Serotyping of *Chlamydia trachomatis* by Indirect Fluorescent-Antibody Staining of Inclusions in Cell Culture with Monoclonal Antibodies

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A new method for determining the serovars of *Chlamydia trachomatis* isolates by utilizing fluorescent-antibody staining of inclusions in cell culture is described. Monoclonal antibodies which have been successfully used previously for serotyping in the microimmunofluorescence test were employed. The cell culture method offers two advantages over the microimmunofluorescence test for many laboratories. It requires less antigen of the new isolate, about 10% cell culture infectivity versus 50% for the microimmunofluorescence test. Although fewer isolates can be typed at one time in cell culture, the technical requirements of the test are less rigorous.

We have reported successful serotyping of *Chlamydia trachomatis* isolates using monoclonal antibodies (MAbs) in the microimmunofluorescence (micro-IF) test (14), including the discovery and establishment of three new *C. trachomatis* serovars (12). The micro-IF test depends on type-specific epitopes residing on the major outer membrane protein, which are exposed on the surfaces of elementary bodies (6, 7). Treatment of elementary bodies with formalin (15) does not alter the serovar specificity of the antigen in the test.

Although the micro-IF test has been useful because of its sensitivity, its serovar-specific reactivity, and its rapidity when performed by experienced workers, it has been difficult to carry out by those who are not experienced with the technique. The concentration of antigen needed for the micro-IF test is roughly equivalent to that of a 50%-infected cell culture monolayer. New *C. trachomatis* isolates may require four or five passages to reach adequate concentration.

This paper reports the use of cell culture *C. trachomatis* inclusions as antigen for serotyping. Chlamydial inclusions have been used for fluorescent-antibody (FA) studies of human sera (5). When chlamydial inclusions are reacted with polyclonal antisera by indirect FA staining, a nonspecific genus reaction, presumably involving lipopolysaccharide reactivity, predominates. However, when the inclusions are reacted with serovar-specific MAbs, which react with major outer membrane protein antigens, a specific reaction can be demonstrated. It was found that the serovar specificity with inclusion staining was similar to that with the micro-IF test when the same set of MAbs was employed.

In order to further simplify the test, we explored using sets of fewer MAbs directed at isolates either from endemic trachoma or from sexually or natally transmitted diseases. The cell culture test requires a greater volume of MAbs than the micro-IF test does, and reducing the number of different MAbs used would save these valuable MAbs.

MATERIALS AND METHODS

MAbs. Most of the *C. trachomatis* MAbs used in this study have been reported (12, 14). Newly prepared MAbs were AA-3 (immunoglobulin G [IgG] derived from A/G-17

fusion), CC-13 (IgG from C/TW-3), EE-5 (IgM from E/UW-5), and DP-2 (IgG from Da/G-1). Others used were *C. trachomatis* species-specific MAbs LV-21 (IgG from L2/434) and KK-12 (IgG from K/UW-31). A single dilution of 1:50 to 1:400 of ascitic fluid of each MAb was used for indirect FA staining of inclusion bodies.

Chlamydia strains. Strains representative of the 18 *C. trachomatis* serovars (12, 14) were A/G-17/OT, B/TW-5/OT, Ba/AP-2/OT, C/TW-3/OT, D/UW-3/Cx, Da/TW-448/OT, E/UW-5/Cx, F/UW-6/Cx, G/UW-57/Cx, H/UW-4/Cx, I/UW-12/Ur, Ia/UW-202/Nasopharyngeal, J/UW-36/Cx, K/UW-31/Cx, L1/440/Bu, L2/434/Bu, L2a/UW-396/Rectum, and L3/404/Bu.

Indirect FA staining of chlamydia inclusions. (i) **Infected coverslips with chlamydia inclusions.** Chlamydia organisms were grown for 3 days in 24-h HeLa 229 cell monolayers on coverslips (12-mm diameter) in flat-bottomed 1-dram (4-ml) vials (2). When inclusions were seen in more than 10% of the cells, the culture fluid was removed and the infected monolayer was rinsed with phosphate-buffered saline and fixed for 10 to 15 min with absolute methanol. Then, the coverslips were removed from the culture vial, air dried, and kept at 4°C in a refrigerator until used.

(ii) **Application of antibody.** The cell-free side of each coverslip with the infected monolayer cells was marked for identification of the infecting strain and into four equal sections to facilitate placing four separate drops of MAb on the cell side. The coverslips, with the cell side facing up, were then placed in a moist chamber (a petri dish with a moistened paper towel underneath). A drop of antibody (3 µl) was delivered with a microtiter pipette to the center of each section of the coverslip. Different MAbs were applied in the same sequence to each of the coverslips which were to be concurrently tested. The coverslips were incubated at 37°C for 30 min.

