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Serovar Determination of *Chlamydia trachomatis* Isolates by Using Type-Specific Monoclonal Antibodies

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A panel of 15 monoclonal antibodies was prepared that could distinguish among the 15 serovars of *Chlamydia trachomatis*. Twelve of these antibodies were specific for a single serovar (A, B, C, D, E, F, G, H, I, K, L_1, and L_2) and three were specific for two serovars (B/Ba, C/J, and C/L_3). Ten of the serovar-specific and two of the bispecific antibodies were shown by immunoblotting to recognize epitopes on the major outer membrane protein. These data provide evidence that such epitopes are closely correlated with and may be partly responsible for the antigenic variations detected by microimmunofluorescence that distinguish the currently recognized serovars. When used in a radioimmunoassay, these antibodies correctly identified the serovar of 17 strains that had been serotyped by the microimmunofluorescence test. In addition, we found that the chlamydial antigen derived from 1.0 cm^2 of an infected HeLa cell monolayer was sufficient to allow serotyping with these antibodies. Thus, these monoclonal antibodies may provide a rapid and reliable alternative to mouse immunization and microimmunofluorescence for serotyping of clinical isolates.

Isolates of *Chlamydia trachomatis* have been grouped into 15 different serovars by a microimmunofluorescence (micro-IF) test, using murine antisera (17). Thirteen of these serovars (A to K and Ba) are classified within the trachoma biovar and generally cause mucosal infections of columnar epithelium resulting in trachoma, inclusion conjunctivitis, pneumonitis, and genital tract disease. The remaining three serovars (L_1, L_2, and L_3) are classified within the lymphogranuloma venereum biovar and produce a characteristic disease (lymphogranuloma venereum) of the lymph nodes. A third biovar, which consists of the mouse pneumonitis agent, is not known to cause human disease. Strains within the mouse biovar, as well as strains within the other species of *Chlamydiae*, *C. psittaci*, have not been well characterized in terms of their antigenic interrelationships.

Serovar determination of clinical isolates, or serotyping, can potentially provide important epidemiological information regarding human chlamydial infections. For example, there are few data documenting the serovar distribution of isolates in different parts of the world and even less information as to whether or not certain serovars are more likely than others to infect particular anatomical sites, such as the conjunctiva, urethra, endometrium, or pharynx. Also unknown is whether individuals who are infected at more than one site are infected with a single or multiple serovars. In addition, we do not know to what extent recurrent chlamydial infections result from relapse of latent or inapparent disease as opposed to reinfection. Another question is whether infection with a strain of any one serovar is more likely to produce protection against reinfection with either the same or different serovars.

Epidemiological studies that relate the infecting serovar to other clinical parameters, such as severity of disease, or body site infected, have been infrequent in spite of recent improvements in culture methods. This has primarily been due to the technical difficulties of serotyping by micro-IF. This method requires immunizing mice with the clinical isolate to produce an antiserum for evaluation of cross-reactivity by titration against the 15 reference serovar antigens (17) and often requires additional testing of the isolate with antisera raised against each of the 15 reference serovars (16).

To provide an alternative method for the serovar determination of clinical isolates, we have produced a panel of monoclonal antibodies that can readily distinguish each of the 15 known *C. trachomatis* serovars. Using these antibodies and a radioimmunoassay (RIA) format, we have been able to type clinical isolates by using antigen derived from as little as 1.0 cm^2 of an infected host cell monolayer. In this report we describe the methods used to create a panel of 12 serovar-specific and 3 bispecific monoclonal antibodies, their antigenic specificity for chlamydial proteins, and the evaluation of an RIA for serotyping, using a group of *C. trachomatis* isolates having known micro-IF serovar designations, and a small group of previously untyped clinical specimens.

