

Sensitivity of Immunofluorescence with Monoclonal Antibodies for Detection of *Chlamydia trachomatis* Inclusions in Cell Culture

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Monoclonal antibodies which recognize the species-specific major outer membrane protein antigen of *Chlamydia trachomatis* were used for immunofluorescence staining of chlamydial inclusions in cell culture. A total of 115 clinical specimens were inoculated onto replicate HeLa 229 cell monolayers and assayed for chlamydial inclusions by immunofluorescence staining and Giemsa staining. Of the isolates, 38 were detected by immunofluorescence staining on passage 1 and 1 was detected on passage 2; 23 isolates on passage 1 and 13 isolates on passage 2 were detected by Giemsa staining. Immunofluorescence staining was significantly more sensitive than Giemsa staining for detecting chlamydial inclusions, particularly from specimens containing low titers of *Chlamydia*.

Chlamydial inclusions in smears or tissue from clinical specimens or in cell culture have conventionally been identified by Giemsa staining, immunofluorescence (IF) staining, or iodine staining methods. Iodine staining has been employed only for *Chlamydia trachomatis* biotypes because *C. psittaci* biotypes do not produce sufficient quantities of glycogen. Unfortunately, iodine-detectable glycogen may only be demonstrated during part of the chlamydial growth cycle (2). Earlier reports have shown that IF staining is more sensitive and specific than Giemsa staining for the direct detection of *C. psittaci* inclusions in clinical specimens and cell culture (12). For the detection of *C. trachomatis* inclusions in conjunctival scrapings, IF staining has also been shown to be superior to Giemsa staining (7). However, comparisons of the sensitivity of these two methods for the detection of *C. trachomatis* inclusions in cell culture have been inconclusive. Thomas et al. demonstrated enhanced sensitivity of IF staining in terms of inclusion counts, but this did not result in a greater rate of chlamydial isolation from clinical specimens (11). In a similar comparison, Darougar et al. observed no marked differences in the number of *C. trachomatis* inclusions identified in cell cultures or in the rates of chlamydial isolation (3).

In this study, we utilized monoclonal antibodies which recognize a species-specific surface antigen of *C. trachomatis* (10) for IF staining of chlamydial inclusions in cell culture. IF staining was shown to be significantly more sensitive and rapid than Giemsa staining for the detection of

C. trachomatis inclusions which were produced from clinical specimens inoculated into cell culture.

MATERIALS AND METHODS

Specimens. A laboratory strain of *C. trachomatis* F/UW-6/Cx (1) or clinical specimens were used in this study. Specimens for isolation of *C. trachomatis* were collected from the urethra and cervix of women attending the Sexually Transmitted Disease Clinic at Harborview Medical Center, Seattle, Wash., or from pregnant women enrolled in a prospective study of chlamydial neonatal infection at the University Hospital, University of Washington, Seattle. Specimens were collected with calcium alginate urethrogenital swabs, placed in 1 ml of sucrose-phosphate-glutamate medium (8), and frozen at -70°C until tested.

Inoculation of cell monolayers. Clinical specimens were inoculated onto DEAE-dextran-pretreated HeLa 229 cell monolayers as previously described (8), except that 0.5 μg of cycloheximide (Sigma Chemical Co., St. Louis, Mo.) per ml was added to the culture medium (9). Each specimen was inoculated onto four separate HeLa cell monolayers and incubated for 48 or 72 h (passage 1). After incubation, two inoculated monolayers on 12-mm cover slips were fixed with methanol and stained with either Giemsa stain or fluorescent antibody. At 72 h the two remaining inoculated monolayers were passaged onto fresh monolayers and reincubated (passage 2). Specimens which demonstrated inclusions on passage 1 by both staining methods were not passaged a second time.

