Comparison of Reticulate and Elementary Body Antigens in Detection of Antibodies Against *Chlamydia trachomatis* by an Enzyme-Linked Immunosorbent Assay

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Reticulate bodies from a type C and elementary bodies from a type L_2 strain of *Chlamydia trachomatis* were isolated and used as antigens in an enzyme-linked immunosorbent assay (ELISA). Results obtained for human sera with these two antigens used in the ELISA were compared with each other and with results obtained for the same sera by the micro-immunofluorescence test. Negative control populations included cloistered nuns and children with respiratory infections. Populations at risk for chlamydial infection consisted of 42 men with nongonococcal urethritis attending a sexually transmitted diseases clinic and 42 college women who had contact with men with nongonococcal urethritis. ELISAs done with the two antigens were equivalent to each other and to the micro-immunofluorescence test in the ability to predict the presence or absence of infection. None of the tests had high predictive values for the men with urethritis. However, the negative predictive value of both the micro-immunofluorescence test and the elementary body ELISA was 0.92 for the college women. Such serological tests may be of value in screening selected populations for subclinical infections with *C. trachomatis*.

Infection with *Chlamydia trachomatis* is now the most common sexually transmitted disease (STD) (3, 5). Although many men (17) and women (18) who are infected with this organism are asymptomatic, considerable evidence is accumulating that such infections are not benign. Included in the disease syndromes attributed to *C. trachomatis* are urethritis, cervicitis, salpingitis, proctitis, conjunctivitis, and pneumonia (3, 5, 16). In addition, subclinical infections appear to be a major cause of infertility in women (6).

Unfortunately, diagnosis of chlamydial infections in most clinical settings is difficult, expensive, or both. Isolation of the organism in tissue culture remains the diagnostic method of choice. However, recovery rates are affected by the manner in which a clinical specimen is obtained, transported, and stored (10, 12) and the method used to infect tissue culture cells (12, 21). Consequently, reliable cultures are generally available only in large medical centers with extensive experience in tissue culture methodology. Other diagnostic techniques, such as direct antigen detection in clinical specimens (19), may offer considerable theoretical promise but require further evaluation in a clinical setting.

The identification of chlamydial antibodies in serum or secretions lacks the specificity of tissue culture identification but may on occasion provide useful information (23, 24). The standard complement fixation test is not sufficiently sensitive for the diagnosis of genital tract infection (15, 23). However, the micro-immunofluorescence (micro-IF) test of Wang and Grayston (22–24), which uses chlamydial elementary bodies (EBs) as a test antigen, has been shown to be both sensitive and specific for the detection of chlamydial antibodies. Similar immunofluorescence tests that use purified reticulate bodies (RBs) (26) or infected cells (13) as the test antigen have also been described.

More recently, detection of chlamydial antibodies by both radioimmunoassay and enzyme-linked immunosorbent assay (ELISA) has been described (2, 8, 9, 20). The relative advantage offered by these solid-phase tests lies primarily in their simplicity. However, EBs from a single strain of chlamydia have always been employed as the test antigen. Although EB antigens from some strains appear to be broadly cross-reactive, such cross-reactivity may not be complete (23, 24). Therefore, an EB antigen from a single immunotype might not detect antibodies against all immunotypes. However, the RB form of *C. trachomatis* appears to express group-reactive

antigens on its surface and to react with antisera directed against all known immunotypes of *C. trachomatis*, as well as some strains of *C. psittaci* (26).

In the present study, EBs and RBs were compared as test antigens in an ELISA, and the results obtained with these ELISAs were compared with the results obtained with the micro-IF test in the determination of serum chlamydial antibodies in selected patients at risk for genital chlamydial infection.

