Reticulate Bodies as Single Antigen in Chlamydia trachomatis Serology with Microimmunofluorescence

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Formalin-fixed, purified reticulate bodies (RB) of Chlamydia trachomatis immunotype C/TW-3/OT were used as a serological test antigen in the microimmunofluorescence test. The sensitivity and specificity of the RB antigen were compared to elementary bodies (EB) used as antigens in the detection of C. trachomatis antibodies in human sera by microimmunofluorescence. RB reacted with all known C. trachomatis immunotypes with the same sensitivity as the homotypic EB. In routine serology with sera and endocervical secretions, the RB antigen had a sensitivity similar to that of the EB in detecting serum antibodies, endocervical secretion antibodies, and antibodies of immunoglobulin M and G classes. No false-positive reactions were detected with control sera. All positive reactions showed type-specific antibodies against an EB immunotype. RB seemed to demonstrate chlamydial group reactivity; sera from 10 psittacosis patients diagnosed clinically and serologically by complement fixation showed five positive, three equivocal, and two negative reactions. By immunofluorescence, RB appeared as distinct rings demonstrating uniform peripheral surface fluorescence at their rims. The EB appeared as pinpoint-sized dots. C/TW-3/OT RB used as a single test antigen should provide a simple and sensitive serological assay for the detection of C. trachomatis antibody.

Numerous serological tests for detecting chlamydial infections have been reported. The complement fixation test has been the standard test for psittacosis and lymphogranuloma venereum (LGV) (13). Complement fixation is a group-specific test that detects antibody common to both Chlamydia psittaci and Chlamydia trachomatis. The test is not useful for non-LGV C. trachomatis infections because of its poor sensitivity (13). The radioisotope precipitation test (3) and the enzyme-linked immunosorbent assay (8), which utilize C. psittaci organisms as test antigen, are group specific. These tests are of limited use in detecting C. trachomatis antibody and have not been used for this purpose in either research or clinical laboratories. Counterimmunoelectrophoresis using a highly purified C. trachomatis species-specific antigen has been used for the serological diagnosis of LGV (1), but was not found useful for other C. trachomatis infections. A sensitive and specific serological test for C. trachomatis antibody has been the microimmunofluorescence test (micro-IF) of Wang and Grayston (16), currently used in several major chlamydial research laboratories. However, due to the difficulty in preparation of multiple immunotype antigens it has not yet been widely used in general clinical laboratories. The test has been simplified by reducing the number of antigens by pooling antigens of related immunotypes, and this method is currently used (18). Thomas et al. (14) used elementary bodies (EB) of a single immunotype strain with broad cross-reactivity in the micro-IF. This latter approach has practical value, because in many instances information of specific immunotypes is not required for diagnosis and treatment of chlamydial infections. Unfortunately, however, no single C. trachomatis strain cross-reacts completely with all 15 C. trachomatis immunotypes. Richmond and Caul (10, 11) and Saikku and Paavonen (12) described a single-antigen indirect immunofluorescence test for detecting antibody, using C. trachomatis inclusions in cultured cells as antigen. These tests are broadly reactive with C. trachomatis and C. psittaci antibodies, suggesting that inclusions contain antigens with both species and genus determinants.

In our previous study of the antigenic analysis of LGV organisms by two-dimensional immunoelectrophoresis, we compared the antigenic profile of reticulate bodies (RB) and found C. trachomatis strains to be antigenically homogeneous (2). We were therefore interested in...
determining whether human *C. trachomatis* serum antibodies would recognize LGV RB in the micro-IF. In these preliminary studies we found broad antigenic cross-reactivity to *C. trachomatis* immunotype antibodies. The RB antigen reacted with sera from 13 *C. trachomatis*-infected patients. Ten of the sera were from infants whose mothers had cervical *C. trachomatis* infections, and three were from trachoma patients. We subsequently tested the RB of another strain, C/TW-3/OT, and obtained the same results. These findings indicated that RB may be used as a single antigen in the micro-IF for chlamydial serology. This report describes the sensitivity and specificity of the RB of strain C/TW-3/OT as a single antigen in comparison to the use of multiple EB antigens in detecting *C. trachomatis* antibodies in human serum, tears, and endocervical secretions.

**MATERIALS AND METHODS**

*C. trachomatis* strains. *C. trachomatis* strains used were A/G-17/OT, B/TW-5/OT, C/TW-3/OT, D/UW-3/Cx, E/UW-5/Cx, F/UW-55/Ur, G/UW-57/Cx, H/UW-43/Cx, I/UW-12/Ur, J/UW-36/Cx, K/UW-31/Cx, L4/400/Bu, L6/434/Bu, and L6/404/Bu (7, 15, 17, 19). All were grown in HeLa 229 cell culture (6).

