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Solid-Phase Enzyme Immunoassay for Chlamydial Antibodies

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An enzyme immunoassay (EIA) for chlamydial immunoglobulin G antibodies was developed by using microtiter wells coated with partially purified reticulate bodies of *Chlamydia trachomatis* serotype L2, grown in McCoy cells, and uninfected McCoy cells as a control. Duplicate testing of a single serum dilution, 1:500, was found to be sufficient. A good correlation between positive reactions was observed in a comparative study of 421 patient sera with the EIA and an inclusion immunofluorescence test. A good correlation between positive reactions was also observed in a comparative study of 140 patient sera with EIA and microimmunofluorescence tests in which chlamydial elementary or reticulate bodies were used as antigens. Sera of 77 healthy control individuals with low titers in inclusion immunofluorescence or complement fixation tests gave negative results in the EIA. Immunoblotting experiments showed that the major antigenic component in the EIA antigen was a protein with an M_r of 39,000.

The significance of chlamydial infections in humans has been increasingly recognized, and convenient diagnostic methods are in great demand. Isolation of chlamydia and serology are complementary to each other and are valuable for diagnosis of infection. Among serological tests, the complement fixation (CF) test (2) has been the time-honored method, but it appears to have only limited value (13). Various modifications of the indirect immunofluorescence antibody test (IFAT) have been developed, including the microimmunofluorescence (micro-IF test) (18) and the inclusion IFAT (I-IFAT) (9). We now report on the feasibility of a solid-phase enzyme immunoassay (EIA) procedure for chlamydial antibodies and the characterization of the EIA antigen. The merit of the EIA was assessed by comparison with the micro-IF and the I-IFAT.

MATERIALS AND METHODS

Serum specimens. The following groups of serum specimens were studied: (i) 188 samples from women with pelvic inflammatory disease (PID), (ii) 231 samples from women and 2 from men with urethritis, cervicitis, or infertility, and (iii) samples from 77 healthy medical students, both female and male. All the sera were stored at −20°C.

Chlamydial antigen and solid-phase EIA. *Chlamydia trachomatis* serotype L2 (strain 434 Bu; kindly provided by Pertti Terho, Department of Virology, University of Turku, Turku, Finland) was grown at 37°C in monolayer cultures of McCoy cells in Glasgow modification of Eagle medium supplemented with 10% fetal calf serum and 10 μg of gentamycin per ml. The reticulate body (RB) antigen (purchased from Orion Diagnostica, Helsinki, Finland) was prepared and purified according to the method of Yong et al. (20).

A solid-phase EIA, described in detail elsewhere (12), was used. Based on preliminary titrations with IFAT-positive and -negative sera, 5 to 10 μg of antigen protein per ml, diluted in 0.01 M phosphate-buffered saline (pH 7.4), was used to coat polystyrene microtiter wells. Phosphate-buffered saline containing 0.02% Tween 20 was used for dilution of serum and enzyme conjugate (alkaline-phosphatase-labeled anti-human immunoglobulin G [IgG] [heavy-chain specific; Orion Diagnostica]), which were added in volumes of 75 μl to each polystyrene well. For the enzyme reaction, disodium *p*-nitrophenyl phosphate (0.2% [wt/vol] in diethanolamine-magnesium chloride buffer) was used as substrate. After 20 min at room temperature, the enzyme reaction was stopped by the addition of 200 μl of 1 N NaOH, and absorbances at 405 nm (A_{405}) were measured with a vertical pathway spectrophotometer (Titertek Multiskan; Eflab, Helsinki, Finland).

All sera were tested in duplicate at dilutions of 1:50, 1:500, 1:5,000, and 1:50,000.

IFAT. The micro-IF test was carried out with formalinized elementary body (EB) antigens of *C. trachomatis* serotypes CJ, A, H, I, K, B, ED, and GF, as described by Wang et al. (19). RB antigen was prepared from *C. trachomatis* serotype C (20). Fluorescein-labeled anti-human IgG (Hyland Laboratories, Inc., Costa Mesa, Calif.) was used as the conjugate, and fourfold dilutions of sera were tested at dilutions of 1:8, 1:32, and 1:128, and greater.

