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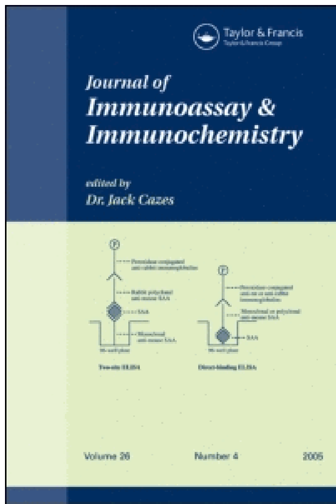


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JOURNAL OF IMMUNOASSAY, 2(3&4), 239-262 (1981)

# DETECTION OF CHLAMYDIA TRACHOMATIS ANTIGEN BY RADIOIMMUNOASSAY

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## ABSTRACT

A sensitive indirect radioimmunoassay (RIA) was developed for detection of chlamydial antigens in infected, irradiated McCoy cell culture. Polystyrene beads were used as the solid phase, guinea pig antichlamydial immunoglobulins were used as the captive antibodies, rabbit antichlamydial immunoglobulins were used as the secondary antibodies, and $^{125}$I- labelled sheep antirabbit immunoglobulin was used as the indicator antibodies. The immunizations were done intracutaneously with purified genital and lymphogranuloma venereum Chlamydia trachomatis strains grown in the yolk sacs of embryonated eggs.

The bound radioactivity was a function of the amount of chlamydial antigen and the method demonstrated the antigen approximately 20 hours post infection. Also noninfectious chlamydial antigen was detectable by RIA. The sensitivity of the assay was about 10 ng/ml for purified antigen and less than 10 inclusions in the cell culture. Each chlamydial serotype could be detected. The RIA was found to be more sensitive than iodine staining and as rapid and sensitive as immunofluorescence method to demonstrate chlamydial infection in cell culture. (KEY WORDS: Chlamydia trachomatis, antigen detection, radioimmunoassay)



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## INTRODUCTION

Human infections due to Chlamydia trachomatis (CT) are clinically important and various disease entities including eye and sexually transmitted diseases are increasingly seen (19). The definitive laboratory diagnosis of chlamydial infection is based on the isolation of the parasite preferably in the cell culture (6). Suggestive evidence for active chlamydiosis may be obtained by staining the inflammatory ocular or urogenital exudates by Giemsa or immunofluorescence (IF) (8) as well as by demonstration of local antibodies (4, 14, 22). The measurement of serum antibody, in particular immunoglobulin M (IgM) response (24, 25) is also possible.

The isolation is, and probably will remain, the basic reference method detecting one single infectious chlamydial elementary body (EB). However, practical drawbacks like the diminished infectivity of specimens sent from long distances to the laboratory limit the usefulness of the isolation method.

The direct demonstration of chlamydial EB:s in clinical specimens has been done by immune electron microscopy method using ferritin labelled antiserum staining (1). This method is time consuming and requires special expertise; it is not suitable for routine purposes. The direct demonstration of enzymes, specific for CT (11) requires further development.

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The detection of viruses and viral antigens directly in gastro-intestinal (7) and respiratory tract (18) specimens by indirect radioimmunoassay (RIA) developed in our laboratory is sensitive and practical for routine use.

In the present report we apply a RIA technique for detection of chlamydial antigens in cell culture specimens. The development and the optimal assay conditions of the RIA test will be described.

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## MATERIALS AND METHODS

### Chlamydia Trachomatis Strains and Propagation

The genital chlamydial strains used were: C (UW-1) [this strain was used instead of J], D (IC-Cal 8), F (MRC-301), G (IOL-200), H (UW-4), I (UW-12), K(UW-31), L1 (440-L), L2 (434-B) and L3 (404-B) (25). These were obtained in early yolk-sac passages from the Institute of Ophthalmology, London, through courtesy of Drs Sohrab Darougar and John Treharne.

