Identification of the Elementary Bodies of *Chlamydia trachomatis* in the Electron Microscope by an Indirect Immunoferritin Technique

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An indirect immunoferritin (IIF) technique is described for recognizing the elementary bodies (EB) of *Chlamydia trachomatis* in unsectioned preparations. Both the EB of a genital strain of *C. trachomatis* grown in irradiated McCoy cells and EB in clinical specimens obtained from patients attending a venereal disease clinic were identified by the IIF test in the electron microscope. Cell culture-grown EB were detected by ferritin staining for up to 4 weeks after the organisms had lost their infectivity for tissue cultures. The IIF test was of comparable sensitivity to isolation methods in detecting chlamydiae in clinical specimens. Other possible applications of the IIF technique are discussed.

Identification of virus particles in clinical material by negative staining and electron microscopy (EM) is a well recognized method in the diagnosis of certain viral infections, where the size and morphology of the virus is sufficiently distinctive, and when sufficient virus particles are present (5). Immunological techniques have also been combined with EM to identify viruses or viral components in clinical specimens; for instance, both hepatitis B antigen in serum and parvoviruses in feces can be visualized by clumping the particles with antiserum (1, 10).

Ferritin-conjugated globulins have been used to detect particular antigens by EM of thin sections of cells (13, 17). The electron-dense ferritin molecules allow antigen-antibody reactions to be identified in the electron microscope in a similar way to that in which globulins conjugated with fluorescent dyes detect these reactions by fluorescence microscopy. In the indirect immunoferritin (IIF) test (4), fixed thin sections of cells are treated with antiserum against the antigen under study and are subsequently treated with ferritin-conjugated globulin directed against the animal species from which the antiserum was obtained.

Chlamydia trachomatis is bacterial rather than viral in origin (9), but since these organisms are obligate intracellular parasites, virological techniques are needed to propagate and study them. The organisms undergo a complex developmental cycle within the cytoplasm of infected host cells, which results in the production of a population of extracellular particles known as elementary bodies (EB). EM studies of thin sections of cells infected with *C. trachom-*

atis have established that the EB consist of spherical particles with a mean diameter of about 300 nm. An electron-dense core, the nucleoid, is separated from the well-defined limiting membrane by a clear zone (2).

The present study describes the recognition of EB of *C. trachomatis* by an indirect IIF test on unsectioned preparations of EB. They have been identified (i) in the supernatant from irradiated McCoy cell cultures infected with a genital strain of *C. trachomatis*, and (ii) in clinical material obtained from the male and female genital tract. The IIF technique provides a rapid test for diagnosing chlamydial infections which is of a comparable sensitivity to isolation methods currently in use.

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MATERIALS AND METHODS

Tissue culture-grown EB. Irradiated McCoy cell cultures (11) were inoculated with a pool of T181, a trachoma-inclusion conjunctivitis E serotype of *C. trachomatis* (15) at a dilution calculated to yield about 1,000 inclusions/cover slip. Cultures were centrifuged at $2,500 \times g$ and incubated at 35 C for 68 h. The cells and supernatant from each culture were then frozen and stored at -70 C until they were processed for EM.

Clinical specimens. Specimens were obtained from patients attending venereal disease clinics. Urethral swabs from men and cervical swabs from women were each placed in 2 ml of sorbitol transport medium (11) and kept at 4 C. On arrival at the laboratory, 0.5 ml was removed from each specimen

and stored at -70°C until examined by EM. The remainder of each specimen was used to attempt isolation of chlamydiae.

Isolation of chlamydiae. Isolation was attempted in both irradiated McCoy cells and McCoy cells treated with cytochalasin B. Details of these isolation procedures have already been described (11, 15).

Antiserum. The antiserum, R367, was provided by L. H. Collier, Lister Institute of Preventive Medicine, London, U. K. It was produced by immunizing a rabbit with a *C. trachomatis* strain MRC 4s propagated in BHK-21 cells (16). Strain MRC 4s (formerly described as LB4) is a trachoma-inclusion conjunctivitis serotype E agent (19). In this laboratory, this serum was found to have a chlamydial antibody titer of 64, tested both by the complement fixation test and by an indirect, single-antigen immunofluorescence (IF) test, in which the inclusions of T181 were used as antigen (12). The similar titers obtained by immunofluorescence and complement fixation tests indicate that these antibodies were directed against the chlamydial group antigen (12). The antiserum was used at 0.1, diluted in complement fixation test diluent (Oxoid Ltd., London, U. K.).

Ferritin-conjugated globulin. Ferritin-conjugated goat anti-rabbit globulin (ferritin conjugate) (Gibco Bio-cult Laboratories Ltd., Paisley, Scotland) was diluted 0.04 in complement fixation test diluent. This stock solution was stored for up to 14 months at 4°C without reduction in its activity. Preliminary experiments showed that diluting the ferritin conjugate in very weak detergent reduced the background ferritin staining; therefore, immediately before use a small volume of the stock solution was diluted in 0.004 Teepol L to bring the final concentration of the ferritin conjugate to 0.08 diluted in 0.008 Teepol L.

