Chlamydia pneumoniae Elementary Body Antigenic Reactivity with Fluorescent Antibody Is Destroyed by Methanol

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Methanol, used for fixing chlamydia organisms to microscopic slides, was shown to eliminate the antigenic reactivity of *Chlamydia pneumoniae* elementary bodies with fluorescent antibodies. Acetone fixation had no adverse effect. *Chlamydia trachomatis* and *Chlamydia psittaci* were not affected by methanol fixation. The effect of methanol on *C. pneumoniae* is another characteristic that differentiates this species from the other two chlamydia species.

Fluorescent antibody (FA) stain is commonly used for laboratory diagnosis of *Chlamydia trachomatis*. The technique is used for the detection of organisms in smears from clinical specimens and for the measurement of antibodies in serum and other body fluids in serologic tests. In these tests, smears of chlamydial organisms are fixed to microscopic slides before reaction with antibodies. The two most commonly used fixatives are acetone and methanol (3, 11, 12).

Chlamydia pneumoniae is a newly described species of chlamydia (5), a primary human pathogen which causes respiratory tract infections (4, 6). So far only one strain or serovar called TWAR has been described.

This report describes the finding that when methanol but not acetone is used as the fixative, the antigenic reactivity of *C. pneumoniae* elementary bodies for FA was destroyed. Methanol did not have a similar effect on the other two species of chlamydia.

The chlamydia organisms used were as follows: *C. trachomatis* combined serovars C plus J plus H plus I, G plus F plus K, and B plus E plus D as three antigens; *Chlamydia psittaci* avian strains PS-1 (parrot) (6), CP-3 (pigeon), and GR-9 (German duck) and mammalian strain GPIC; and *C. pneumoniae* TWAR, AR-39, and AR-388 (isolated from patients in Seattle), TW-183 (from a patient in Taiwan) (4), and AC-43 (from a patient in Japan) (16). The antibodies used were murine monoclonal antibodies CF-2 (genus specific [to lipopolysaccharide of chlamydia]), LV-21 (species specific [to *C. trachomatis*]), KB-8 (specific to *C. trachomatis* serovars E and L1), PP-13 (specific to strains PS-1 and CP-3), 2A-9 (specific to strain GR-9), GP 80a (specific to strain GPIC), and RR-402 and TT-205 (specific to the TWAR strain of *C. pneumoniae*). Also tested were patients' sera which were known to have immunoglobulin M (IgM) and/or IgG TWAR antibodies. Chlamydial strains CP-3 and GR-9 and monoclonal antibody 2A-9 were kindly provided by Arthur A. Andersen, U.S. Department of Agriculture, Ames, Iowa, and monoclonal antibody GP 80a was provided by You-xun Zhang, Rocky Mountain Laboratory, Hamilton, Mont. The other chlamydia strains and monoclonal antibodies have been previously described and used for immunotyping (6, 14).

All chlamydia strains were grown in HeLa 229 cell culture (8). Whole chlamydia organisms (elementary bodies), either treated with 0.02% formalin (15) or alive, were mixed with

3% normal yolk sac suspension to facilitate adherence, spotted onto slides with pen points, air dried, and treated for 15 min (or as indicated) with a fixative. The fixatives used were ethyl alcohol (95% purity), methyl alcohol (100% purity, American Chemical Society), acetone (99.6% purity, American Chemical Society), or a mixture of methanol and acetone (1:1). Details of the microimmunofluorescence technique have been previously reported (12). Serial twofold dilutions of murine monoclonal antibodies (ascitic fluid) (1:50 to 1:6,400) and of the patient serum (1:8 to 1:1,024) were tested.

Table 1 shows an example of the results of microimmunofluorescence tests with formalin-treated chlamydia elementary body antigens fixed with the different chemicals and tested concurrently. The use of methanol as a fixative, either alone or as part of mixture with acetone, was found to abolish the immunofluorescence of TWAR elementary bodies with antibody RR-402 and the patient's serum. Ethanol reduced but did not abolish the antibody titer with TWAR antigen. This reaction to alcohols was unique to *C. pneumoniae* TWAR and was not shared by the other chlamydia species. As a fixative, acetone was found to be as good or better than any of the alcohol-containing fixatives for the *C. trachomatis* and *C. psittaci* strains tested.

Direct FA staining of TWAR elementary bodies with fluorescein isothiocyanate (FITC)-conjugated TWAR monoclonal antibody also disappeared following methanol fixation.

Table 2 shows the effect of length (from 5 s to 15 min) of exposure to methanol. Four *C. pneumoniae* TWAR isolates, two from patients in Seattle and one each from patients in Taiwan and Japan, were tested in quadruplicate. One isolate was treated with formalin, and the others were nontreated (live) organisms. *C. trachomatis* antigens were concurrently tested as controls. The deleterious effect of fixation with methanol for as little as 5 s to TWAR but not to *C. trachomatis* can be seen. While not all indirect FA staining of all TWAR strains was abolished with short exposures to methanol, staining was either abolished or degraded compared with acetone fixation and the effect increased with time.