Monoclonal antibodies. Hybrid cell lines (*C. trachomatis* 2C1 and 1H8) which secrete monoclonal antibodies that recognize the species-specific major outer membrane protein of *C. trachomatis* were used to produce ascitic fluids in mice as previously described

(10). For the direct IF staining technique, immunoglobulins from ascitic fluid 2C1 were affinity purified on a protein A-sepharose column (Pharmacia Fine Chemicals, Uppsala, Sweden) (4). Fluorescein isothiocyanate-conjugated monoclonal antibodies (2C1) were prepared by the method described by Goding (6). A 1:50 dilution of ascitic fluid (1H8) or fluorescein isothiocyanate-conjugated monoclonal antibody (20 $\mu\text{g/ml}$) was used for the indirect and direct IF staining techniques, respectively.

Giemsa staining and IF staining. May-Grunwald-Giemsa staining (5) was performed as follows. A saturated solution of May-Grunwald stain (Hopking & Williams, Chadwell Heath, Essex, England) in methanol was applied to cover slips for 5 min, washed with water, and stained with 0.08% Giemsa stain (Fisher Scientific Co., Pittsburgh, Pa.) for 10 min. Cover slips were then washed with water, dehydrated sequentially with acetone, acetone-xylene (1:1), and xylene, and mounted with Permount (Fisher Scientific Co.).

Both indirect and direct IF staining techniques were assessed. Indirect IF staining was performed with an anti-mouse immunoglobulin G fluorescein conjugate (Hyland Diagnostics, Deerfield, Ill.). The direct staining procedure was performed with fluorescein isothiocyanate-conjugated monoclonal antibodies. Evans blue (0.2%) counterstain was applied in the final step.

Examination of cell cultures. Stained cell monolayers were examined for chlamydial inclusions by bright-field or fluorescent microscopy (fluorescent microscope from Wild, Heerbrugg, Switzerland) at a 100 \times magnification. Inclusions observed at a 100 \times magnification were verified for typical morphology at a 400 \times magnification. The total number of inclusions per cover slip was recorded. Inclusion counts were performed independently by two experienced observers; the results obtained by one observer were not revealed until those of the other were recorded.

Statistics. The paired-sample *t* test and McNemar's test of proportions were used to determine statistical significance.

RESULTS

Standard control cultures. Replicate monolayers infected with serial dilutions of a laboratory strain of *C. trachomatis* (UW-6) were examined for inclusions at 18, 24, 48, and 72 h. Although Giemsa-stained inclusions were recognizable at 48 h, the limited development of these inclusions made examination difficult and imprecise compared with the inclusions observed at 72 h.

IF staining revealed small inclusions in infected cultures after 18 and 24 h. Within 48 h, fluorescent chlamydial inclusions displayed a sharply defined mass within the cytoplasm of HeLa 229 cells. Although IF was evident as early as 18 h, typical inclusion morphology was not definitively displayed until 48 h. The number and morphology of inclusions observed at 48 h by IF staining were not significantly different from those observed at 72 h. Nonspecific IF staining was not observed with uninoculated HeLa cell monolayers. Thus, for the subsequent comparison of IF staining and Giemsa staining

for the identification of chlamydial inclusions from clinical specimens in cell culture, we evaluated cultures at 72 h with Giemsa staining. IF-stained cultures were evaluated at 48 or 72 h, depending on a previously defined work schedule for these clinical specimens.

Evaluation of clinical specimens. A total of 53 specimens were studied by indirect IF staining, and 62 specimens were studied by direct IF staining. No significant differences were observed between the sensitivity of the two IF staining techniques; thus, these data were pooled for analysis.

Inclusions were detected by either IF staining or Giemsa staining in 39 of the 115 specimens after passage 2. Inclusions from every specimen in which Giemsa staining revealed inclusions were detected by IF staining. Of the 39 isolates, 38 (97%) on passage 1 and 1 on passage 2 were detected by IF staining. However, only 23 (59%) isolates on passage 1 and 13 (33%) on passage 2 were detected by Giemsa staining. (The three remaining samples in which inclusions had been detected by IF staining but not by Giemsa staining on passage 2 were reexamined. One and two inclusions per cover slip were detected in two of these specimens, but none was detected in the third specimen. After passage 3, inclusions were detected in the third sample.) The difference in sensitivity between IF staining and Giemsa staining for the detection of inclusions on passage 1 was significant ($P < 0.0005$).