Growth and preparation of RB. *C. trachomatis* TW-3 was grown in HeLa 229 cell culture as previously described (6). One-day-old HeLa cell monolayers in 32-ounce (ca. 0.95-liter) prescription bottles were inoculated with about 10 inclusion-forming units of organisms per cell to assure infection of 100% of the cells. Infected cells were harvested at 27 to 28 h postinfection. Cells were disrupted by sonication for 30 s with an intermediate probe (Biosonik III; Bronwill Scientific, Rochester, N.Y.) at 60 intensity. After one cycle of differential centrifugation (600 × g for 10 min and 30,000 × g for 90 min), the resuspended pellet was layered over 10 ml of 35% Renografin (methylglucamine diatrizoate, 76% for injection; Squibb and Sons, New York, N.Y.) and centrifuged at 22,000 × g for 1 h in a Spinc 25.1 rotor in a Spinc model L ultracentrifuge (Beckman Instruments Inc., Palo Alto, Calif.).

The resulting pellet was suspended in buffer, layered over a 35 to 60% linear Renografin gradient, and centrifuged at 42,000 × g for 1.5 h. A single band appeared in the middle of the gradient. The material in the band was collected with a capillary pipette, diluted with 10 mM N-tris[hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)—saline (pH 7.0), and centrifuged at 30,000 × g for 30 min. The RB were fixed immediately by resuspending in 10 mM TES-saline containing 6% Formalin. The suspension was centrifuged at 30,000 × g for 20 min and then resuspended in 0.02% Formalin in 10 mM TES-saline. The final product from 12 to 18 infected culture bottles was suspended in 1.0 to 1.5 ml of 0.02% Formalin. The Formalin-fixed RB were kept at 4°C until used. Samples used for infectivity assays and electron microscopy were taken before Formalin fixation. For use in the micro-IF the antigen solution was mixed with an equal volume of a solution of 3% normal yolk sac in phosphate-buffered saline (20). The methods for applying RB antigen to glass microscope slides, acetone fixation, and fluorescence microscopy were as for the standard micro-IF test (16).

Human sera, tears, and endocervical secretions. Human sera were obtained from the serum bank of S.-P. Wang. These sera were from previous seroepidemiological studies of trachoma and related *C. trachomatis* infections (4, 5, 16). In these studies, patients, their contacts, or both were known to have had various *C. trachomatis* infections which had been diagnosed by isolation, serology, distinctive clinical syndromes, or a combination of these determinations. Sera and endocervical secretions from patients attending a venereal disease clinic in Seattle were obtained from ongoing studies with D. H. Martin and K. K. Holmes of the Department of Medicine, University of Washington. Tear specimens were obtained from a trachoma study conducted in Taiwan (4). Tears were collected onto filter paper (5 by 20 mm2) and eluted in 0.2 ml of phosphate-buffered saline. Endocervical secretions were collected by cotton swab and eluted in 0.3 ml of phosphate-buffered saline. Sera from psittacosis patients diagnosed clinically and serologically by complement fixation were obtained from Julius Schachter, University of California, San Francisco, and from Tiara Fukushima, Utah State Department of Social Services, Division of Health. These sera had no demonstrable micro-IF antibodies to any *C. trachomatis* immunotypes. Normal sera were obtained from staff and students at the University of Washington, Seattle, who were not in chlamydial research.

*C. trachomatis* serology. Serology was performed using the micro-IF test as described by Wang and Grayston (16). RB antigens were applied to the microslide along with the EB antigens of the particular immunotypes that were to be compared. Twofold serial serum dilutions or three fourfold dilutions starting with 1:8 (18) were used as indicated. Tears and endocervical secretions were tested in the same way as serum. The dilution factors of the eluate were estimated to be 1:10 for tears and 1:15 for endocervical secretions. Fluorescein-conjugated goat anti-human immunoglobulins (immunoglobulin M [IgM], IgA, and IgG), as well as class-specific goat anti-human IgM (μ-chain specific) and IgG (γ-chain specific), were obtained from Hyland Laboratories, Los Angeles, Calif. Only typical fluorescence associated with evenly distributed EB or RB was considered positive (15). The highest serum dilution that gave a definite fluorescence was taken as the endpoint. Titers of 1:8 or greater were regarded as positive. Duplicate slides were tested in each experiment.