Chlamydia elementary bodies fixed with acetone do react with FITC-conjugated genus-specific monoclonal antibodies, although the reaction is less distinctive than with species-specific monoclonal antibodies. There was complete loss of staining of TWAR isolates with the *Chlamydia*

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TABLE 1. Results of the microimmunofluorescence test with chlamydia elementary body antigens treated with different fixatives^a

Fixative	Chlamydia serovar or strain	Antibody titer ^b					
		Murine monoclonal antibody ^c				Patient serum	
		KB-8	RR-402	PP-13	2A-9	IgM	IgG
Ethyl alcohol	CT(BED) ^d	800	0	0	0	0	0
	AR-39	0	200	0	0	16	16
	PS-1	0	0	3,200	0	0	0
	CP-3	0	0	3,200	0	0	0
	GR-9	0	0	0	400	0	0
Methanol	CT(BED)	1,600	0	0	0	0	0
	AR-39	0	0	0	0	0	8
	PS-1	0	0	1,600	0	0	0
	CP-3	0	0	1,600	0	0	0
	GR-9	0	0	0	1,600	0	0
Acetone	CT(BED)	1,600	0	0	0	0	0
	AR-39	0	1,600	0	0	32	64
	PS-1	0	0	3,200	0	0	0
	CP-3	0	0	3,200	0	0	0
	GR-9	0	0	0	1,600	0	0
Methanol-acetone mixture	CT(BED)	800	0	0	0	0	0
	AR-39	0	0	0	0	0	0
	PS-1	0	0	3,200	0	0	0
	CP-3	0	0	3,200	0	0	0
	GR-9	0	0	0	1,600	0	0

^a The elementary bodies had been treated with formalin.^b An antibody titer of 0 is shown for <50 and <8 antibodies for murine monoclonal antibodies and patient serum, respectively.^c KB-8 was used against *C. trachomatis* serovar E, RR-402 was used against *C. pneumoniae* TWAR, PP-13 was used against *C. psittaci* PS-1, and 2A-9 was used against *C. psittaci* GR-9.^d CT(BED), *C. trachomatis* combined serovars B, E, and D.

genus-specific monoclonal antibody CF-2 with as little as 5-s fixation with methanol.

Methanol has been recommended for direct FA detection of *C. trachomatis* organisms in smears of clinical specimens (3, 11). Although methanol fixation does not appear to affect detection of TWAR inclusions in cell culture (8), this study

shows that it should not be used to fix TWAR elementary bodies which are to be used as antigen in direct or indirect FA staining.

The microimmunofluorescence test has been extensively used for chlamydial serology (12) and has been found to be extremely useful for studies of TWAR infections (7, 9, 10,

TABLE 2. Effect of time of methanol fixation on TWAR antigens in the microimmunofluorescence test^a

Fixative and length of fixation	Reaction ^b of antibody ^c and antigen ^d																	
	RR-402 ^e and TWAR antigen:				TT-205 ^e and TWAR antigen:				LV-21 ^f and <i>C. trachomatis</i> antigen:			CF-2 ^g and TWAR antigen:				CF-2 ^g and <i>C. trachomatis</i> antigen:		
	1	2	3	4	1	2	3	4	I	II	III	1	2	3	4	I	II	III
Methanol																		
5 s	±	o	+	±	±	±	±	o	++	++	++	o	o	o	o	+	+	+
10 s	±	o	±	o	o	o	±	o	++	++	++	o	o	o	o	+	+	+
15 s	o	o	±	o	o	o	±	o	++	++	++	o	o	o	o	+	+	+
30 s	o	o	±	o	o	o	±	o	++	++	++	o	o	o	o	+	+	+
60 s	o	o	o	o	o	o	±	o	++	++	++	o	o	o	o	+	+	+
5 min	o	o	o	o	o	o	o	o	++	++	++	o	o	o	o	+	+	+
15 min	o	o	o	o	o	o	o	o	++	++	++	o	o	o	o	+	+	+
Acetone (15 min)	++	++	++	++	++	++	++	++	++	++	++	+	+	+	+	++	++	++

^a Each monoclonal antibody used at a 1:100 dilution of ascitic fluid. Each test done in quadruplicate.^b Symbols: ++, strong positive fluorescence; +, positive fluorescence; ±, weak fluorescence; o, no reaction.^c Monoclonal antibodies RR-402 and TT-205 are specific to *C. pneumoniae* TWAR; LV-21 is specific to *C. trachomatis*; CF-2 is specific to the *Chlamydia* genus.^d TWAR antigen abbreviations: 1, formalin-treated AR-39; 2, AR-388; 3, TW-183; 4, AC-43. *C. trachomatis* antigen abbreviations: I, *C. trachomatis* serovars C, J, H, and I; II, *C. trachomatis* serovars G, F, and K; III, *C. trachomatis* serovars B, E, and D.^e All *C. trachomatis* antigens did not react with monoclonal antibody RR-402 or TT-205.^f All TWAR antigens did not react with LV-21.^g All reactions to CF-2 were atypical fluorescence.

13). For fixation of TWAR organisms on slides, acetone should be used. While inclusions are occasionally seen on direct FA-stained smears of clinical specimens, stained elementary bodies are almost always the reason a smear is positive. We have found direct FA staining of throat swabs to be less sensitive than cell culture for identifying TWAR infection (6). However, the technique has not been reported for nasopharyngeal or lower respiratory tract specimens. Smears for detection of TWAR organisms by FA staining should be fixed with acetone.

The unique characteristics of the *C. pneumoniae* species in the sensitivity of its antigens to alcohol is another distinguishing trait of this species compared with the other chlamydia species. We have previously shown that the morphology of TWAR elementary bodies is distinctive with the aid of electron microscopy (2) and that the TWAR major outer membrane protein appears to be less antigenically complex and lacks the molecular weight heterogeneity of those proteins in the other species (1). The chemical reaction that abolished FA reactivity of the TWAR antigens is unknown. The antigen recognized by TWAR-specific monoclonal antibodies has not been characterized. The findings reported here provide further evidence of differences in the antigens of *C. pneumoniae* compared with those of *C. trachomatis* and *C. psittaci*.

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