Formalinized *Chlamydia trachomatis* Organisms as Antigen in the Micro-Immunofluorescence Test

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*Chlamydia trachomatis* organisms grown in HeLa 229 cell cultures were purified and formalinized for use in the micro-immunofluorescence test. As test antigens, they were stable when stored unfrozen at 4°C for a long period of time without loss of type specificity and sensitivity.

Laboratory techniques for the diagnosis of *Chlamydia trachomatis* infection are in increasing demand. An effective serological test is desirable in addition to the isolation of the organisms by an appropriate method of cell culture. The serological test should be sensitive and specific. The micro-immunofluorescence (micro-IF) test meets these criteria (1). We have worked to simplify the test procedure to increase its practicability for serodiagnosis (13). The micro-IF test has not been widely used because of the difficulty in preparation of test antigens. The many immunotypes must be obtained and grown in cell culture or embryonated eggs. It has been considered necessary to use frozen live organisms for antigens. This discourages serological testing in most clinical laboratories. The use of a single easily grown strain of antigen (5) seriously compromises the sensitivity and specificity of the test. Pooling antigens (6, 13) does not reduce the problems of antigen preparation.

In studies of immunological classification of *Neisseria gonorrhoeae* by the same technique (14), it was observed that the Formalin treatment of organisms stabilized their immunotype specificity. The formalinized *Neisseria* were stable when stored at refrigerator temperature for many months. Encouraged by these findings, we examined some of our old stock of trachoma vaccines which were prepared in 1966 for purposes of animal immunization (8, 11). After various purification procedures, they had been formalinized and stored unfrozen at 4°C for more than 12 years. We were surprised to find that these formalinized vaccines were still reactive with excellent fluorescence and type specificity in the micro-IF test.

Cell cultures have generally replaced chicken embryo yolk sac cultures for isolation and growth of *C. trachomatis* organisms. HeLa 229 cell culture is particularly useful for this purpose (3). In this report we describe the procedures for the preparation of formalinized elementary body antigens from HeLa 229 cell culture-grown *C. trachomatis* organisms and the stability and quality of such antigens in terms of specificity and sensitivity in the micro-IF test. It is hoped that this will contribute to the further simplification of the chlamydial serology.

The *C. trachomatis* strains used include representative strains of each immunotype: 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-43/Cx, I/UW-12/Ur, J/UW-36/Cx, K/UW-31/Cx, L₀/440/Bu, L₀/434/Bu, and L₀/404/Bu. Most of these strains have been described in our previous reports (4, 9, 10, 12). Each strain has been successfully adapted to infect more than 80% of HeLa 229 monolayer cells (without centrifugation for infectivity enhancement) in a 32-ounce (ca. 950-ml) bottle culture (3).

The organisms are grown for 72 h (and therefore mostly at the elementary body stage) in 32-ounce bottles of HeLa 229 cell culture. When more than 80% of the cells are found to have inclusion bodies by direct visualization under the inverted microscope, they are harvested and suspended in phosphate-buffered saline, pH 7.0 (PBS). This is sonicated for 20 s (Biosonik III Sonicator, Bronwill Scientific, Rochester, N. Y.) and submitted to one cycle of differential centrifugation (500 × g for 10 min and 30,000 × g for 20 min). The resulting pellet is suspended in PBS at 10 ml per culture bottle. This suspension is sonicated before further purification by centrifugation (20,000 × g for 40 min) through a layer of 10 ml of 30% Renografin (Squibb & Sons, Inc., Princeton, N.J.). The organisms in the resulting pellet are washed with PBS and centrifuged (30,000 × g for 20 min) to remove the renografin. The pellet, containing relatively

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purified organisms, is then resuspended in PBS containing 0.02% final concentration of freshly prepared Formalin (Formalin–PBS) at 2 ml/bottle. A homogeneous suspension of the organisms is achieved by sonication for a few seconds. The density of the organisms at this step has previously been shown (3) by electron microscopy (8) to be approximately 5 x 10^8 organisms per ml.

To be used as test antigens in the micro-IF test, normal yolk sac (NYS) suspension in Formalin–PBS is added to make the final concentration of NYS 2 to 5%. The purpose of adding NYS is to facilitate the adhesion of the organisms on microscope slide. The optimum density of organisms to be used in the micro-IF test is about 10^9 organisms per ml after the addition of NYS. The formalinized antigens are stored unfrozen at 4°C. After addition of NYS the storage at 4°C is restricted to 2 to 3 weeks.