**RESULTS**

RB preparation. Density gradient-purified RB were homogeneous when examined by electron microscopy. There were, however, small numbers of EB and intermediate forms detectable. Low titers of infectious organisms were found by infectivity assays. In micro-IF, the RB were coccoid in appearance and demonstrated uniform peripheral surface fluorescence, in con-
trast to the pinpoint-sized dots of the EB. The RB varied in size, approximately two to three times larger than the EB, corresponding to the sizes reported for each, i.e., approximately 800 nm for the RB and 350 nm for the EB (13). When homotypic serum was tested, small numbers of EB were seen, again indicating some contamination. However, when heterotypic serum was tested the presence of EB was less evident. The Formalin-fixed RB antigen was stable for 1 year at 4°C.

**Sensitivity of RB in testing human sera.** Fifty-two different human sera with known antibody titers against EB immunotypes were tested against the RB antigen. These sera included each of 15 known immunotypes except Ba. Pooled EB antigens were used for types C and J, E and D, and G and F as in the simplified micro-IF test (18). Titers of 1:8 or greater were regarded as positive. RB reacted with approximately the same titer as the homotypic EB for all *C. trachomatis* immunotypes (Fig. 1). However, occasionally immunotype I (one of three tested) and immunotype K (two of eight tested) sera showed less reaction to RB than to the respective homotypic EB. The correlation coefficient of antibody titers between RB and EB was \( r = 0.9518 \) and \( P < 0.001 \).

**Antibody to RB in control sera.** Fifty-one normal sera were tested against RB. Five (9.8%) had titers of 1:8 or greater, four had titers of 1:8 to 1:16, and one had a titer of 1:64. All five had antibodies to EB: two of immunotype B and one each of A, CJ, and GF.

**Reaction of RB to sera of psittacosis patients.** Ten sera from psittacosis patients were tested against RB; two were negative, three were equivocal, and five were positive. By micro-IF the three equivocal results demonstrated atypical staining of RB, with fluorescence appearing as slightly undulating circles, resulting in a "patchy" appearance rather than sharp, clear, round cocci. The reciprocal titers of the five positive cases were 8, 16, 64, 64, and 64. No direct correlation with the CF titer of each serum was made, since the two tests were not conducted at the same time.

**Detection of antibodies in tears and endocervical secretions with RB.** Thirty-five tear specimens and 17 endocervical secretions were tested simultaneously against homotypic EB. Antibody titers against EB are shown on the abscissa, and those against RB are shown on the ordinate. Both the abscissa and ordinate are represented in terms of the reciprocal of twofold serum dilutions. (○) Isolation-positive cases of *C. trachomatis* organisms; (●) isolation-negative cases. The correlation coefficient between RB and EB is \( r = 0.9518 \); \( P < 0.001 \).
Two sera were tested against RB as well as against multiple EB antigens. Three twofold dilutions were used (1:10, 1:20, and 1:40) for tears, and three fourfold dilutions (1:15, 1:60, and 1:240) were used for endocervical secretions. Twenty-three of 35 tear specimens (66%) were positive for EB, and 21 of 35 (60%) were positive for RB (Table 1). Of 23 positive tear specimens, 15 (65%) were type C, 1 was type GF, and the rest were type B. Nine tear specimens had higher titers to EB than to RB: two with two-tube differences and seven with a one-tube difference. Two specimens were EB positive and RB negative. The remaining 12 had equal titers to EB and RB. Nine of 17 (53%) endocervical secretions were positive for EB, and 10 of 17 (59%) were positive for RB. Seven endocervical secretions showed equal titers for RB and EB, two specimens had a one-dilution higher titer with RB than with EB, and one specimen was RB positive and EB negative. The immunotypes found were three ED and six GF.

Routine serology and detection of IgM and IgG antibodies of patients attending a venereal disease clinic. The routine serological test used three fourfold serum dilutions, 1:8, 1:32, and 1:128. Sixty-three patients including 49 males and 14 females were tested, some more than once, giving a total of 97 tests. The 15 cases with positive isolations all showed antibodies against RB and EB. RB reacted more often than EB, 78/97 (80%) versus 68/97 (70%) respectively (Table 2), but these differences were not statistically significant. The majority of the sera tested were specific for ED and GF immunotypes, and the remaining were specific for CJ, I, and K immunotypes. Forty-seven sera had equal titers for RB and EB. RB titers were higher than EB titers in two sera and lower in seven sera. Two sera were positive to EB and negative to RB, whereas 12 sera were positive to RB and negative to EB. Six of the 12 sera that reacted only to RB were from patients who either demonstrated lymphocyte blast transformation to C. trachomatis antigen (unpublished data from David Martin) or had sera positive for EB at least once during follow-up. Sixty of the above sera were also tested for IgM and IgG antibodies (Table 2). IgM was detected in three sera by both RB and EB and in one each by RB or EB, whereas IgG was detected by both in 54 of 60 sera.

