A Novel Method for Isolation of *Chlamydia pneumoniae* by Treatment with Trypsin or EDTA

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To establish a novel method for the efficient isolation of *Chlamydia pneumoniae*, experiments were performed to determine the effects of EDTA or trypsin treatment of *C. pneumoniae* on its adsorption and inclusion body formation. Treatment of *C. pneumoniae* with 0.1% trypsin or 1 mM EDTA significantly increased inclusion body-forming activity from 8,000- to 10,000-fold higher than that of the control. *C. pneumoniae* was successfully isolated in cultured cells which were inoculated with clinical specimens after treatment with 0.1% trypsin.

Currently *Chlamydia pneumoniae* is considered an important pathogen of acute respiratory diseases (3, 9, 13, 14) and middle ear diseases (15). However, reports of successful isolation of *C. pneumoniae* are relatively limited (3, 6, 9, 10, 13–15). The following procedures are routinely employed to infect cells: low-speed centrifugation of chlamydial suspensions into cell monolayers (2) and DEAE-dextran treatment of cell monolayers before infection (2, 11). Nevertheless, high-titer preparations of *C. pneumoniae* are generally difficult to obtain and in vitro passages of fresh isolates are often unsuccessful, although it was reported that the attachment and penetration of *Chlamydia psittaci* and *Chlamydia trachomatis* in cells were highly efficient (12). It was previously reported that Sendai virus grown in cultured cells is inactive in hemolysis, cell fusion, and infectivity, and treatment of the cell-grown virus with trypsin enhanced all of these activities (5, 16). It has also been reported that gram-negative bacteria can be divided according to pH-dependent EDTA bactericidal activity into four groups (7, 8); for example, *Vibrio cholerae* and *Escherichia coli*, for which the most potent bactericidal activity was observed with 10 mM EDTA-2 Na saline solution, at pHs 5.0 and 9.0, respectively, and *Proteus mirabilis* and *Shewanella putrefaciens* were EDTA insensitive and EDTA sensitive, respectively, at any pH tested. These findings led us to investigate conditions for successful isolation of *C. pneumoniae* by determining the effects of trypsin or EDTA treatment of *C. pneumoniae*, based on the hypothesis that the infection efficiency of *C. pneumoniae* could be increased by altering the chlamydia cell surface after mild treatment with EDTA or trypsin.

The TW-183 reference strain and a clinical isolate of the KIE-130 strain (15) of *C. pneumoniae* and a human lung cell strain of HL cells (10), a generous gift from C. C. Kuo of the University of Washington, Seattle, were used. The following solutions were employed: Eagle’s minimal essential medium (MEM) containing 8% fetal bovine serum as a cell growth medium, MEM supplemented with 8% fetal bovine serum and 0.5% glucose for adsorption of *C. pneumoniae*, MEM with 10% fetal bovine serum and 1 μg of cycloheximide/ml as a maintenance medium for inoculated cells, and phosphate-buffered saline (PBS) supplemented with 7.5% sucrose and 0.072% glutamine (PSG) as a transport medium and a diluent for *Chlamydia* preparation. A total of 65 specimens (20 middle ear aspirates from patients with middle ear diseases and 30 throat swabs and 15 sputa from patients with acute respiratory diseases) were randomly selected from the file of stocked specimens which had been sent to our laboratory for *Chlamydia* isolation. All specimens were collected in 2 ml of PSG, suspended by using a Vortex mixer, and sonicated at 50 Hz for 10 min. As determined by a routine procedure (6), these specimens were all inclusion negative in isolation tests in shell vials containing HL cells. The specimens were tested at 72 h postinfection after three passages and were stored at −80°C until use.

Cultivation and isolation of *C. pneumoniae* were performed similarly, if not identically, to previously reported methods (6, 10). Briefly, the culture medium was harvested from HL cell culture 72 h after infection with *C. pneumoniae* and sonicated at 50 Hz for 30 s, followed by centrifugation at 500 × g for 10 min. The supernatant was again centrifuged at 15,000 × g for 30 min, and the pellet was resuspended with PSG and stored at −80°C until use. In routine isolations, HL cells were treated with DEAE-dextran before infection, but this procedure was omitted for titration and isolation of chlamydia treated with trypsin and EDTA.