MATERIALS AND METHODS

Chlamydial strains and purification. *C. trachomatis* strains A/G-17/OT, B/TW-5/OT, Ba/Ap-2/OT, C/TW-3/OT, D/UW-3/Cx, E/UW-5/Cx, F/UW-94/Ur, G/UW-57/Cx, H/UW-4/Cx, I/UW-12/Ur, J/UW-36/Cx, K/UW-53/Cx, L$_1$/440/Bu, L$_2$/434/Bu, and L$_3$/404/Bu were kindly provided by C.-C. Kuo, Seattle, Wash. EB antigens for use in the micro-IF test were prepared as described by Kuo et al. (7), except that the organisms were grown in cycloheximide-treated McCoy cells (14) rather than in HeLa cells. RBs were harvested from McCoy cells 28 h after infection with strain TW-3 by a modification of the procedure of Yong et al. (26). After the cells were disrupted by sonication followed by differential centrifugation (500 × *g* for 10 min and 30,000 × *g* for 30 min, respectively), they were partially purified by centrifugation through a 30% Renografin (methylglucamine diatrizoate, 76% for injection; E. R. Squibb & Sons, New York, N.Y.) cushion at 22,000 × *g* for 40 min. They were then fixed in 6% Formalin in Hanks balanced salt solution, pelleted, resuspended in 0.02% Formalin in phosphate-buffered saline (PBS) (4 mM KH$_2$PO$_4$, 16 mM Na$_2$HPO$_4$, 115 mM NaCl, pH 7.3), and stored at 4°C until used.

EBs from strain L$_2$/434 were harvested from McCoy cells 48 h after infection and subjected to differential centrifugation as described above. The pellet (yield from three 150-cm^2 flasks) was then suspended in 25 ml of 30% (vol/vol) Percoll (Pharmacia Inc., Uppsala, Sweden)–10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)–145 mM NaCl (pH 7.4) and centrifuged at 30,000 × *g* for 30 min at 4°C (SS-34 angle head rotor; Du Pont Co., Wilmington, Del.). Two bands were resolved in the self-generating Percoll gradient. The band at the bottom of the gradient consisted of purified EBs and about 5% RBs, as determined by electron microscopy. The band of chlamydial particles was diluted sixfold in HBS (10 mM HEPES–145 mM NaCl [pH 7.4]) and centrifuged at 30,000 × *g* for 30 min at 4°C. The EB pellet was washed again in HBS, suspended in HBS containing 0.05% Formalin, and stored at −70°C.

EB and RB protein concentrations were determined by the method of Bradford (1), with bovine serum albumin used as a standard after samples were boiled for 1 min in 0.1% sodium dodecyl sulfate.

Attempts to purify RBs in Percoll instead of Renografin were unsuccessful, presumably due to osmotic lysis.

Chlamydial serology. The micro-IF test was performed with EB antigens from representative strains of all known immunotypes of *C. trachomatis* (except Ba) as described by Wang et al. (24) and with an RB

antigen from strain TW-3 as described by Yong et al. (26).

The RB and EB ELISAs were performed with standard ELISA technology (25). Based on protein content, RBs from strain TW-3 or EBs from strain L$_2$/434 were diluted to the appropriate concentration in 0.5 M Na$_2$CO$_3$ (pH 9.6), and 0.2 ml of this suspension was used to coat each well of a 96-well polystyrene microtiter plate (Dynatech Laboratories Inc., Alexandria, Va.). After incubation for 18 h at room temperature, the antigen suspension was aspirated, and each well was washed three times with PBS (pH 7.3)–0.05% (vol/vol) Tween 20 (Sigma Chemical Co., St. Louis, Mo.) (PBST).

A 0.3-ml amount of PBST, 0.5% with respect to bovine serum albumin, was added to each well, and the plates were incubated for 1 h at room temperature. Each well was then washed once with 0.3 ml of PBST, and 0.2 ml of a 1:100 dilution of serum in PBST was added to each well. Incubation was carried out at 37°C for 1 h, the serum was aspirated from each well, and each well was washed three times with 0.3 ml of PBST. To each well was then added 0.2 ml of a 1:1,000 dilution in PBST of alkaline phosphatase-conjugated goat anti-human immunoglobulin G (IgG) (Sigma). After further incubation for 1 h at 37°C, the conjugate was removed by aspiration, and each well was washed five times with 0.3 ml of PBST. Next, 0.2 ml of *p*-nitrophenyl phosphate (1 mg/ml)–50 mM Na$_2$CO$_3$–1 mM MgCl (pH 9.8) was added to each well, and incubation was continued at 37°C for 30 to 40 min. A well containing a strongly reactive control serum was monitored until an absorbance of 1.0 at 405 nm was reached. The reaction was then stopped with the addition of 25 µl of 5 N NaOH to each well, and the absorbance at 405 nm was determined in a spectrophotometer.