Negative staining of the tissue culture-grown EB. The cells and supernatant from chlamydia-infected irradiated McCoy cell cultures stored at -70°C (see above) were frozen and thawed twice to rupture the cells and release intracellular EB into the supernatant. Each culture was made up to 2 ml with sterile phosphate-buffered saline, pH 7.2, and then filtered through a $0.8\text{-}\mu\text{m}$ membrane filter (Millipore Corp.) with the use of a Sweeney filter holder. The filtrate was centrifuged at $49,000 \times g$ for 20 min on a Sorvall RC 2B centrifuge at 4°C . The supernatant was removed and the pelleted material was washed twice by resuspending the pellet in 2 ml of sterile phosphate-buffered saline and then repeating the centrifugation procedure.

The final pellet was resuspended in about 0.1 ml of phosphate-buffered saline and a drop of this suspension was placed on dental wax. A Formvar-coated grid was floated on the drop and left for 6 min at room temperature. The grid was then removed and the excess moisture was blotted off, but the grid was not allowed to dry. It was washed in a drop of distilled water placed on dental wax, the excess water was blotted off, and the grid was then floated on a drop of phosphotungstic acid (0.1%, pH 6.5). After 30 s in this stain at room temperature the grid was blotted dry and examined at $\times 25,000$ in an AEI EM801 electron microscope.

One chlamydia-infected McCoy cell culture provided sufficient material for at least eight grids.

Preparation of material for the IIF test. Each clinical specimen and the cells and supernatant from the chlamydia-infected irradiated McCoy cell cultures were processed, as described above for negative staining of tissue culture-grown EB, by filtering, centrifuging the filtrate at $49,000 \times g$ and washing the pellet twice in sterile phosphate-buffered saline. The final pellet was resuspended in 2 ml of absolute methanol to fix the EB and the centrifugation procedure was again repeated. The deposited material was resuspended in glass-distilled water; one drop was used to resuspend the clinical material and 0.1 ml to resuspend the cell culture preparation. One drop of resuspended material was applied to a Formvar grid and allowed to air dry; the grid was then washed under a gentle stream of glass-distilled water for 30 s and excess fluid was then blotted off.

Only one grid was prepared from each clinical specimen.

The IIF test. Each grid was floated on a drop of antiserum (diluted 0.1) placed on dental wax and incubated for 2 h at 37°C in a moist chamber. The grid was then washed well for 30 s under running glass-distilled water, blotted but not allowed to dry, and then floated on a drop of ferritin conjugate placed on dental wax and incubated for 25 min at 37°C in a moist chamber. The grid was then washed thoroughly again in running glass-distilled water, blotted dry, and examined at $\times 25,000$ in the AEI EM801 electron microscope. A particle was defined as ferritin stained when there was an obvious difference between the amount of ferritin attached over the whole surface of the particle compared with the amount of background ferritin attachment.

Grids from all clinical specimens were each scanned until a ferritin-stained particle between 200 to 500 nm in diameter was found (positive specimen) or for 25 min if no ferritin-stained particles were found (negative specimen). This scanning procedure was designated EM scan 1. The grids were then recoded and each was scanned as before (EM scan 2). Both EM scans were carried out with no knowledge of the isolation results on these specimens.

RESULTS

Tissue culture-grown EB. The grids prepared from chlamydia-infected irradiated McCoy cell cultures which were negatively stained contained numerous particles between 200 and 500 nm in diameter with a dense central nucleoid surrounded by a clear zone and bounded by a well-defined limiting envelope (Fig. 1). These particles were similar in size and morphology to the EB described in thin sections of cells infected with *C. trachomatis* (2). Since such particles were not seen in grids prepared from uninfected irradiated McCoy cell cultures it was felt they were probably the EB of T181.

Ferritin staining of such particles was achieved when the IIF test was carried out,

FIG. 1. Negatively stained elementary body of *C. trachomatis* from irradiated McCoy cell culture. The bar in the electron micrographs represents 100 nm.

provided that the EB were fixed in methanol before treatment with antiserum (Fig. 2). Such fixation disrupted the outer envelope (Fig. 2 and 3). If the material was not fixed, or if milder fixatives such as glutaraldehyde were used, the particles were not disrupted to such an extent and ferritin staining did not occur. When a control rabbit serum was used in the IIF test instead of the specific chlamydial antiserum R367, ferritin staining of methanol-fixed particles was not achieved (Fig. 3). It was felt therefore that the IIF test provided a specific immune EM technique for identifying EB.

When the cells and supernatant from several irradiated McCoy cell cultures, heavily infected with T181, were harvested and pooled after 68 h of incubation and then left at room temperature, viability of the pool declined rapidly. Chlamydiae could not be reisolated from this material 5 days or more after the cultures were harvested. However, EB were identified by the IIF technique in samples taken from the pool for as long as 36 days after harvesting. It appears, therefore, that EB can be identified in the IIF test when they are no longer viable in tissue culture.