MATERIALS AND METHODS

Chlamydiae. *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-6/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, and *C. psittaci* strain meningopneumonitis (MN)/CAL-10 were originally provided by C.-C. Kuo, University of Washington, Seattle. Strains G/392 and L_2/470/Bu were kindly provided by J. Schachter, University of California, San Francisco. Strains A/IOL-1590 and A/T-16 were provided by J. Treharne, London Institute of Ophthalmology, London, and strain H/CDC-21 was provided by R. Harrison, Centers for Disease Control, Atlanta, Ga. All other strains used in this study were derived from clinical specimens cultured in our laboratory and are designated as Indiana University (IU) isolates. Clinical isolates were first grown in vials and then passed to bottles as described by Kuo

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† The creative energy and kindness of Pertti Terho, recently deceased, will be missed.

et al. (8). Large-scale growth was with 150-cm^2 HeLa 229 cell monolayers, using 90% Eagle minimum essential medium–10% fetal calf serum (pH 7.3) supplemented with 4 mg of glucose, 1 μg of cycloheximide, 100 μg of streptomycin, and 100 μg of vancomycin per ml.

Elementary bodies (EBs) of each strain were purified by differential centrifugation followed by Percoll density gradient centrifugation (11). Purified EBs were either stored frozen at −70°C or fixed in 0.05% (vol/vol) Formalin 100 at a protein concentration of 0.2 mg/ml and stored at 4°C. Protein concentrations were estimated by fluorescence detection with *o*-phthalaldehyde (Fluoraldehyde; Pierce Chemical Co., Rockford, Ill.) (1), using bovine serum albumin as a standard after samples had been boiled for 1 min in 0.1% (wt/vol) sodium dodecyl sulfate (SDS).

Monoclonal antibodies. Murine monoclonal antibodies directed against chlamydia were produced essentially as described by Kohler and Milstein (7). Female BALB/c mice were immunized intraperitoneally with formalinized EBs (100 μg of protein) in complete Freund adjuvant. After 10 days the mice were given a booster injection in the same manner and sacrificed 3 days later. Single-cell suspensions of splenocytes were mixed with Sp2/0-Ag 14 cells at splenocyte/Sp2/0 ratios of 1:5, cosedimented, and fused by brief treatment with polyethylene glycol 1500. Hybridomas were selected with HAT (hypoxanthine-aminopterin-thymidine) medium, and culture fluids were screened against purified homologous EBs by solid-phase RIA. Cultures producing antibody were further screened by RIA against three or four EB preparations from heterologous serovars. Cultures producing antibody that did not cross-react with heterologous EBs were cloned in soft agar or by limiting dilution, rescreened by RIA, and established as ascites tumors in pristane-primed BALB/c mice.

RIA. Antigens were adsorbed to Immulon-2 (Dynatech Laboratories, Inc., Alexandria, Va.) strip wells in 100 μl of 0.5 M sodium carbonate (pH 9.6) for 16 h at room temperature. Percoll-purified EBs at 0.5 μg of protein per well were used to screen monoclonal antibodies and to evaluate a single antigen with multiple antibodies. Excess protein-binding sites in the wells were blocked by the addition of 300 μl of 4 mM KH_2PO_4–16 mM Na_2HPO_4–115 mM NaCl–0.05% (vol/vol) Tween 20–2% (vol/vol) fetal bovine serum (pH 7.3) (PBST-FBS) by incubation for 1 h at 37°C. After blocking, 100 μl of monoclonal antibody (diluted in PBST-FBS) was added per well and incubated for 2 h at 37°C. Nonbound antibody was removed by washing four times with PBST. Next, 100 μl of rabbit anti-mouse immunoglobulins (DAKO, Glostrup, Denmark) diluted 1:1,000 in PBST-FBS was added per well followed by incubation for 2 h at 37°C. After washing four times with PBST, 100 μl of PBST-FBS containing 75,000 cpm of [^{125}I]protein A was added per well with incubation for 2 h at 37°C. Wells were washed four times with PBST and counted individually for 1 min in a gamma counter. The working dilution for each antibody was determined by titration to optimize counts bound and specificity.