(iii) **Application of fluorescein-conjugated anti-mouse immunoglobulins.** The coverslips were rinsed in three changes of distilled water and dried at room temperature (or in a 37°C incubator for 10 min). A mixture of fluorescein isothiocyanate-conjugated polyvalent anti-mouse immunoglobulins (Sigma Chemical Co.; product no. F-1010) at a dilution of 1:20 and Evans blue as a counterstain at a final concentration of 0.1% was used for indirect FA staining. The conjugate dilution (3-µl drop) was delivered by a micropipette onto the

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TABLE 1. Serotyping of *C. trachomatis* serovars based on indirect FA staining of inclusion bodies^a with MAbs

MAb	Reaction to <i>C. trachomatis</i> serovar																	
	C	J	Ia	I	A	H	G	L3	K	F	B	Ba	L2	L2a	E	L1	D	Da
<i>C. trachomatis</i> species specific																		
KK-12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LV-21	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>C. trachomatis</i> type or subspecies specific																		
LL-33	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-
GG-11	-	+	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-
BB-11	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
CC-1	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PE-5	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AC-11	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
LA-10	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
KK-1	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
FC-2	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
DD-1	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
BB-3	-	+	-	-	-	-	+	-	+	+	+	+	+	+	-	-	-	-
LV-23	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-
LV-27	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
KB-8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
JG-9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
DP-2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+

^a Inclusions in methanol-fixed coverslips of infected HeLa 229 cells.

center of each section of the coverslip where antibody had been applied. The coverslips were incubated at 37°C for 30 min in a moist chamber, and the process of rinsing and drying was repeated as before. The coverslips (with cell side down) were mounted on microscope slides with mounting fluid (90% glycerol in 0.1 M Tris buffer, pH 8.5). The slides were read immediately or were stored at 4°C for a few days before being read.

(iv) **FA microscopy.** An FA microscope (WILD M20 research microscope) with transilluminating light was used. A mercury-vapor-type HBO lamp provided the light source. The primary filters were composed of a heat-absorbing filter and a blue-light passage filter, BG-12, and the secondary filters were GG-13 for selective UV protection and a yellow filter, WILD 8075. Monocular observation was made under dark field with 10× ocular and 10× objective lenses. A 4× objective lens was used to locate the stained area of the cell monolayer, which had a reddish background due to the counterstain. A 40× objective lens was used for more detailed observation when necessary. Only inclusions with typical morphology and fluorescence associated with cells were considered positive.

micro-IF test. Formalinized whole elementary bodies of chlamydia organisms (15) were used as antigen. The test procedures have been published previously (10, 11).

RESULTS

Titration of MAbs in the micro-IF test and by inclusion staining. In order to compare antibody titers in both the micro-IF and the inclusion-staining systems, serial twofold dilutions (1:50 to 1:6,400) of two MAbs, LV-21 and AC-11, were concurrently tested in both systems. In the micro-IF test, formalinized antigens of serovars C, A, I, D, and Ba were used, while for inclusion staining, serovar A-infected coverslips were used. LV-21, a *C. trachomatis* species-specific MAb, reacted in micro-IF to all tested antigens to end titers of 1:800 (with C, A, I, and D) or 1:1,600 (with Ba),

while AC-11 reacted, as expected, only to serovar A (1:6,400) and serovar I (1:3,200). Both MAbs reacted to serovar A inclusions at titers similar to those found by micro-IF in the concurrent tests. In both tests, the optimal reaction was found with MAb dilutions of 1:100 to 1:400.

Inclusion staining with MAbs for *C. trachomatis* serovars. Infected cell cultures on coverslips were prepared from each of the 18 *C. trachomatis* serovars, and inclusion staining was done with 2 *C. trachomatis* species-specific and 16 *C. trachomatis* serovar- and subspecies-specific MAbs. A single dilution of ascitic fluid of each MAb (1:50 to 1:400, depending on the micro-IF titer) was used for the test. The results are shown in Table 1. The inclusions of all serovars were reactive with *C. trachomatis* species-specific MAbs. The range of serovar reactions to each type- or subspecies-specific MAb was similar to that demonstrated by micro-IF with elementary body antigens (14). An exception was observed with a subspecies-specific MAb, BB-3. BB-3 at a dilution of 1:400 reacted to serovars J and G, in addition to the expected F, K, B, Ba, L2, and L2a, as previously observed with the micro-IF test. Nevertheless, the combined results of inclusion reactivities to the 16 *C. trachomatis* type- and subspecies-specific MAbs did identify the 18 *C. trachomatis* serovars.