The 15 specimens which revealed inclusions on passage 1 by IF staining but not by Giemsa staining all contained fewer than 20 inclusions per cover slip. A comparison of the isolation rates on passage 1 between the IF and Giemsa methods of those specimens which contained fewer than 20 inclusions per cover slip showed 22 positive specimens by IF staining and 7 positive specimens by Giemsa staining (Table 1). Thus, in 68% of these low-titer specimens, inclusions were not detected by Giemsa staining on passage 1.

The number of inclusions per specimen detected by IF staining was significantly greater than that detected by Giemsa staining (IF/Giemsa = 2.3; $P < 0.05$). The enhanced sensitivity of IF staining was most evident in those passage 1 samples which contained fewer than 200 inclusions per cover slip (IF/Giemsa = 3.0; $P < 0.001$). In contrast, samples containing more than 200 inclusions per cover slip showed no significant differences in counts between the two staining methods (IF/Giemsa = 1.1).

DISCUSSION

For the detection of *C. trachomatis* inclusions from clinical specimens we have routinely used May-Grunwald-Giemsa staining of HeLa 229

TABLE 1. Number of specimens from which *C. trachomatis* inclusions were detected by IF staining or Giemsa staining^a

Cell culture passage	All positive specimens		Specimens with <20 inclusions per cover slip		Specimens with <10 inclusions per cover slip	
	IF	Giemsa	IF	Giemsa	IF	Giemsa
1	38	23	22	7	15	3
2	1	13	1	13	1	10

^a The difference in total positive specimens between the IF and Giemsa methods reflects three specimens for which either reexamination or a 3rd passage was required to detect inclusions by Giemsa staining.

cell monolayers at 72 h after inoculation, followed by a second passage of negative specimens onto fresh monolayers. For the past 10 years this procedure has been effectively utilized for the isolation of *C. trachomatis*. We tried fluorescent-antibody techniques previously (8), but the poor growth of *C. trachomatis* biotypes in the available culture systems seriously limited practical attempts to grow enough antigen for the adequate immunization of large laboratory animals to obtain antiserum for use in IF staining. The limitations inherent in obtaining conventional antiserum are avoided by utilizing monoclonal antibodies which are available in potentially unlimited quantities and which recognize a *C. trachomatis* species-specific major outer membrane surface antigen.

The threefold increase in sensitivity of IF staining over Giemsa staining for the number of inclusions counted in cell culture agrees with the results obtained by Thomas et al. (11). The fact that another study (3) found no marked differences in sensitivity between IF staining and Giemsa staining can probably be understood in terms of a high multiplicity of inclusions per sample. In our study and in that of Thomas et al., as the number of inclusions per sample increased, the disparity between the IF and Giemsa methods rapidly diminished. The most stringent comparison of these methods should thus be made with samples containing only a few organisms per sample, which tests the ability of these methods to demonstrate a greater rate of chlamydial isolation. Such a comparison has not been reported. This information can be derived from our study by comparing passage 1 samples producing fewer than 10 inclusions per cover slip by IF staining. In only three of these samples were inclusions detected by Giemsa staining (Table 1; $P < 0.005$). Furthermore, after passage 2, inclusions in three of the IF-positive specimens were not detected by the initial Giemsa reading.