Immunoblotting. The details of the immunoblotting methods have been described previously (11). Briefly, proteins of purified EBs were solubilized by boiling in the presence of SDS and 2-mercaptoethanol, resolved by SDS-polyacrylamide gel electrophoresis on 12.5% polyacrylamide slab gels and electrophoretically transferred to a nitrocellulose membrane (NCM). After blocking excess protein-binding sites on the NCM with a solution containing Tween 20, individual 0.5-cm-wide strips of the NCM were probed with dilutions of ascites fluids containing monoclonal antibodies. The NCM strips contained 10 μg of protein for each of the different serovars. The antibody dilutions used were equivalent to the working dilutions used in the RIA. Bound immunoglobulin was detected by probing with ^{125}I-labeled protein A from *Staphylococcus aureus*, radiolabeled goat anti-mouse immunoglobulin G (γ chain specific), radiolabeled goat anti-mouse immunoglobulin M (μ chain specific), or rabbit anti-mouse immunoglobulins followed by radiolabeled protein A. After washing, the NCM strips were dried and used to expose Kodak X-OMAT AR film. Proteins on the NCM were visualized by staining with amido black 10B (11).

Micro-IF typing. Antisera against intact chlamydial EBs from laboratory strains or clinical isolates were produced by immunizing BALB/c mice intraperitoneally with 100 μg of protein of purified EBs that were fixed in 0.01% (vol/vol) formalin and emulsified in Freund complete adjuvant. Mice were injected with 100 μg of EB protein intravenously without adjuvant on day 7, and sera were harvested 3 days later. These sera were used to type clinical isolates as described by Wang et al. (17).

Monoclonal antibody typing. Fresh clinical isolates were grown in vial culture until >80% of the host cells contained chlamydial inclusions. When four or more of such heavily infected vials could be obtained for a single isolate, one of them was processed for serotyping while the others were harvested and stored at −70°C. For most isolates that were positive in primary culture, sufficient material for typing could be obtained after three to five passes in vial culture and rarely required eight or more passes.

For serotyping, the monolayer from one vial (1.0 cm^2) was removed into 1.0 ml of 0.5 M sodium carbonate (pH 9.6) and sonicated for 10 s, using a probe tip to completely disrupt the infected cells and release the chlamydial particles. Samples were then centrifuged for 10 min with a table-top microcentrifuge (Fisher Scientific Co., Cincinnati, Ohio) at about 13,000 × *g*. The pellets containing the chlamydia were suspended in 2.0 ml of 0.5 M sodium carbonate (pH 9.6) and dispersed by sonication for 10 s as before. Eighteen Immulon-2 strip wells were sensitized with 100 μl of chlamydia antigen per well, and the RIA was performed as described above where individual wells were treated with each of the 15 serovar-specific and bispecific monoclonal antibodies, buffer as a background control, and a genus-specific and *C. trachomatis* species-specific monoclonal antibody to verify the presence of sufficient chlamydial antigen.

RESULTS

Monoclonal antibody specificity. After screening multiple fusions from mice immunized with representative strains of each of the recognized *C. trachomatis* serovars, 15 monoclonal antibodies were selected as possible serotyping reagents on the basis of their limited cross-reactivities with heterologous serovars. These monoclonal antibodies were then evaluated by RIA for reactions with all of the different serovars (Fig. 1). Twelve of the antibodies reacted in a serovar-specific manner with the homologous EB antigen. The remaining three antibodies reacted with two different EB antigens: one with B and Ba, one with C and J, and another with C and L_3. Attempts to obtain serovar-specific monoclonal antibodies for Ba, J, and L_3 have been unsuccessful to date.