MAbs designed for serotyping of ocular trachoma isolates from areas of trachoma endemicity. The great majority of *C. trachomatis* organisms responsible for trachoma in areas of endemicity have been found to be serovars A, B, Ba, and C. Serovar Da also causes endemic trachoma (12). Six MAbs, KK-12 (*C. trachomatis* species specific), AA-3 (type specific to serovar A), BB-3 (specific to B, Ba, L2 and L2a), DD-1 (type specific to B), CC-13 (specific to C and J), and DP-2 (specific to Da) were selected for the test. The results of inclusion staining for ocular trachoma serovars are shown in Table 2.

MAb KK-12 reacted with all *C. trachomatis* strains. The absence of reaction indicates that the antigen preparation of that particular strain is not satisfactory for the typing test.

TABLE 2. Indirect FA staining of inclusion bodies with six MAbs designed for serotyping of ocular isolates from areas of trachoma endemicity

MAb	Reaction to <i>C. trachomatis</i> serovar				
	A	B	Ba	C	Da
KK-12	+	+	+	+	+
AA-3	+	—	—	—	—
BB-3	—	+	+	—	—
DD-1	—	+	—	—	—
CC-13	—	—	—	+	—
DP-2	—	—	—	—	+

This is usually due to an inadequate number of inclusions in the preparation. It could also mean that the strain being typed is not a *C. trachomatis* strain. A positive reaction to BB-3 and DD-1 indicates serovar B, a positive reaction to BB-3 but not to DD-1 indicates serovar Ba, a positive reaction to CC-13 indicates serovar C, and a positive reaction to DP-2 indicates serovar Da. Although BB-3 reacts to serovars B, Ba, L2, L2a, and possibly others and CC-13 reacts to serovars C and J, the serovars L2, L2a, J, and others are very unlikely to be causes of ocular trachoma in an area of endemicity. To determine the serovar of an isolate reacting with KK-12 but with none of the other MAbs by this method requires further studies using a complete set of *C. trachomatis* MAbs (Table 1).

MAbs designed for serotyping genital *C. trachomatis* isolates. The *C. trachomatis* organisms responsible for genital infections and infections in infants have been found to be the following serovars, which are listed in the order of their frequency: E, F, D, J, G, Ia/I, K, B/Ba, H, and Da (13). Serovars A and C, the most frequent isolates from trachoma in areas of endemicity, are virtually never isolated from sexually transmitted diseases. Among the LGV biovar strains, serovar L2 is the most common.

An approach similar to that used for endemic trachoma isolates requires 14 MAbs because of the increased number of "genital" serovars. LV-21 is used to assess the quality of the inclusion preparation. CC-1 reacts to serovars C and J, but an isolate of genital origin is almost certainly a J strain. LA-10 reacts only to serovar H, KK-1 reacts to K, FC-2 reacts to F, DD-1 reacts to B, EE-5 reacts to E, JG-9 reacts to D and possibly to Ba, DP-2 reacts to Da, and LV-27 reacts to L2. Both KB-8 and EE-5 react to E, but only KB-8 reacts to L1. GG-11 reacts to G and L3 and weakly to J, Ia, and H. PE-5 reacts to I and Ia; it is the only MAb that reacts to I. The cross-reactivity of GG-11 to J and Ia serves to differentiate J from C and Ia from I. BB-11 reacts to all B-complex serovars (B, Ba, E, D, Da, L1, L2, and L2a). With these 14 MAbs, isolates that react only to BB-11 and LV-21 are serovar Ba or L2a. Serovars L3 and L2a are rare in our experience, and LGV isolates are usually obvious from patient history and growth characteristics.

DISCUSSION

This study has shown that *C. trachomatis* inclusions used as antigens in indirect immunofluorescence staining can give a serovar-specific reaction if a specific MAb is used. A set of MAbs previously used for immunotyping the 18 *C. trachomatis* serovars in the micro-IF test were shown to give the same reactions with inclusions, allowing a serovar-typing test to be developed with inclusions.

Typing was successful with as little as 10% of cells infected. However, it is possible that this use of less antigen may make identification of dual *C. trachomatis* serovar infections less frequent. It is also true that retesting isolates for which results are unclear is more cumbersome with inclusion typing.

On the basis of the clinical disease and location being studied, immunotyping can be further simplified. This is especially true for endemic trachoma, for which six MAbs can identify all causative serovars.

We would not expect the inclusion-typing method to replace the micro-IF method in laboratories that routinely perform micro-IF tests or for reference purposes. However, it should make immunotyping accessible to many other laboratories that isolate chlamydia. While serotyping has been proven useful for epidemiological studies (1, 3, 4, 9, 11, 14), we believe that it is also useful for confirmation of the identity of isolates. Occasionally, inexperienced personnel can mistakenly identify artifacts as chlamydia. Further passage and serovar identification avoid any mistaken identity.

Utilizing cell culture inclusions as antigens with our MAbs but with a microtiter plate culture method, Suchland and Stamm have presented a preliminary report of successful immunotyping of 920 *C. trachomatis* isolates (8).

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