Table 1 shows the results of comparative reactivities of Formalin-treated and nontreated antigens in the micro-IF tests with mouse antisera and trachoma patient sera. The antigen preparations from the same strain (either TW-3 or TW-5) were derived from the same culture bottles, different only in Formalin treatment. The mouse antisera were prepared by intravenous immunization (7), and the patient sera were derived from a trachoma family study in Taiwan (2), from patients whose eye infections were proven by isolation of the organisms. As test antigens, all preparations from both TW-3 (type C) and TW-5 (type B) reacted equally well to homologous type antibodies, but were not reactive to heterologous antibodies. There was no difference in terms of specificity or sensitivity between Formalin-treated and nontreated antigens.

In a separate experiment (data not shown in table), different concentrations of Formalin (0.2, 0.1, 0.05, 0.025, and 0.0125%) were tested for their effect on TW-3 and TW-5 test antigens. No appreciable difference could be seen in the reactivities of the treated antigens during storage at 4°C in sequential follow-ups for up to 9 months.

To assess the reactivity of Formalin-treated HeLa-grown antigens in the micro-IF test after storage for a length of time at 4°C, the stored antigens were compared with our conventional standard antigens (9, 12). Table 2 shows examples of such comparisons. UW-36 (type J), UW-5 (type E), and UW-3 (type D) antigens were prepared from HeLa-grown organisms, formalinized as described, and stored unfrozen for 6 months at 4°C. The control antigens used were prepared from the highly infected yolk sacs of the same strains. A 2 to 3% infected yolk sac suspension was distributed in vials and frozen at ~65°C for 4 years. They were thawed just before use. All the antigen preparations were tested simultaneously in the micro-IF test with homologous and heterologous mouse antisera. Mouse antiserum to UW-209 is a type D antiserum. As would be expected, there were extensive cross-reactions between antigens and antisera of types E and D. There was no difference in terms of sensitivity or specificity between the different elementary body antigen preparations from the

**Table 1. Comparison of reactions in the micro-IF test of Formalin-treated and nontreated antigens with mouse antisera and trachoma patient sera**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>C/TW-3</th>
<th>B/TW-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse antisera</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Anti-TW-3</td>
<td>128</td>
<td>0</td>
</tr>
<tr>
<td>Anti-TW-5</td>
<td>0</td>
<td>128</td>
</tr>
<tr>
<td>Patient sera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>YB-10</td>
<td>128</td>
<td>0</td>
</tr>
<tr>
<td>YB-29</td>
<td>128</td>
<td>0</td>
</tr>
<tr>
<td>YB-31</td>
<td>0</td>
<td>128</td>
</tr>
<tr>
<td>YB-38</td>
<td>0</td>
<td>128</td>
</tr>
</tbody>
</table>

* Both antigens were grown in HeLa 229 cells.
* +, Formalin treated; -, untreated.
* 0 = <8.

**Table 2. Comparison of reactions in the micro-IF test of Formalin-treated and nontreated antigens after storage**

<table>
<thead>
<tr>
<th>Mouse antiserum</th>
<th>J/UW-36</th>
<th>E/UW-5</th>
<th>D/UW-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-J/UW-36</td>
<td>128</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Anti-E/UW-5</td>
<td>0</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>Anti-D/UW-209</td>
<td>0</td>
<td>64</td>
<td>128</td>
</tr>
</tbody>
</table>

* +, Test antigen was prepared from growth in HeLa 229, formalinized, and stored at 4°C for 6 months.
* -, Test antigen was prepared from growth in egg yolk sac, was not formalinized, and was stored frozen at ~65°C for 4 years.
* 0, Titer < 8.
same strain. As test antigens, the preparations of formalinized antigens showed a more homogeneous density of elementary bodies.

Antigen preparations have been made for each of the 15 immunotypes, formalinized, and stored at 4°C for at least 10 months in our laboratory. They have been successfully used for immunotyping of new strains and for detection of type-specific antibodies in serum and secretions including, by specific immunoglobulin classes, immunoglobulin M, immunoglobulin G, and secretory immunoglobulin A.

The preparation of formalinized antigens from HeLa 229-grown organisms further simplifies the C. trachomatis micro-IF test. The ability to successfully stabilize the type-specific surface antigens with Formalin without loss of specificity or sensitivity provides the opportunity for reference laboratory or commercial distribution of the antigens. Provision of micro-IF antigen will greatly increase the availability of laboratory diagnosis of C. trachomatis infection. The use of formalinized organisms also eliminates the hazard of laboratory infection.

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LITERATURE CITED