**DISCUSSION**

Human sera of all known C. trachomatis EB immunotypes reacted with C/TW-3/OT RB, even though it is likely that different antigenic determinants are recognized. The RB antigen was as sensitive as multiple EB antigen, resulting in a further simplification of the micro-IF test. If needed, positive sera can be retested for immunotype specificity by the standard micro-IF test. The larger size and peripheral surface fluorescence of the RB greatly simplifies immunofluorescence reading compared to conventional micro-IF serology. In addition, the antigen can be stored at 4°C for at least 1 year, making the RB antigen extremely useful in the serological screening of sera for chlamydial antibodies even in laboratories not specialized in chlamydial research. The stability of the Formalin-fixed antigen may make commercial availability possible. A 1.0-ml stock antigen solution can last up to 1 year when an average of 45 sera are tested daily.

A few I and K immunotype sera did not react with RB. However, those that reacted with RB

![Table 2. Sensitivity in micro-IF of C trachomatis C/TW-3/OT RB and various immunotype EB for serum antibodies in patients at a venereal disease clinic and their sexual contacts](image-url)
had the same titers as against their homotypic EB. The I and K nonreactors may have been due to variation in host antibody response. Since immunotypes I and K constitute only 5% of all the non-LGV C. trachomatis infections (20), and since only a fraction of these are nonresponders to RB, the loss of ability to detect these immunotypes would be minimal.

Although antibody response to RB is group specific, the detection of psittacosis antibodies with RB appeared not to be as efficient as was the detection of C. trachomatis antibodies, and the fluorescence observed was different in some reactors, resulting in a patchy appearance. Further confirmation is necessary to define the significance of RB reaction to psittacosis antibodies.

Although we did not compare the RB test with other single-antigen immunofluorescence tests, some indirect comparisons can be made. Thomas et al. (14) compared the sensitivity of a single EB antigen, SA2(f), which is an L2 immunotype, to multiple EB immunotype antigens in micro-IF serology of a venereal disease population. They obtained a sensitivity of 95.5% with this population. However, the SA2(f) strain, as well as the majority of chlamydial genital strains E, D, G, and F, belongs to the B-complex strains of C. trachomatis. These strains show a greater degree of cross-reactivity with each other than with C-complex strains (20). The sensitivity of the SA2(f) antigen may decrease when tested in areas where trachoma is endemic, since a large portion of these C. trachomatis infections would be due to A and C immunotypes to which the L2 strain shows little cross-reactivity (20).

Chlamydial serology using immunofluorescence staining of C. trachomatis inclusions in infected cells as reported by Richmond and Caul (10, 11) and Saikku and Paavonen (12) is a simple and sensitive screening method. However, the relative sensitivity of inclusion staining compared to micro-IF is not known, since a direct comparison using EB antigen was not made by these authors. It is quite possible that immunofluorescence staining of inclusions which result in broad serological cross-reactions may be measuring the same chlamydial antibodies as our staining of RB, since the inclusions contain the organisms in various stages of their developmental cycle. It has also been shown that group-specific CF antigen persisted in cell cultures at all times during the developmental cycle of the organism (9), which appears to explain the group-specific reactivity of inclusions.

Immunofluorescence serology methods using RB, EB of a single strain, or single-strain inclusions in infected cells all exploit the capacity of these antigens to cross-react broadly with antibodies primarily from the same species of organisms. The sensitivity of these tests is likely very similar. The advantage of the RB antigen over the EB antigen is that the RB form is easier to read because of its larger size. The major disadvantage of the use of inclusions as test antigen is the requirement for chlamydial-infected cells; also, the procedure is more cumbersome and more expensive than the application of prepared antigens on microscope slides. A single antigen applied directly on microscope slides allows the results obtained with the micro-IF technique to be used to full advantage, i.e., a large number of sera can be screened at once and endpoint titers can be obtained, if desired. Staining of inclusions in infected cells would require large quantities of cells to obtain the same results, since one antigen dot on a microscope slide is equal to one cell monolayer. As many dots can be placed on a slide, many sera can be screened easily and simultaneously from a single slide.

C/TW-3/OT RB harvested at 27 to 28 h were contaminated with a trace amount of EB. The transition of RB to EB in the developmental cycle is a continuous process with regard to size and also in density. These two populations could not be separated by density gradient centrifugation. However, contamination with trace EB did not interfere with micro-IF serology. Although we have studied the RB of only two C. trachomatis strains, trachoma C/TW-3/OT and LGV L2/434/Bu, the RB of any strain would probably give comparable results. It seems likely that the same application may be extended to C. psittaci serology when the RB of a psittacosis strain are used. The use of a single RB antigen (C/TW-3/OT) in the micro-IF assay should be a useful, simple, and sensitive test for C. trachomatis serology.

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LITERATURE CITED