Clinical specimens. Forty-five clinical specimens were examined by both the IIF test and by isolation techniques. One specimen was toxic to the McCoy cell cultures and an inadequate preparation for EM was obtained from another specimen. Valid comparisons were therefore possible on 43 specimens: chlamydiae were isolated from 16 (37%) of these specimens

and 13 (30%) positive specimens were recognized in the IIF test; the difference between these two results is not statistically significant. The findings in EM scan 1, EM scan 2, and the overall EM results were all compared with the isolation results and are shown in Table 1. (The overall EM results were obtained by counting any specimen positive that was found positive in either of the EM scans, as well as specimens found positive in both scans.)

FIG. 2. Elementary body of *C. trachomatis* fixed in methanol and ferritin stained by IIF technique.

FIG. 3. Elementary body of *C. trachomatis* fixed in methanol showing disruption of outer envelope due to fixation, and absence of ferritin staining when control serum was used in IIF test.

TABLE 1. Recognition of *C. trachomatis* in clinical specimens: comparison of results obtained in the IIF test with results obtained by isolation of chlamydiae in McCoy cell cultures

EM scan	No. of clinical specimens				% Match ^b	P
	Positive in IIF test		Negative in IIF test			
	Chlamy- diae isolated ^a	Chlamy- diae not isolated	Chlamy- diae not isolated	Chlamy- diae isolated		
1	9	2	25	7	79	<0.01
2	10	2	25	6	81	<0.001
Overall results	11	2	25	5	84	<0.001

^a In one specimen chlamydiae were isolated only in McCoy cells treated with cytochalasin B. In all other specimens chlamydiae were isolated in both irradiated McCoy cells and McCoy cells treated with cytochalasin B.

^b Percentage of co-positive and co-negative specimens.

The strength of association between the IIF test results and the results obtained in tissue culture was expressed as the percentage of specimens that were positive or negative in both systems (percentage match). The statistical significance of this association, expressed by the probability, was demonstrated by calculating the chi-squared (χ^2) value on these results. There was a good percentage match and a highly significant association between the IIF test results and the isolation results (Table 1). As a method of diagnosing chlamydial infections, therefore, the IIF test compared well with the isolation methods. Also, the results obtained by scanning the grids were reproducible, since there was a 93% match between the results of the two EM scans, and this association was highly significant ($P < 0.001$).

DISCUSSION

To demonstrate specific ferritin-staining by the IIF technique, the level of background staining must be low. In practice, this has been difficult to achieve when the technique has been carried out on other antigens in fixed thin sections of cells (8, 14). However, in this work, where the IIF test was carried out on unsectioned preparations of chlamydial EB, background staining was reduced by diluting the ferritin conjugate in very dilute detergent: ferritin conjugate diluted in 0.008 Teepol L markedly reduced background ferritin staining, probably by reducing the surface tension of the ferritin conjugate so that nonspecific adherence to the Formvar grid was minimal. Specific ferritin staining of *C. trachomatis* EB could then be demonstrated with the IIF technique, both on tissue culture-grown EB and on EB in clinical specimens obtained from the human genital tract.

The IIF test compared well with the methods

of detecting chlamydiae by isolation in irradiated McCoy cells and in McCoy cells treated with cytochalasin B, and in practice this test should be of particular value in two situations: first, in providing a rapid diagnosis of chlamydial infections. Up to 10 specimens could conveniently be processed for EM at a time, and the preparatory procedures (excluding scanning time) were carried out in about 4 h. A result can be obtained therefore within 1 day of receiving a specimen, whereas results from culturing organisms are obtained at the earliest after 2 to 3 days. Second, the EB in some clinical specimens (for instance, those delayed in transit to the laboratory) may no longer be viable, and to test these the EM method would be preferable to isolation techniques, since the IIF test detected tissue culture-grown EB for up to 4 weeks after they were no longer infectious in tissue culture.

The IIF test may also yield information on chlamydial antigen-antibody reactions: the test described in this work used group-specific antibody, and ferritin staining was achieved only after the EB were treated with a fixative which disrupted the cell wall. This argues that the group antigen is not situated on the surface of the elementary body. This observation does not conflict with studies by other workers on the location of the chlamydial group antigen: silver-methenamine staining of thin sections of EB of *C. psittaci* suggested the group antigen was situated at the periphery of the EB, particularly on the inner layer of the limiting membrane (6).

If ferritin staining of EB can be achieved with type-specific chlamydial antibodies, as well as with group antibody, more information should be obtained on the type-specific chlamydial antigens. Type-specific antibodies which neutralize the infectivity of trachoma orga-

nisms for the owl monkey eye have been demonstrated in pooled plasma and eye secretions from patients with trachoma (3). These antibodies are presumably directed against surface antigens. Immunofluorescent tests have also demonstrated type-specific activity in acetone-fixed preparations of EB (7). The IIF test, carried out with type-specific sera on fixed and unfixed preparations of EB, may yield more information on the site and nature of these type-specific antigens.

In addition, if ferritin staining of EB can be achieved with type-specific sera, the IIF test may provide a quick alternative method to the micro-immunofluorescence test (18), the method currently in use for distinguishing between the different immunological types of *C. trachomatis*.

Work on these aspects of the IIF technique is in progress at present in this laboratory.

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