The I-IFAT was performed according to the method of Saikku and Paavonen (11) by using the L2 strain of *C. trachomatis* inclusions in the dog kidney cell line (KoMu), kindly provided by N. Oker-Blom, Depart-

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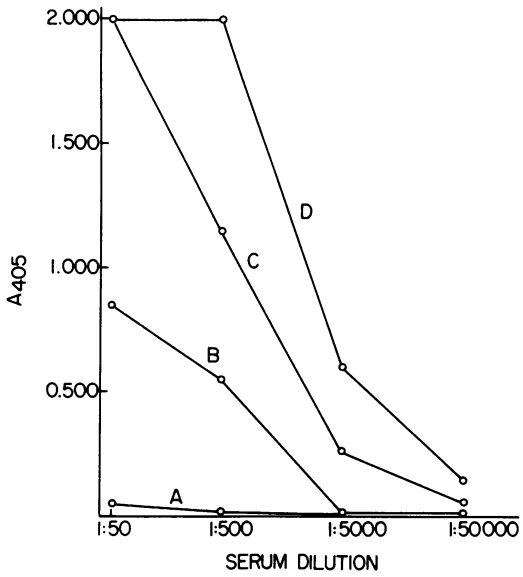

FIG. 1. EIA absorbance curves of different dilutions of representative sera with high (1,024; D), intermediate (256; C), low (64; B), and negative (<8; A) I-IFAT titers. The A_{405} values are the values obtained with chlamydial antigen after substraction of those obtained with the control antigen.

ment of Virology, University of Helsinki. The sera were tested at dilutions of 1:8, 1:32, 1:128, 1:512, and greater with fluorescein-labeled anti-human IgG (Wellcome, Beckenham, United Kingdom) as the conjugate.

CF test. The CF test was performed in microtiter plates as described elsewhere (10). The CF antigen, kindly provided by C. Mordhorst, State Serum Institute, Copenhagen, Denmark, was an ether-acetone extract of yolk sacs infected with *C. trachomatis* serotype E.

Immunoblotting of proteins. The procedure described by Towbin et al. (16) was used for immunoblotting of proteins. Polypeptides separated on sodium dodecyl sulfate-polyacrylamide (8%) slab gel electrophoresis according to the method of Laemmli (7) were transferred electrophoretically to nitrocellulose sheets (type HAWP filter; Millipore Corp., Bedford, Mass.), the protein binding sites of the sheet were saturated with bovine serum albumin, and the sheets were stained by using the immunoperoxidase method. Both the human and rabbit sera were diluted in Tris-buffered saline containing 10% fetal calf serum. The species-specific anti-IgG peroxidase conjugates (γ-chain specific; Orion Diagnostica) were used at a dilution of 1:100 in Tris-buffered saline.

Rabbit antisera. *C. trachomatis* UW200 (serotype D) and UW213 (serotype G), both from cases of Reiter's syndrome, were grown in yolk sacs of chicken embryos. The infected yolk sacs were homogenized, and elementary bodies were purified (5) by pelleting in an ultracentrifuge through a 30% Renografin cushion, and immunization of the rabbits was carried out according to the schedule of Kenny (6).

RESULTS

Standardization of EIA. An optimal antigen concentration was selected for coating the microtiter wells. Figure 1 shows an antibody assay for different dilutions of four representative sera with high, medium, low, and negative IFAT titers. The A_{405} values obtained with wells coated with control antigen prepared from uninfected McCoy cells were negative (<0.100) at all serum dilutions. It appeared that the 1:500 serum dilution was optimal to observe differentiation of high and low titers; this dilution was routinely used to test for antibody in the sera.

It was found that 7 to 8% of sera reacted with the control antigen. All sera was therefore tested concomitantly against both the chlamydial and control antigens, and the differences in A_{405} values were used for expression of EIA results.

Correlation of EIA with I-IFAT. A total of 421 sera from patients with PID, urethritis, cervicitis, or infertility were tested in parallel by EIA and I-IFAT.