The serovar-specific reactivity of 12 of these monoclonal antibodies permits those 12 serovars to be identified unambiguously. The remaining three (Ba, J, and L_3) can be identified since these antigens react with one of the bispecific

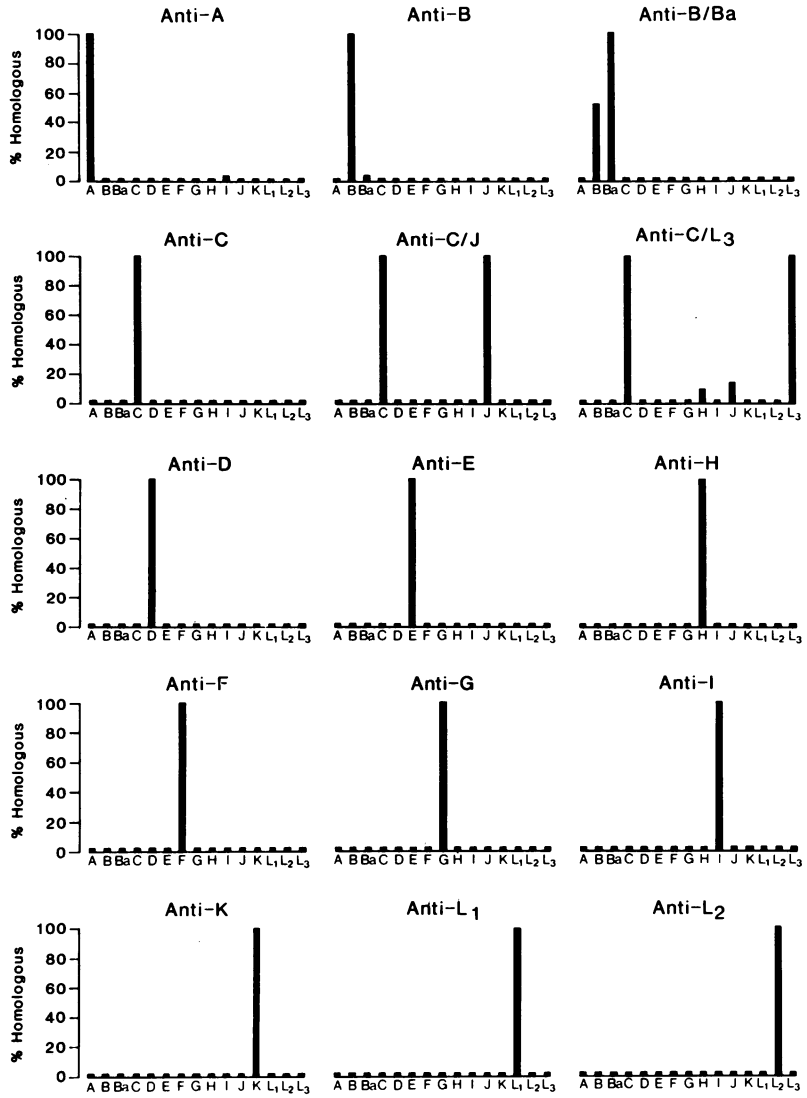

FIG. 1. After titration to establish the optimum signal to noise ratio, each monoclonal antibody was tested by RIA with purified EBs from each serovar. The counts bound to the heterologous antigens were expressed as a percentage of those bound to the homologous antigen.

antibodies (B/Ba, C/J, or C/L_3) but not with the serovar-specific antibodies for either B or C. For example, the Ba antigen reacts with the B/Ba but not the B-specific antibody, J reacts with the C/J but not the C-specific antibody, and L_3 reacts with the C/L_3 but not the C-specific antibody. The C/L_3 antibody also cross-reacts weakly with H and J. Potential confusion in typing these latter serovars was eliminated by measuring the reactivity of the test strain relative to that of standardized amounts of the homologous strains. Thus, for a J strain the relative reactivity compared with the reference C strain will be much higher with the C/J antibody than with the C/L_3 antibody. Similarly, an H strain will have a higher reactivity with the H antibody relative to the reference H strain than with the C/L_3 antibody relative to the standardized C and L_3 reference strains.

