stain, rinsed for 10 s in deionized water, and allowed to completely air dry. A cover slip was placed on the slide with mounting medium (Syva Co.) and sealed with fingernail polish to prevent slipping of the cover slip and drying out of the mounting medium. Slides were examined under a transmitted fluorescence microscope at ×500 and ×1,250. Slides were called positive only when at least three of the characteristic apple green elementary bodies were seen (18, 28).

For each of the above methods, positive and negative control specimens were included and examined. One trained observer read all cultures and another read all cytology. All positive specimens and a sample of negative specimens were evaluated independently by a second observer.

Statistical testing. Two standard statistical tests were used to evaluate the sensitivity and specificity of the various methods: sensitivity = number of true-positives/(number of true-positives + number of false-negatives) and specificity = number of true-negatives/(number of true-negatives + number of false-positives).

True-positive specimens were those specimens that were positive with both the method being tested and the method used as the standard. Similarly, true-negative specimens were negative with both methods. False-positive specimens were positive with the test method but negative with the standard method and, therefore, were regarded as being truly negative. False-negative specimens were negative with the test method but positive with the standard method and were regarded as being truly positive.

RESULTS

Altogether, 110 paired specimens were examined after the initial ocular inoculation. Compared with tissue culture using the monoclonal antibody or monoclonal antibody-stained cytology, Giemsa cytology was very insensitive (sensitivities, 30 and 24%, respectively), although it was highly specific (100% for each) (Table 1). Again, with culture as the standard, monoclonal antibody-stained cytology was highly sensitive (94%), but it did not appear to have a high specificity (70%). On two of the three occasions where cultures were positive and monoclonal antibody-stained cytology was negative, it was noted that insufficient cells were obtained for adequate cytological examination. This was not noted as a problem with Giemsa-stained specimens, but is more likely to be a limitation of cytology in general rather than a specific problem with monoclonal antibody staining. Monoclonal antibody staining of tissue culture detected far more positive specimens than were detected by iodine staining (Table 2). The specimens that were positive by monoclonal antibody staining were all identified by the first-passage culture, whereas 4 of 11 (36%) positive iodine-stained cultures were not detected until the second passage.

To examine the apparent lack of specificity of monoclonal antibody-stained cytology, the frequency with which each test was positive was plotted over time for each of the two groups of monkeys (Fig. 1). Chlamydial infection was detected by either Giemsa cytology or iodine-stained culture only during the first 3 to 4 weeks after initial inoculation and was

TABLE 1. Frequency of agreement of three methods of diagnosing chlamydial infection and sensitivity and specificity of each test pair

Test pair	Test standard (no.)				Sensitivity (%)	Specificity (%)
	True positive	False positive	True negative	False negative		
Giemsa cytology, culture	15	0	60	35	30	100
Giemsa cytology, monoclonal antibody-stained cytology	15	0	48	47	24	100
Monoclonal cytology, culture	47	18	42	3	94	70
Culture, antibody-stained monoclonal cytology	47	3	42	18	72	93

TABLE 2. Chlamydia-positive specimens detected by tissue culture with monoclonal antibody staining and with iodine staining

Passage	No. of positive specimens	
	Monoclonal antibody staining	Iodine staining
First	50	7
Second	0	4

positive for only about one-half of the animals. Both monoclonal antibody-stained cytology and culture with monoclonal antibody staining detected infection in almost all monkeys for the first 6 weeks. After 6 weeks, however, chlamydia could not be detected by culture, but the monoclonal antibody-stained cytology continued to detect chlamydial infection for a further 4 weeks. This finding suggests that monoclonal antibody-stained cytology may detect lower levels of infection than can be detected by culture.

DISCUSSION

In this study, we compared two well-established methods of detecting chlamydial infection with a newly developed immunofluorescent cytology technique, using a monoclonal antibody. Giemsa cytology is a well-accepted and standard technique (11, 29), and finding the characteristic inclusions is diagnostic of chlamydial infection. Yoneda and co-workers (31) have shown that the associated inflammatory cytological features can be a useful guide to infection, and Hardy and co-workers (8) considered the cytology findings to be indicative of infection even if inclusions were not seen. However, in our study, smears were only called positive if definite and characteristic intracytoplasmic inclusions were seen (29).