Statistical analysis of the results (Fig. 2) showed a good correlation between the two

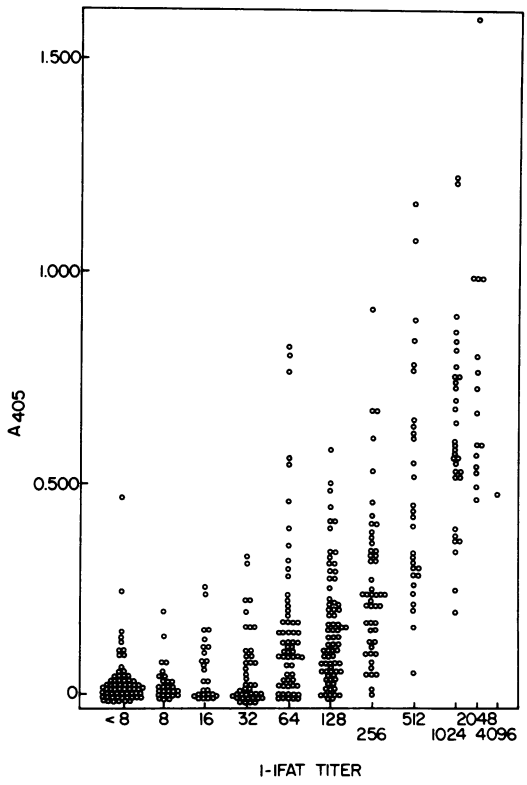

FIG. 2. Correlation between EIA and I-IFAT tests for chlamydial antibodies in 421 patient sera. Linear regression analysis gave the following values: correlation coefficient (r) = 0.686 ($P < 0.001$) and y intercept = −0.067.

TABLE 1. Chlamydial serum antibodies determined by the CF, EIA, and I-IFAT tests in 77 healthy medical students

Group	No. of samples	No. of samples with an I-IFAT titer of:		
		1:<16	1:16	1:32
CF				
1:<8	65	42	20	3
1:8	12	5	7	0
EIA				
Negative	77	47	27	3
Positive	0	0	0	0

tests. It is also apparent from the figure that most sera with low I-IFAT titers (≤16) were negative (A_{405} < 0.200) in EIA, whereas very few I-IFAT-negative sera (<8) were EIA positive (A_{405} > 0.200).

When the sera of 77 healthy medical students were tested for chlamydial antibodies with the CF, EIA, and I-IFAT tests, it was found (Table 1) that all sera were EIA negative, whereas the CF and I-IFAT tests gave weakly positive titers for 16 and 39%, respectively.

Correlation of EIA to EB and RB micro-IF. A group of 140 sera from PID patients were tested for chlamydial antibodies in parallel by EIA and micro-IF with both EB and RB antigens. Statistical analysis of the results (Fig. 3) showed a good correlation between the two tests, with no apparent difference between the two micro-IF tests. The correlation between the tests was similar to that of the I-IFAT with the EIA.

EIA in diagnosis of chlamydial infections. A total of 130 paired sera were available from the patients studied. Results on all these serum specimens are included in Fig. 2. Of these 130 paired sera, 16 pairs showed a significant change in titer of chlamydial antibodies in EIA or I-IFAT or both. Moreover, in 9 of the 130 paired sera (6.9%), an increase in A_{405} to >0.200 was recorded for control antigen. The 16 cases and their antibody titers are shown in Table 2. Four of the cases could be diagnosed by EIA alone, four by I-IFAT alone, and in eight cases titer changes were shown by both tests.

Preliminary characterization of the EIA antigen. The chlamydia EIA antigen preparation was electrophoresed in polyacrylamide gels, and the proteins were transferred to nitrocellulose sheets and stained with human sera from patients and controls and hyperimmune rabbit sera. As shown in Fig. 4, the positive sera reacted mainly with a polypeptide of M_r = 39,000; in addition, several higher-molecular-weight polypeptides were stained. The staining patterns obtained with patient and hyperimmune rabbit sera were nearly identical; no reactivity was obtained with control sera.

DISCUSSION

This report describes the development of a solid-phase EIA for chlamydial IgG antibodies with a partially purified RB fraction of *C. trachomatis* serotype L2, grown in McCoy cells. In preliminary experiments, it was obvious that concurrent use of a control antigen was required, because some of the sera showed significant reactivity with the control antigen and even a "seroconversion," presumably due to autoantibodies, known to occur in chlamydial infections (1). We initially tested all of the 421 patient sera at four dilutions (1:50, 1:500, 1:5,000, and 1:50,000), but could then conclude that a single dilution, 1:500, tested in duplicate, was sufficient for practical purposes, as illustrated in Fig. 1.