To determine whether the epitopes recognized by these antibodies are located on chlamydial proteins, each of the monoclonal antibodies was evaluated by immunoblotting, using equivalent amounts of homologous EB antigens (Fig. 2). The protein specificity of each antibody was determined by associating bound radioactivity with amido black-stained proteins on individual NCM strips. All except the H, I, and C/J-specific antibodies gave reactions with this method. For each, these reactions were with the major outer membrane protein (MOMP) of the homologous strain. Retesting of the nonreactive antibodies at lower dilutions also gave negative results. The absence of any reaction with the H, I, and C/J antibodies suggests that the epitopes recognized by these antibodies are sensitive to heat, SDS, or reduction, are not resolved by SDS-polyacrylamide gel electrophoresis, or do not transfer to or adsorb to the NCM. The B/Ba antibody reacted with the MOMPs of both the B and Ba strains (Ba is shown), and the C/L_3 antibody likewise reacted with the MOMPs of both the C and L_3 strains (L_3 is shown). Faint reactions were consistently observed with proteins below MOMP (about 30,000 daltons [30K]) for the D- and C/L_3-specific antibodies and may reflect some minor degradation of MOMP. In addition, the C/L_3 antibody reacted with a band just below the MOMP. This antigen may represent an internally disulfide-cross-linked form of monomeric MOMP (5).

Serovar determination. The panel of monoclonal antibod-

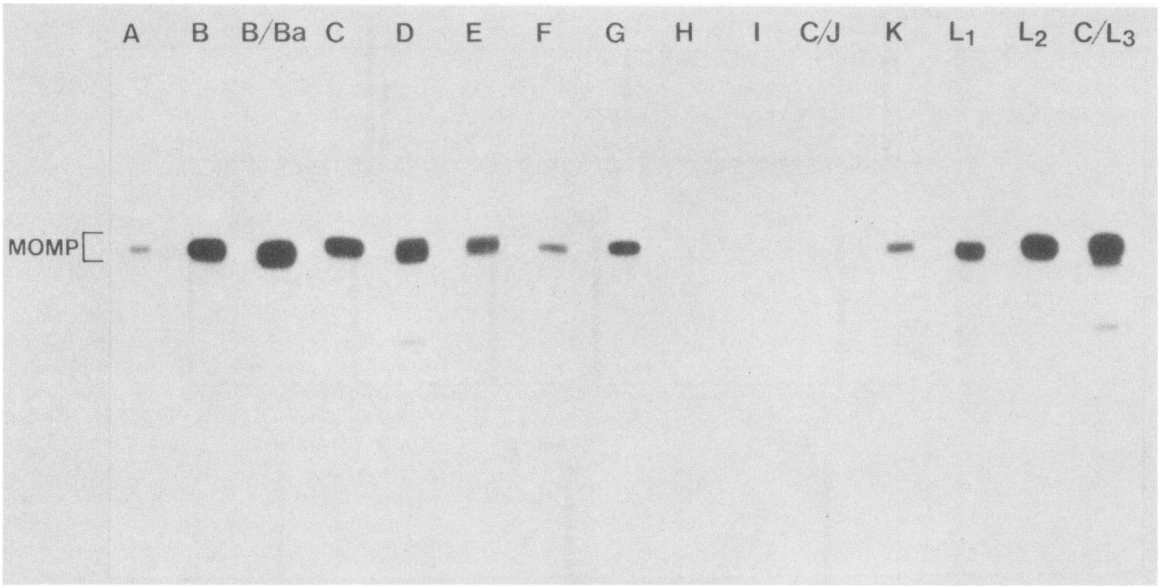

FIG. 2. Autoradiogram of 15 individual NCM strips, each representing a different serovar, after incubation with homologous monoclonal antibody and radiolabeled probe. The general location of the MOMPs is indicated by a bracket. The letters at the top indicate the specificity of the monoclonal antibody.

ies was used in an RIA to evaluate a group of 17 additional laboratory strains and clinical isolates that had been typed by the micro-IF test. Each of the isolates was grown in quantity, and purified EBs were used as antigens. The reactions of the 15 monoclonal antibodies with these antigens are expressed qualitatively in Table 1. Fifteen of the strains reacted strongly with only a single antibody; the remaining two reacted with both the B- and B/Ba-specific antibodies. The latter reactions indicate a B antigen and are consistent with the micro-IF typing of these strains. For each of the strains tested there was perfect agreement between typing by the micro-IF test and typing by the panel of monoclonal antibodies.