A control well without antigen was run for each serum tested, as were control wells without serum (both with and without antigen) for each plate tested.

Chlamydial cultures. Isolation of *C. trachomatis* from clinical specimens was done as described by Kuo et al. (7), except that cycloheximide-treated McCoy cells (14) were substituted for HeLa cells and inclusions were identified by indirect immunofluorescence (21) using hyperimmune rabbit serum raised against HeLa cell-grown strain L$_2$/434 EBs and adsorbed with a crude HeLa cell homogenate (2 h, 4°C). McCoy cell monolayers were inoculated with clinical specimens that had been stored at −70°C. They were incubated for 72 h at 37°C, fixed in methanol, and allowed to air dry. They were then overlayed with a 1:80 dilution of the anti-L$_2$/434 rabbit serum in PBS (pH 7.6), incubated in a moist petri dish at room temperature for 30 min, rinsed with PBS, air dried at 37°C, overlayed with fluorescein-conjugated sheep anti-rabbit immunoglobulin (Wellcome Reagents, Ltd., Beckenham, England) diluted 1:20 in PBS, incubated for 30 min at room temperature in a moist petri dish, rinsed with PBS, dried at 37°C, and examined for fluorescent inclusions with a Leitz SM-LUX epifluorescent microscope (E. Leitz Inc., Rockleigh, N.J.). Inclusions from representative strains of all 15 immunotypes were easily identifiable when stained by this method (W. J. Newhall V, unpublished data).

Populations evaluated. Serum was obtained from Catholic nuns who were members of a cloistered
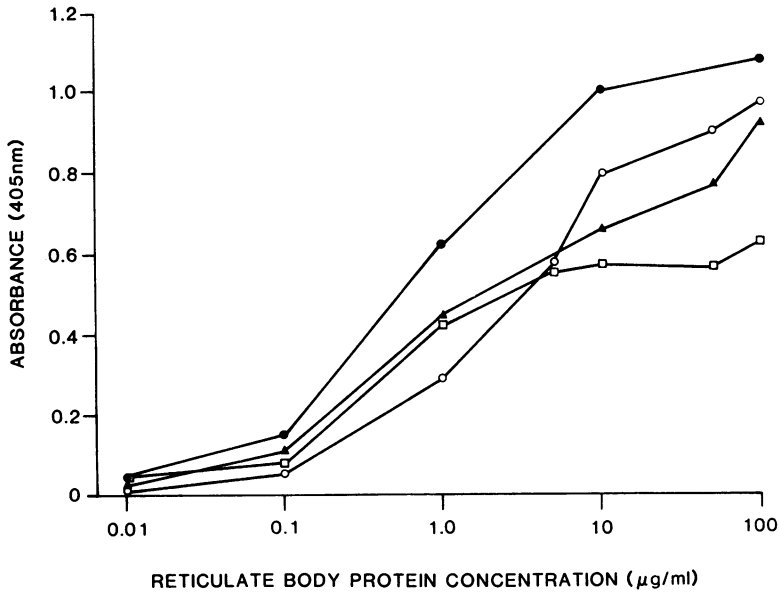
RETICULATE BODY PROTEIN CONCENTRATION (μg/ml)

FIG. 1. Absorbance in ELISA as a function of RB antigen protein concentration with sera from patients with known chlamydial infections. Clinical lymphogranuloma venereum in a man with a micro-IF titer of 1:1024 against type L_2 (▲); culture-positive pregnant woman with a micro-IF titer of 1:512 against type I (○); culture-positive pregnant woman with a micro-IF titer of 1:512 against type B (□); and culture-positive man with NGU with a micro-IF titer of 1:512 against types G/H (●).

order. The mean age of the sisters was 52.1 years, with a range of 35 to 74 years. Their mean time in the order was 29.5 years, with a range of 8 to 54 years. Serum was also obtained from 10 children with signs and symptoms of an acute upper respiratory infection for whom an etiological diagnosis had not been made. Their mean age was 23.5 months, with a range of 9 to 37 months. Cultures for *C. trachomatis* were not obtained from these two population groups.