The correlations of the EIA results with those of the I-IFAT and the micro-IF tests with their EB or RB as antigens were all good, with

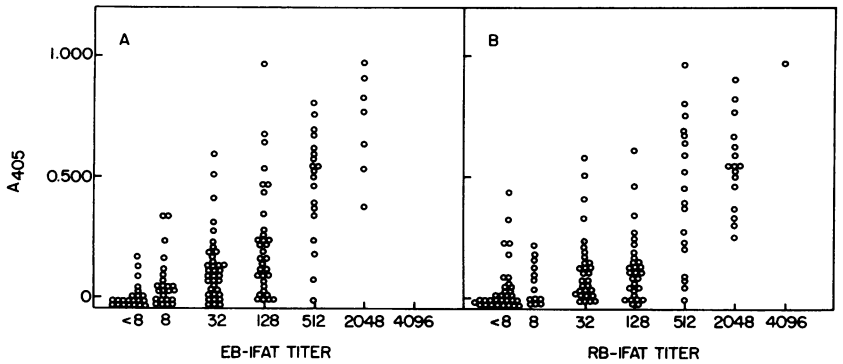

FIG. 3. Correlation between EIA and EB (A) and RB micro-IF (B) tests for chlamydial antibodies in 140 patient sera. Linear regression analysis gave the following correlation coefficients: EB, r = 0.705 (P < 0.001); RB, r = 0.671 (P < 0.001).

TABLE 2. Changes in chlamydial antibodies in paired sera of patients as determined by the EIA and I-IFAT tests[a]

Patient no.	Diagnosis	Serum sample	Days after onset	EIA	I-IFAT
1	PID	1	14	0.316	128
		2	23	**0.848**	**512**
2	Pyosalpinx	1	9	0.984	512
		2	23	1.162	**2,048**
3	PID	1	18	0.513	512
		2	34	**1.223**	1,024
4	Pyosalpinx	1	15	0.829	1,024
		2	65	0.676	**256**
5	PID	1	16	0.593	2,048
		2	48	**0.378**	1,024
6	PID	1	9	0.230	128
		2	38	**0.636**	**512**
7	PID	1	11	0.009	8
		2	112	**0.588**	**128**
8	PID	1	3	0.042	128
		2	126	**0.557**	**1,024**
9	PID	1	7	0.392	128
		2	24	**1.079**	**512**
10	PID	1	2	0.225	64
		2	41	**0.434**	128
11	Perihepatitis	1	2	0.252	1,024
		2	9	**0.468**	**4,096**
12	PID	1	10	0.290	512
		2	51	**0.582**	**2,048**
13	PID	1	24	0.224	32
		2	82	**0.679**	32
14	PID	1	x	0.314	32
		2	$x + 7$	**0.776**	**512**
15	PID	1	8	0.112	64
		2	18	0.250	**256**
16	NGU	1	x	0.019	16
			$x + 32$	0.122	**64**

[a] Patients 1 to 15 were females, and patient 16 was a male. NGU, Nongonococcal urethritis. The EIA results are given as A_{405} of 1:500 serum dilutions and those of I-IFAT as reciprocal titers; the diagnostic changes ($E_{405} \geq 0.200$ or $\geq$ fourfold change in titer) are in boldface type.

correlation coefficients from 0.681 to 0.700 ($P < 0.001$). The EB micro-IFAT test is generally regarded as an established reference method and is particularly suitable for serotyping immune responses and for epidemiological surveys (19). A major advantage of the I-IFAT test over the micro-IF tests is the simplicity of antigen preparation (11). The subjectivity in interpretation is a natural drawback of all IFAT tests, but it is eliminated in the EIA, in which the spectrophotometric recordings may even be checked visually.