Serotyping clinical isolates. The feasibility of serotyping clinical isolates without large-scale growth and purification was assessed by using antigen from a single well-infected vial monolayer to sensitize wells in the RIA. This method was applied to a group of 16 chlamydia genital isolates. With the monoclonal antibodies, all 16 isolates typed unambiguously: 5-D, 4-E, 6-F, and 1-I. Although the sample size is small, this distribution is consistent with that previously observed for genital isolates (9). Thus, these data provide an example of the potential utility of this kind of rapid serotyping assay.

DISCUSSION

To provide a reproducible and universally applicable method of typing clinical isolates of *C. trachomatis*, we have developed a panel of 12 serovar-specific and 3 bispecific monoclonal antibodies that can distinguish each of the currently recognized serovars.

Monoclonal antibodies that recognize serovar-specific epitopes of *C. trachomatis* have been described by several investigators. Matikainen and Terho (10) identified a monoclonal antibody that is specific for the MOMP of L_1 serovar strains, and Caldwell and Hitchcock (2) have identified an L_2 MOMP-specific monoclonal antibody. Serovar-specific monoclonal antibodies recognizing A, B, C, D, F, and L_2 have been identified by Stephens and co-workers (12–14). The antigen(s) recognized by these latter antibodies could not be identified by immunoblotting but some were found to be heat stable, periodate resistant, and pronase sensitive, suggesting a protein antigen. In addition, two monoclonal antibodies specific for serovar A have been described by Clark et al. (4). Although the reactive antigens were not identified, both appeared to react with surface-exposed epitopes when viewed by immune electron microscopy.

In the process of screening numerous hybridomas for antichlamydial antibodies, we have identified genus, species (*C. trachomatis*), and subspecies reactivities (B. E. Batteiger, P. Terho, W. J. Newhall, C. E. Wilde, and R. B. Jones, manuscript in preparation). Fifteen antibodies were identified that were specific for one or two different serovars. Twelve of these antibodies recognized epitopes on the MOMP, while the antigen specificity of the other three remains unknown. These data provide conclusive evidence for the presence of serovar-specific epitopes on most MOMPs and strengthens the hypothesis that the MOMPs are the predominant antigens responsible for the serovar specificity observed in the micro-IF test (3, 14). The finding of so many MOMP epitopes that are expressed both on purified organisms and on a protein that has presumably lost much of its tertiary and secondary structure by heating in SDS suggests that these epitopes may be determined by amino acid sequence. However, we cannot rule out the possibility that these epitopes are due to protein conformation since some of the protein on the nitrocellulose membrane may have renatured after transfer. Possible explanations for the nonreactivity of the H-, I-, and C/J-specific antibodies by immunblot are that the reactive antigens are conformational MOMP epitopes that do not renature or are amino acid sequences that became buried during denaturation in SDS. Alternatively, the epitopes recognized by these antibodies could be present on other proteins and are heat or SDS labile, or are on macromolecules that do not resolve by SDS-polyacrylamide gel electrophoresis or are not present

TABLE 1. Monoclonal antibody reactions with *C. trachomatis* isolates previously serotyped by micro-IF