The methods for culturing chlamydia have evolved over the years, and the culture method used in this study combined a number of these advances (1, 10, 12). For example, the use of a monoclonal antibody to identify chlamydial inclusions has been shown to be more sensitive than either iodine or Giemsa staining of cultures (15, 16). In this study, it also proved to be more sensitive than iodine staining.

The chlamydial monoclonal antibodies used for both culture confirmation and monoclonal cytology were raised against the species-specific major outer membrane protein of *C. trachomatis* and are already well characterized (10, 17).

The monkey model of chlamydial eye infection is now well established and is a good model for the spectrum of human eye disease (20). Compared with studying a patient population, the animal model provides an excellent system in which to assess the effectiveness of different diagnostic methods for several reasons. In the animal model, one knows exactly the timing of infection and the type of organism. There is the ability to schedule and collect multiple specimens at will as well as the opportunity to follow the infection over its entire course while controlling the occurrence of reinfection. In a patient population, infections are likely to be of varying duration at the time of presentation, and, therefore, specimen collection could be haphazard. Prior antibiotic exposure may be difficult to determine and the withholding of treatment may be unethical. Finally, follow-up may be uncertain.

In this study, Giemsa cytology was less sensitive than tissue culture in detecting chlamydial infection although it was highly specific. These findings agree with those of previous studies (13, 23). Similarly, Giemsa cytology was