Treatment of *Chlamydia* preparations with trypsin (Difco Laboratories, Detroit, Mich.) and EDTA (Dojin Chemicals, Kumamoto, Japan) was carried out as follows: 100 μl of *C. pneumoniae* preparation, containing about 5 × 108 inclusion-forming units (IFU)/ml, was harvested from HL cell cultures, distributed in a microcentrifuge tube, and sonicated at 50 Hz for 30 s followed by centrifugation at 500 × g for 10 min. The supernatant was again centrifuged at 15,000 × g for 30 min, and the pellet was resuspended with PSG and stored at −80°C until use. In routine isolations, HL cells were treated with DEAE-dextran before infection, but this procedure was omitted for titration and isolation of chlamydia treated with trypsin and EDTA.

Supporting Information Available

A detailed summary of the preliminary results is available. It can be downloaded from http://www.microbiol.org.
...sions was performed at 72 h after infection by using a previously reported method (10). Immunofluorescent staining was performed first with monoclonal antibody against \textit{C. pneumoniae} (RR40Z; DAKO, Glostrup, Denmark) and then with fluorescein isothiocyanate-labelled anti-mouse immunoglobulin G (ZYMED). The titer (measured in IFU) was about 5 \times 10^8 IFU/ml. Experiments were performed in duplicate, and titers were expressed as IFU/ml.

First, the effect of trypsin or EDTA on inclusion body formation of \textit{C. pneumoniae} was studied. IFUs of both strains increased significantly after treatment with appropriate concentrations of either trypsin or EDTA at pH 7.2. As shown in Table 1, the maximal IFUs for the TW-183 and KIE-130 strains were 2 \times 10^6 and 1 \times 10^7 IFU/ml, respectively, after treatment with 0.1\% trypsin, and 8 \times 10^6 and 2 \times 10^7 IFU/ml with 1 mM EDTA, respectively. IFUs of both strains decreased on treatment with concentrations of trypsin above 0.1\% and of EDTA above 1 mM. It was also found that the inclusions which formed were approximately 1.5-fold larger in diameter at an average of three inclusion bodies per cell compared with controls in which only one inclusion was usually observed (data not shown). There was no difference in the enhancing effect according to the pH of trypsin and EDTA solutions tested at pHs 5.0, 7.2, and 9.0 (data not shown).

To determine the numbers of the elementary body (EB) adsorbed on the cells, cells on coverslips were infected with KIE-130 strain (about 5 \times 10^5 IFU/slip) which had been treated with 0.1\% trypsin in PBS or 1 mM EDTA in 0.85\% NaCl, incubated for 30 min at 37°C, and then centrifuged at 1,500 \times g for 30 min. PBS and 0.85\% NaCl solutions were used as controls. The cells were fixed and stained after the 30-min incubation and after the 30-min centrifugation. The numbers of EB particles on 200 cells per slip were counted under a fluorescence microscope, and an average count per two slips was determined. Although the data are not shown, higher percentages, 25 and 18\%, of cells inoculated with the trypsin or EDTA preparation, respectively, were EB antigen positive, while only 12 and 10\% of the control cells were positive after the 30-min incubation; 60\% (trypsin) and 44\% (EDTA) of cells with more than two EB particles per cell were observed to have more than 10 EB particles positive compared to 33 and 30\%, respectively, in the control. On the other hand, after centrifugation, there were rapid increases of cells (from 45 to 69\% with trypsin and from 47 to 72\% with EDTA) with no antibody-reacting EB particles. The reason for the rapid loss of reactivity to antibodies is unknown. The above values were drawn from duplicate experiments in which 200 cells per slip were counted on two slips per criterion and showed similar, if not identical values, although statistical analysis was not performed. These results indicate that mild treatment with 0.1\% trypsin or 1 mM EDTA enhanced the adsorption efficiency of EB particles to a host cell and suggest both that the treated EB particles were more rapidly phagocytosed than were the non-treated particles and that trypsin treatment rather than EDTA resulted in more efficient adsorption of EB particles on HL cells.