The diagnosis of *C. trachomatis* infection is currently based on cell culture isolation. The isolation results are usually assessed by two cell culture passages (6 days) with either Giemsa or iodine staining for the detection of chlamydial

inclusions. IF staining with the reagent used in this study is now being compared with iodine staining for the isolation of *C. trachomatis* in cycloheximide-treated McCoy cells at another laboratory. The preliminary results are similar; they show a higher sensitivity for IF staining than for iodine staining, both in the rate of isolation and in the number of inclusions counted (Walter Stamm, personal communication). The IF staining method did not significantly enhance the sensitivity of the isolation procedure if negative cultures were passaged a second time. However, IF staining with monoclonal antibodies that recognize species-specific antigens provides a reproducibly sensitive and specific assay for detecting *C. trachomatis* inclusions in cell culture after only one passage. Furthermore, the time required to scan cover slips stained with fluorescent antibody (average time, 5 min) was less than half the time required to scan Giemsa-stained cover slips (average time, 12 min). These savings in time and material can substantially lower the cost of isolation and provide for earlier diagnoses.

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LITERATURE CITED

- Alexander, E. R., S. P. Wang, and J. T. Grayston. 1967. Further classification of TRIC agents from ocular trachoma and other sources by the mouse toxicity prevention test. *Am. J. Ophthalmol.* 63:1469-1478.
- Bernkopf, H., P. Mashiah, and Y. Becker. 1962. Correlation between morphological and biochemical changes and the appearance of infectivity in FL cell culture infected with trachoma agent. *Ann. N.Y. Acad. Sci.* 98:62-81.
- Darougar, S., R. M. Woodland, T. Forsey, S. Cubitt, J. Allarni, and B. R. Jones. 1977. Isolation of *Chlamydia* from ocular infections, p. 295-298. In D. Hobson and K. K. Holmes (ed.), *Nongonococcal urethritis and related infections*. American Society for Microbiology, Washington, D.C.
- Ey, P. L., S. J. Prowse, and C. R. Jenkin. 1978. Isolation of pure IgG₁, IgG_{2a}, and IgG_{2b} immunoglobulins for mouse serum using protein A-sepharose. *Immunochemistry* 15:429-436.

5. **Furness, G., D. M. Graham, and P. Reeve.** 1960. The titration of trachoma and inclusion blenorrhoea viruses in cell cultures. *J. Gen. Microbiol.* **23**:613-619.
6. **Goding, J. W.** 1976. Conjugation of antibodies with fluorochromes: modifications to the standard methods. *J. Immunol. Methods* **13**:215-226.
7. **Hanna, L., M. Okumoto, P. Thygeson, L. Rose, and C. R. Dawson.** 1965. TRIC agents isolated in the United States. X. Immunofluorescence in the diagnosis of TRIC agent infection in man. *Proc. Soc. Exp. Biol. Med.* **119**:722-728.
8. **Kuo, C. C., S. P. Wang, B. B. Wentworth, and J. T. Grayston.** 1972. Primary isolation of TRIC organisms in HeLa 229 cells treated with DEAE-dextran. *J. Infect. Dis.* **125**:665-668.
9. **Ripa, K. T., and P.-A. Mårdh.** 1977. Cultivation of *Chlamydia trachomatis* in cycloheximide-treated McCoy cells. *J. Clin. Microbiol.* **6**:328-331.
10. **Stephens, R. S., M. R. Tam, C. C. Kuo, and R. C. Nowinski.** 1982. Monoclonal antibodies to *Chlamydia trachomatis*: antibody specificities and antigen characterization. *J. Immunol.* **128**:1083-1089.
11. **Thomas, B. J., R. T. Evans, G. R. Hutchinson, and D. Taylor-Robinson.** 1977. Early detection of chlamydial inclusions combining the use of cycloheximide-treated McCoy cells and immunofluorescence staining. *J. Clin. Microbiol.* **6**:285-292.
12. **Woodland, R. M., H. El-Sheikh, S. Darougar, and S. Squires.** 1978. Sensitivity of immunoperoxidase and immunofluorescence staining for detecting chlamydia in conjunctival scrapings and in cell culture. *J. Clin. Pathol.* **31**:1073-1077.