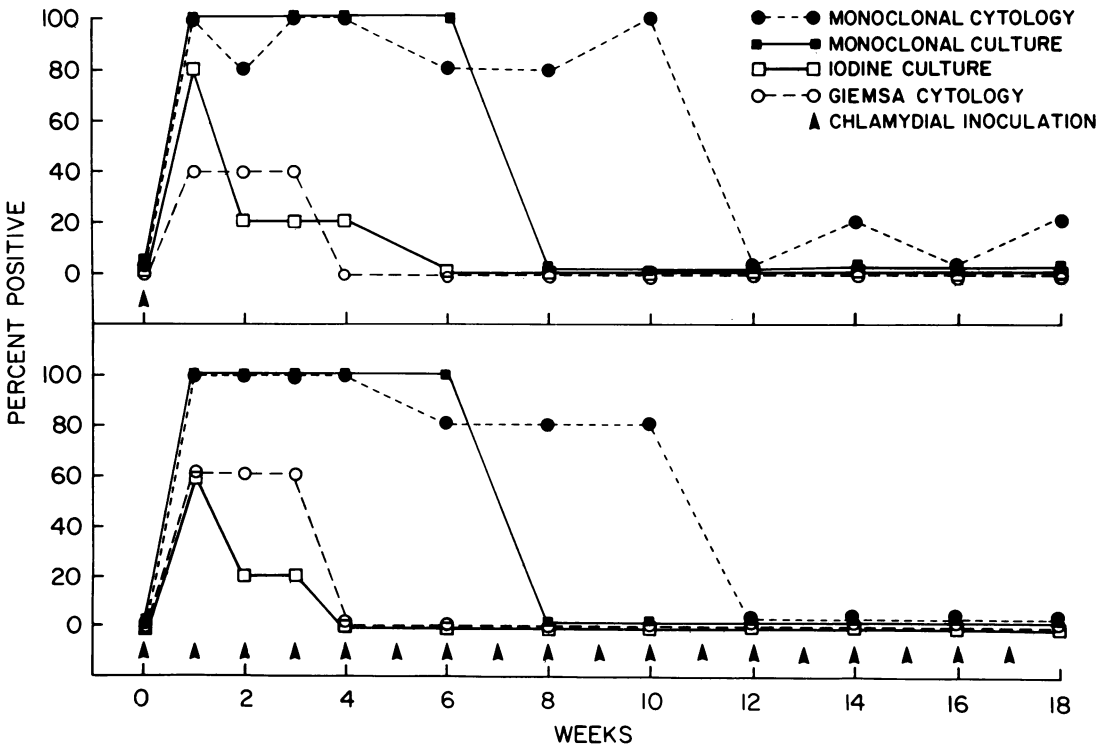

FIG. 1. Frequency of positive detection of chlamydial infection by four different methods (monoclonal antibody-stained cytology, monoclonal antibody-stained tissue culture, iodine-stained tissue culture, and Giemsa-stained cytology) used in two groups of experimentally infected monkeys. There were five monkeys in each group.

inferior to monoclonal antibody-stained cytology. Previous studies of immunofluorescent cytology with polyclonal antibodies have shown less difference between the sensitivity of Giemsa cytology and that of fluorescent-antibody cytology than we observed (2, 7, 9, 31). This perceived decrease in the sensitivity of Giemsa cytology is possibly due to a great increase in the sensitivity of the immunofluorescent test because it used a monoclonal rather than a polyclonal antibody. In general, monoclonal antibodies demonstrate extremely precise specificity, react with uniform avidity, and can be purified to homogeneity.

Chlamydial tissue culture is the presently accepted standard for detecting chlamydial infection (3). Compared with culture, monoclonal antibody-stained cytology had a high sensitivity but only a moderate specificity. This perceived lack of specificity could occur for several reasons, including a true lack of specificity of the antibody. This is unlikely, as previous studies have shown that the monoclonal antibody has high antigenic specificity (17). A similar lack of test specificity would be seen if the organisms detected by cytology were nonviable or if the method under review (in this case, monoclonal antibody-stained cytology) was, in fact, more capable of detecting infection than the "standard" chlamydial culture. Our data suggest that the latter may be the case. We and others (10, 15) have found a difference in the rates of identification of chlamydia by iodine and monoclonal antibody-stained cultures. This indicates that the iodine-stained culture may not detect the presence of viable organisms which can be detected by monoclonal antibody-stained cultures. It also seems reasonable to suppose that viable organisms may persist below the level at which monoclonal antibody-stained culture can detect them. Although the numbers of organisms or inclusions were not specifically recorded on each cytology smear in this study, the number of inclusions found in monoclonal antibody-stained culture was greater than six per well for each animal 1 week after infection, but in every case remained between one and five after that until the cultures became negative. In another group of monkeys that received a single ocular inoculation with chlamydia, the numbers of elementary bodies seen on monoclonal antibody-stained cytology were 15.6 at day 7, 26.8 at day 14, 11.6 at day 21, 1.6 at day 28, 2.4 at day 35, and none thereafter (unpublished data). The infection was of shorter duration in this group of monkeys as they had been previously infected and were partially immune. Therefore, it appears likely that the monoclonal cytology is a more sensitive technique and detects lower levels of chlamydial infection than can be detected by current tissue culture methods.

An interesting feature of the monkey model of trachoma is that chlamydia cannot be detected by culture or by cytology in the monkeys receiving weekly inoculations with chlamydia after about 10 weeks despite the presence of continued reinoculation with live organisms. Continued reinoculation is needed to maintain the persistent active disease (21). This phenomenon has parallels in human trachoma for chlamydia cannot be detected in many individuals with active trachoma, including some of those with the most severe disease (4, 22). It seems possible that chlamydial infection may exist at levels so low as to be undetectable by current methods and this "undetectable" infection causes continued disease. It is unknown whether a similar phenomenon occurs in human genital tract or other extraocular infection, but from what is known of ocular infection, it seems likely. Studies are now indicated to examine the role of chlamydial monoclonal antibody-stained cytology in the diagnosis of human ocular and extraocular infection. If the monoclonal antibody-stained cytology is, in fact, more sensitive than culture, it might enhance our knowledge of the role of chlamydia in genital infection as well as provide a quick, cheap, sensitive, and specific test for the detection of chlamydial infection in ocular and extraocular specimens.

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