Isolation of \textit{C. pneumoniae} from clinical specimens was attempted to evaluate the practical use of trypsin treatment of chlamydia. Since the enzymatic activity of trypsin in chlamydia preparations was inhibited by the addition of calf serum instead of removal of trypsin by centrifugation (data not shown), trypsin treatment was chosen for chlamydia isolation. The 65 specimens sent for \textit{C. pneumoniae} isolation were treated with the addition of 0.1\% trypsin for 30 min at 37°C. Heat-inactivated calf serum was then added to a final concentration of 10\%. The treated specimens without centrifugation were inoculated directly onto cells by centrifugation, and the cells were examined after a 72-h incubation at 37°C for the presence of the inclusion bodies by fluorescence microscopy. Samples were repeatedly inoculated into the fresh culture, and no inclusion detected after three blind passages was considered isolation negative for both trypsin-treated and untreated specimens. PCRs with two different primer pairs (1) were used as an alternate method for confirming specimens as being \textit{C. pneumoniae} positive. Samples that were negative by the HM-1–HR-1 primer pair were reexamined by nested PCR first with the HL–HR-1 pair and then with the HM–HR-1 primer pair. As shown in Table 2, and of 115 of 65 specimens were inclusion body positive by the ordinary isolation method and the trypsin treatment method at the first passage, respectively, but only 8 strong-positive specimens were serially passaged by the trypsin method. Only nine specimens were PCR positive. All PCR negative samples were analyzed by nested PCR, resulting in 13 positive specimens. The 13 specimens that were positive by nested PCR include the specimens positive by the trypsin method and the initial PCR. All PCR-positive specimens yielded products (229 or 437 bp [1]) that were detected by agarose gel electrophoresis. In addition, it is noteworthy that one throat swab specimen that was positive only in the first passage by the ordinary isolation method was also positive for isolation by the trypsin method and detection by two PCR methods. Among three positive samples (two middle ear aspirates and one throat swab), two were positive by both the trypsin method and the initial PCR, and one was positive by the trypsin method only.

<table>
<thead>
<tr>
<th>Specimens</th>
<th>No. of positive specimens isolated*</th>
<th>DNA detection of positive specimens (no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ord. method</td>
<td>Tryp. method</td>
</tr>
<tr>
<td>Middle ear aspirates (n = 20)</td>
<td>0</td>
<td>4 (2)</td>
</tr>
<tr>
<td>Throat swabs (n = 30)</td>
<td>1 (0)</td>
<td>5</td>
</tr>
<tr>
<td>Sputa (n = 15)</td>
<td>0</td>
<td>2 (1)</td>
</tr>
<tr>
<td>Total (n = 65)</td>
<td>1 (0)</td>
<td>11 (8)</td>
</tr>
</tbody>
</table>

*Numbers in parentheses are those of positive samples only in the first passage.

b HM-1–HR-1 primer pair used.

The table above shows the isolation and detection of \textit{C. pneumoniae} from clinical specimens. The effectiveness of trypsin treatment was evaluated by measuring the number of elementary bodies (EB) adsorbed on cells. The isolation efficiency was improved by treating specimens with mild trypsin or EDTA. The use of nested PCR confirmed the positive results obtained by the trypsin method.
rates and one sputum) at the first passage by the trypsin method, the aspirate was positive only by PCR with the HM-1–HR-1 primer, and all three samples were positive by nested PCR. Among the three methods employed in these experiments, nested PCR gave the highest positivity rate, but the rate after trypsin treatment was comparable with the value of PCR with the HM-1–HR-1 primer pair.

There is a previous report on the effects of proteolytic treatment of chlamydia EB which found that surface proteolysis of Lymphogranuloma venereum did not impair infectivity but drastically cleaved the major outer membrane protein (4). In this study, it was unexpectedly demonstrated that treatment of C. pneumoniae with 0.1% trypsin or 1 mM EDTA resulted in a remarkable increase of its inclusion-forming activity through high adsorption and phagocytosis on HL cells. The mechanism of the enhancement phenomenon is unclear at present, but disaggregation of the clumps of EB particles and/or inclusion bodies by trypsin, EDTA, or sonication is not likely because the chlamydia suspension was routinely prepared after sonication and treatment of C. pneumoniae with trypsin or EDTA not only enhanced the inclusion-forming activity but also the development of large multiple inclusion bodies in the cells. Alternatively, the effects of trypsin or EDTA on the EB particles may cause EB particles on the HL cell surface to be phagocytosed. However, cells previously treated with trypsin or EDTA before infection resulted in a slight increase (trypsin) or remarkable decrease (EDTA) of inclusion-forming activity (data not shown), although there was no additive or synergic effect of the two reagents on chlamydia cells. An alternative explanation is that a masking factor(s) on the EB cell surface, which interferes with EB attachment to host cells by some unknown mechanism, might be removed by trypsin or EDTA. The chlamydia preparation in cell cultures may consist of EB particles with varying amounts of the masking substance. The trypsin- or EDTA-inducible EB particles may be partially covered with the masking substance(s) and be converted to fully adsorbable EB particles after treatment.

In light of these observations and interpretations, analysis of the masking and unmasking of the inclusion-forming activity as well as the larger inclusion body formation of C. pneumoniae may provide important clues for the study of the entry mechanism and low attachment of C. pneumoniae. Trypsin treatment may be a useful procedure for the isolation of C. pneumoniae from clinical specimens.

REFERENCES