A notable difference in correlation between the EIA and I-IFAT was that a large number of sera with low I-IFAT titers were EIA negative. The significance of these sera with low I-IFAT

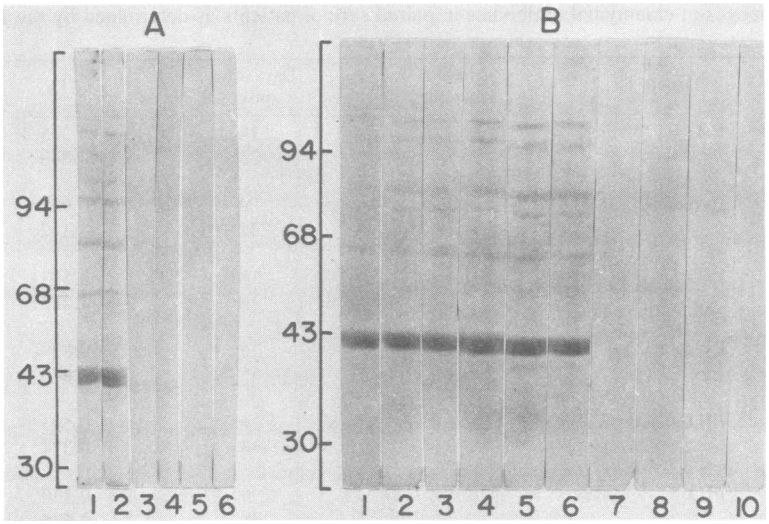

FIG. 4. Immunoblotting of the EIA chlamydial antigens. The polypeptides were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose sheets, and stained with human (A) or rabbit (B) sera by the immunoperoxidase procedure. The following human sera from PID cases were used at a 1:50 dilution: lane 1, I-IFAT titer 1:1,024; lane 2, I-IFAT 1:512; lane 3, I-IFAT 1:64; lane 4, I-IFAT 1:64; and lanes 5 and 6, I-IFAT 1:<8. The rabbit sera (B) used were anti-UW213 serum diluted 1:100 (lane 1), 1:200 (lane 2), and 1:500 (lane 3), anti-UW200 serum diluted 1:100 (lane 4), 1:200 (lane 5), and 1:500 (lane 6), and preimmune rabbit sera diluted 1:100 (lanes 7 and 8) and normal rabbit sera diluted 1:100 (lanes 9 and 10).

titers remains open; however, some of them were also positive in the EB micro-IF test. Some of the low titers may represent interbacterial antigenic cross-reactivity as described between *Chlamydiae* and *Acinetobacter* (17) and *Coxiella* (15). Analysis of the data by using serial serum dilutions might have clarified the issue, but since the low I-IFAT titers have only questionable clinical significance (excluding male nongonococcal urethritis), this was not attempted.

Serological diagnosis of chlamydial infections is not readily accomplished in localized genital infections, as these induce weak antibody responses and are often reinfections (14). In complicated generalized infections, serology has its place (10a, 13); the present study material did not include complicated ocular infections or neonatal pneumonitis or pneumonia, but was mainly from genital infections. In complicated genital infections such as PID, the EIA as well as the I-IFAT gave high titers and occasionally gave seroconversions as well. It appears from the results that the two tests correlated well in these high titers. Determination of IgM antibodies to chlamydiae by the EB micro-IF test increases the number of serological diagnoses (3, 19; P. Saikku and J. Paavonen, manuscript in preparation). The solid-phase EIA test should be applicable to IgM antibody determinations.

The immunoblotting technique indicated that a polypeptide with an M_r of 39,000, apparently identical to the major outer membrane protein (4), was a major antigenic component in the EIA antigen preparation. In addition, several polypeptides of higher molecular weight were antigenic in the test, and the fact that both high titer human sera and rabbit hyperimmune sera stained them suggests that they are specific. Since our patient material was from genital infections associated with *C. trachomatis* and contained no verified cases of *Chlamydia psittaci* infections, the proportion of the common CF antigen (13) in the EIA antigen preparation could not be estimated. It may be assumed to comprise a large fraction because of its abundance in chlamydiae (8).

We conclude that the present EIA procedure provides a sensitive and simple serological assay for the detection and quantitation of chlamydial antibodies. Unlike the I-IFAT test, the EIA results may be recorded both spectrophotometrically and visually. The EIA procedure seems particularly well suited for large-scale assays and may prove useful as the primary routine technique for diagnosis of chlamydial infections.

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