Urethral cultures for *C. trachomatis* and serum were obtained from 42 men attending an inner-city STD clinic for whom a diagnosis of nongonococcal urethritis (NGU) was made. The criteria employed for a diagnosis of NGU were: presence of a urethral discharge, dysuria, or both; gram-stained material from an endourethral swab that revealed >4 polymorphonuclear leukocytes per field and no gram-negative intracellular diplococci at 400× magnification; and a urethral culture negative for *Neisseria gonorrhoeae*. All patients were in good health except for urethritis.

In addition, 42 college women referred to a student health service because of sexual contact with a man with NGU were evaluated for evidence of chlamydial infection. All were asymptomatic and in good health. Evaluation included chlamydial serology and endocervical cultures for chlamydiae. The sensitivity, specificity, and predictive value of serological tests for these groups were calculated by standard methods (4).

RESULTS

Characteristics of ELISA. Titrations were performed for different concentrations of RB and EB antigens and dilutions of sera that were either negative or strongly positive in the micro-IF test. An example of one such experiment is

shown in Fig. 1. The four sera used in this experiment were used to standardize both the RB and EB ELISAs. Similar curves were obtained with the EB antigen, except that it required two to four times more EBs (based on protein concentration) than RBs to produce the same absorbance with a given serum.

Antigen excess was not observed at 100 μg of protein per ml with either EBs or RBs. Antigen concentrations of 10 μg/ml for EBs and 2.5 μg/ml for RBs were chosen for use in subsequent experiments. They gave equivalent absorbances with the high-titer sera shown in Fig. 1, and serial twofold dilutions from 1:50 to 1:12,800 of these strongly reactive antisera showed a linear relationship to absorbance. Based on similar titrations with multiple sera, 1:100 was chosen as the working dilution for unknown sera.

Analysis of population groups and comparison of test results. Sera from 14 of 15 nuns and from all 10 children were negative in the micro-IF test (titer, <1:8) and were used in a negative control panel. The results for RB and EB ELISAs are shown in Fig. 2. The mean plus two standard deviations for absorbance values obtained with the sera from the nuns and children was designated as the division between a positive and a negative test result.

With sera from the two population groups at risk for acquiring a chlamydial infection, the results obtained with the RB and EB ELISAs were compared with each other, with results
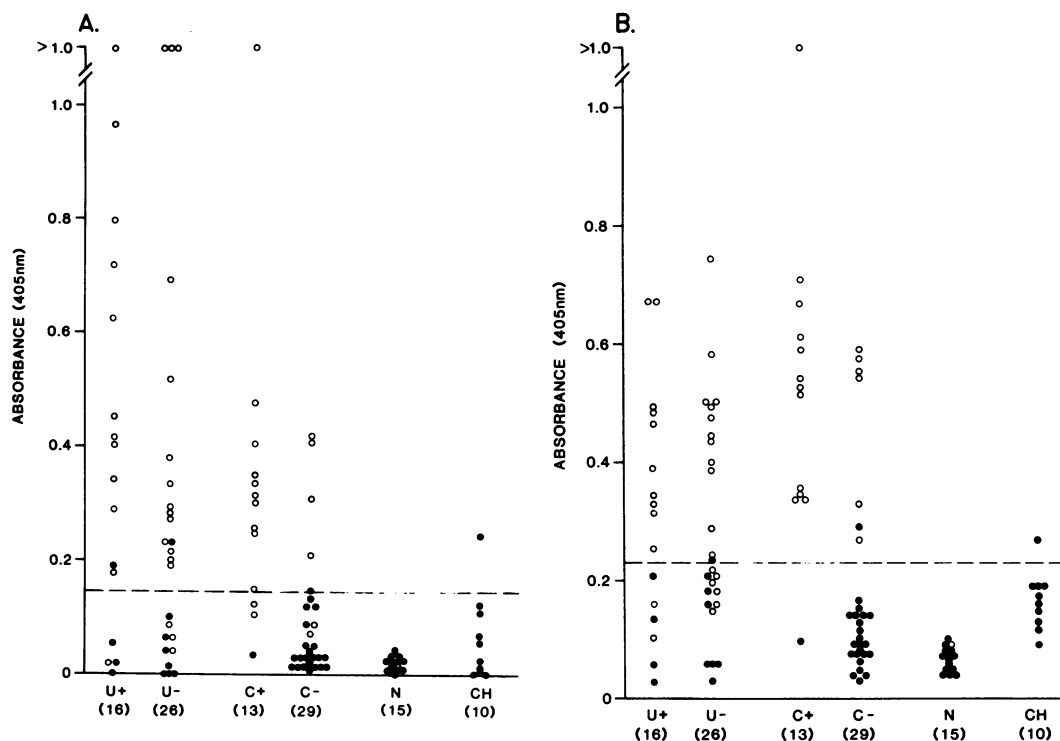
FIG. 2. Absorbance values in RB (A) and EB (B) ELISAs with sera from different populations. Culture positive (+) or negative (−); men with urethritis (U); female contacts to men with NGU (C); nuns (N); and children (CH). Serum positive (O) or negative (●) in micro-IF test. Dotted lines indicate mean plus two standard deviations for absorbance values for sera from the 15 nuns and 10 children.