Serotype/strain	Monoclonal antibody reactivity[a]														
	A	B	B/Ba	C	D	E	F	G	H	I	C/J	K	L_1	L_2	C/L_3
A/G17	+++ +++ +++	−	−	+	−	−	−	−	−	−	−	−	−	−	+
A/IOL-1590	+++ +++ +++	−	−	−	−	−	−	−	−	−	−	−	−	+	−
B/IU-B107	−	+++ +++ +++	+ +++ +	−	−	−	−	−	−	−	−	−	−	−	−
B/IU-B123	−	+++ +++ +++	+ +++ +	−	−	−	+	−	−	−	−	−	−	−	−
D/IU-L253	−	−	−	−	+++ +++ +++	−	−	−	−	−	−	−	−	−	−
E/IU-S51	−	−	−	−	−	+++ +++ +++	−	−	−	−	−	−	−	−	−
E/IU-S54	−	−	−	−	−	+++ +++ +++	−	−	−	−	−	−	−	−	−
F/UW-94	−	−	−	−	−	−	+++ +++ +++	−	−	−	−	−	−	+	−
F/IU-527	−	−	−	−	−	−	+++ +++ +++	−	−	−	−	−	−	+	−
G/392	−	−	−	−	−	−	−	+++ +++ +++	−	−	−	−	−	−	−
H/CDC-21	−	−	−	−	−	−	−	−	+++ +++ +++	−	−	−	−	−	+
H/IU-B51	−	−	−	−	−	−	−	−	+++ +++ +++	−	−	−	−	−	+
J/IU-B24	−	−	−	−	−	−	−	−	−	−	+++ +++ +++	−	−	−	+
L_2/470	−	−	−	−	−	−	−	−	−	−	−	−	−	+++ +++ +++	−
L_2/IU-229	−	−	−	−	−	−	−	−	−	−	−	−	−	+++ +++ +++	−
L_2/IU–233	−	−	−	−	−	−	−	−	−	−	−	−	−	+++ +++ +++	−
L_2/IU–239	−	−	−	−	−	−	−	−	−	−	−	−	−	+++ +++ +++	−

[a] Each antibody reaction with the test strain was related to that with the homologous prototype strain as a ratio. The antibody giving the highest ratio was assigned nine +'s. The reaction ratios of the other antibodies were then expressed as a percentage of the highest ratio, with one + being equal to about 11%.

on the nitrocellulose. Further studies will be required to distinguish among these possibilities.

Interestingly, two of the antibodies (D and C/L_3) reacted with MOMP, but also with a 30K component. This reaction could represent proteolytic breakdown of MOMP or may indicate the presence of another protein that shares these serotype-specific epitopes with MOMP. Based on its molecular mass, this 30K band may be analogous to the type-specific antigen isolated by Hourihan and co-workers (6).

Two sets of serovars that are classically difficult to distin-

guish by micro-IF are C/J and B/Ba. The identification of monoclonal antibodies that specifically recognize each of these sets indicates that these micro-IF relationships are likely due to the sharing of specific epitopes. The identification of a MOMP epitope shared by the C and L_3 serovars, in contrast to the others, defines a specific relationship that had not been characterized earlier. However, such a relationship is not entirely unexpected since L_3 has been shown to bridge the B and C complexes antigenically (16). Thus, the C/L_3 epitope described here may represent part of the molecular basis for this bridge. Undoubtedly as more antibodies are produced, many additional relationships among the different serovars will be observed.

As a first step in the evaluation of these 15 monoclonal antibodies for serotyping, we screened by RIA a small number of isolates from diverse sources that had been typed previously by the micro-IF test. The monoclonal antibody method correctly identified the serovar of each strain. Thus, these antibodies appear capable of providing reliable serotyping results. For such an assay to be useful for serotyping the large number of clinical isolates encountered in various epidemiological studies, it must be able to use a small amount of antigen. We found that, after minor processing, the antigen from a single culture vial was sufficient to obtain typing information.

These results are highly encouraging; however, the possibility exists that there are more than 15 *C. trachomatis* serovars or that there are as yet unrecognized subtypes. Such strains may not be typable with the monoclonal antibodies we are currently using due to the precise specificities of such antibodies. However, the identification of such strains would direct the development of additional reagents and should lead to a sharper definition of the antigenic relationships among the different isolates of *C. trachomatis*. Additional studies are currently under way to validate further the use of these antibodies for serotyping purposes. If these studies confirm the reliability of this method, this panel of antibodies should provide a powerful tool for typing of clinical isolates and may find applications in a wide variety of epidemiological studies.

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