obtained with the micro-IF test, and with the known culture status of the patients. Although the relative concentrations of RB and EB antigens employed gave comparable absorbance values with high-titer reference sera, the absolute absorbance values obtained with the patient panel was higher in the EB ELISA for the majority of sera. However, it was not unusual for a particular serum to give high absorbance values with the RB antigen and only moderate values with the EB antigen or vice versa.

Serum from one child gave a positive reaction with the RB antigen but was negative with the EB antigen, and serum from another child was positive with the EB but not the RB antigen. None of the sera from the nuns gave a positive reaction with either antigen.

In the men with NGU there was no meaningful correlation between the results of any of the serological tests and the culture status. Twelve of 16 (75%) culture-positive men also had positive results in the micro-IF test, versus 18 of 26 culture-negative men (69%). Similar results were obtained in both the RB and EB ELISAs. On the other hand, a good correlation was observed between serological and cultural results in the NGU contacts. Twelve of the 13 culture-positive women (92%) were seropositive in the micro-IF

test, versus 6 of 29 (21%) culture-negative women (P = 0.001, Fisher's exact test). Similar results were also obtained for these patients with the EB ELISA, whereas the RB ELISA appeared to be slightly less sensitive.

The correlations among the results obtained with the different serological tests and the culture results are given in Table 1. All three tests were in agreement for 66 of the 84 patients (79%) tested. Based on culture results, all three tests gave false-positive and false-negative results at

TABLE 1. Correlation between results of serological tests and culture status in men with NGU and female contacts of men with NGU

Serological tests results[a]	Culture	
	Positive	Negative
M+ R+ E+	20	15
M+ R+ E−	1	4
M+ R− E+	2	3
M+ R− E−	1	2
M− R+ E+	0	0
M− R+ E−	1	2
M− R− E+	0	2
M− R− E−	4	27

[a] Positive (+) or negative (−) in micro-IF (M), RB ELISA (R), or EB ELISA (E).

TABLE 2. Predictive power of chlamydial EB ELISA in two populations at risk

Population	EB ELISA results				
	Prevalence (no. positive cultures/no. tested)	Sensitivity	Specificity	Predictive value	
				Positive	Negative
Men with NGU (n = 42)	0.38	0.65	0.50	0.46	0.68
Women with NGU contacts (n = 42)	0.31	0.92	0.76	0.63	0.96

similar ratios. The micro-IF was positive slightly more often than either ELISA, but not significantly so. In these patients there was no correlation between the absolute titer in the micro-IF test and the presence of infection as determined by culture.

Calculated differences in sensitivity, specificity, and predictive value for the three tests were not statistically significant by chi-square analysis. These values are given for the EB ELISA in Table 2.

DISCUSSION

Based on the limited sample size evaluated, the RB and EB ELISAs appear to be roughly equivalent to each other and to the micro-IF test in detecting antichlamydial IgG in infected adults. Thus, in spite of theoretical concerns, it would seem that there are sufficient group- or species-reactive antigens present in both the EB and RB antigen preparations used in the ELISAs to detect antibodies against most infecting strains.

Less RB than EB antigen was required for equivalent binding of antibody from highly reactive sera, consistent with the hypothesis that a greater number of common determinants are expressed on the RB than the EB. However, EB yields are 5- to 10-fold greater per tissue culture flask than RB yields, and EB preparations are more consistent from experiment to experiment. Thus, for practical reasons EBs seem to be preferable to RBs for use as the antigen in an ELISA.

Although some semiquantitative measure of the amount of antibody present, such as a titer, may be helpful in selected instances (23), a major advantage of the single-dilution ELISA is that it does not require the titration of sera. This reduces the work load and the unit cost per test. Similarly, although antichlamydial IgM is more specific than IgG for diagnosing current infection, it is more frequently absent than present and not very sensitive for detecting genital tract infection in adults (15, 23). Consequently, only IgG was measured in the present study.

The role of serological testing in the diagnosis of genital chlamydial infections remains controversial. The relatively high background rate of

26% seropositivity in normal adults (24) and up to 87% (15) in selected patient populations makes interpretation difficult. This is especially true in a sexually active group of patients, such as those attending STD clinics. For example, in the present study the positive and negative predictive values for the EB ELISA were only 0.46 and 0.68, respectively, for men with NGU. Presumably this reflects both the high background seropositivity rate in patients who attend STD clinics (24) and the absence of chlamydial antibody in many men experiencing their first episode of NGU (23).

On the other hand, quite different results were obtained in the college women we evaluated. Their background seropositivity rate (24% for the EB ELISA in culture-negative women) was similar to that reported for adults in general. This may reflect fewer sexual partners over their lifetime and thus an overall lower exposure rate than that usually seen in patients attending STD clinics (11, 15). However, the prevalence of infection (31%) was consistent with their having had recent contact with men with NGU. This combination of factors resulted in positive and negative predictive values of 0.63 and 0.96, respectively. Admittedly, this is a highly selected population. In unselected college women with a prevalence of infection closer to 5% (11), the predictive value of a positive serological test would be much lower. However, the negative predictive value of the test would remain high so long as its sensitivity remained high (4).

Although additional studies in larger population groups are needed to define the diagnostic role of chlamydial serological tests, it would seem that in selected groups of patients a negative test by micro-IF or ELISA may be as effective at excluding a diagnosis of infection as a negative culture, given that the sensitivity of the latter is only about 90% (15).

Approximately 20% of men with a first episode of chlamydial NGU are seronegative by micro-IF (23), and thus a negative serology test in men with NGU would not be helpful. A similar false-negative rate has not been reported in women, although some women would also be expected to be seronegative shortly after acquiring a first chlamydial infection. No attempt was

made in the present study to distinguish between source and secondary contacts. Some of the NGU contacts were presumably secondary, yet only 1 of the 13 culture-positive women was seronegative by either micro-IF or the EB ELISA. Whether the relative numbers of seronegative, culture-positive NGU contacts would increase with a larger sample size is unknown.

In spite of these problems in interpretation, serological testing may prove sufficiently convenient and cost effective to be of value in the initial screening of selected populations in whom there is a low background of seropositivity. Examples could include middle-class women who are pregnant or seeking contraceptives for the first time. A positive serological test would only indicate exposure, not active infection, and would be an indication for further evaluation (i.e., culture). A negative serological test, on the other hand, would provide reasonable evidence that subclinical infection was not present unless it had been very recently acquired. The importance of diagnosing subclinical infections in pregnant women is primarily to prevent transmission of the infection to their infants (16), whereas in sexually active young women early diagnosis and treatment may prevent later salpingitis and infertility (5, 6). In populations of women in whom the prevalence of infection and seropositivity is high, culture remains the only practical means for screening.

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