These prototype strains were initially passaged in irradiated (5 000 rad) McCoy cell vial culture using centrifuge assisted inoculation (15 000 xg for 60 min at 35°C, 20). The growth medium was BHK medium (Gibco) supplemented with 10% foetal calf serum

(FCS) (Flow) and antibiotics. The vials were incubated for 72 h in 5% $CO_2$ atmosphere at 35°C. The cells on a circular 13mm in diameter cover glass were fixed with methanol and were stained with iodine. Chlamydial inclusions were counted and this represented the number of inclusion forming units (IFU) of the inoculum.

For the immunization genital chlamydial strains were grown in yolk sacs of fertile hens' eggs and EB:s were purified by Renografin$^R$ gradient method (21). The protein content of purified EB preparation was measured by the method of Lowry et al. (13).

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For antigen detection tests and for antibody determinations these strains were also grown in McCoy cell bottle culture and purified by Renografin$^R$ gradient method (21). L2 strain was harvested from infected McCoy cells also at 27 hours post infection and reticulate bodies (RB) were purified after Yong et al. (27). In addition to McCoy cells human amnion cells were cultured for L2 (9).

Immunization of Animals

Purified chlamydial strains were pooled according to their protein content. Adult rabbits were immunized intracutaneously four times at 3-week intervals. A total amount of 1 200 µg egg-grown genital antigen pool was used mixed with Freund's incomplete adjuvant. Guinea pigs were immunized similarly with 1 000 µg of antigen.

GP 3 was immunized with egg-grown L 1, L2 and L 3 antigens, GP 170 and 171 with genital antigen pool (vide supra).

RIA Reagents

Rabbit and guinea pig immunoglobulin fractions were prepared from pretested hyperimmune sera (see results) by precipitation of serum with an 18% (w/v) final concentration of sodium sulphate, followed by chromatography on a Sephadex G 25 column.

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Polystyrene (PS) beads (Precision Plastic Ball Co., Chicago, IL) used in RIA were incubated overnight (16 to 18 hours) at 20$^oC$ with guinea pig immunoglobulins, 0.5 µg/bead (or as indicated) in carbonate buffer (pH 9.6). The label used was $^{125}$iodinated sheep antirabbit IgG purified by immunosorbent chromatography as previously described (7).

Diluent for the RIA test was PBS [0.1 M $Na_2HPO_4$- $KH_2PO_4$ buffer (pH 7.2) containing saline ( 0.8% NaCl and 0.02% KCl)], 20% inactivated FCS, 2% Tween 20 and 0.1% $NaN_3$.

RIA Procedure, Antibody Assay

The antibody titer was determined by RIA essentially as described

earlier (22). Briefly, the antigen coated (2.5 µg/bead) PS beads were incubated with 200 µl of dilutions of guinea pig or rabbit antichlamydial antiserum for 2 h at 37°C. When guinea pig serum was tested a further 2 h incubation at 37°C was carried out with rabbit antiguinea pig serum (1:2 000, Dako). To each tube, 200µl $^{125}$I-labelled sheep anti-rabbit IgG antibody was added and incubated for 2 h at 37°C. The beads were washed, transferred to clean tubes and the bound radioactivity was measured with 1270 Rack Gamma II or LKB 1280 counter.

The serum antibody titer was defined as the dilution that resulted in bound radioactivity two times buffer blank value or at least 150 cpm bound.

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## Antigen Assay

A volume of 200 µl of the specimen (unless otherwise stated) was pipetted into disposable 5 ml PS tubes and a PS bead coated with guinea pig immunoglobulin (GP 3, 0.5µg/bead or GP 3 & GP 170 & GP 171, 0.2 µg/bead each) was added to each tube. After an overnight incubation at 37°C the medium was aspirated and the beads were washed twice with 5 ml tap water. A 200 µl volume of antichlamydial rabbit (R4) immunoglobulin (8µg/ml) was added to each tube and the beads were incubated at 37°C for 2 hours. The beads were washed and a 200 µl volume of $^{125}$I-labelled sheep antirabbit IgG was added.

The tubes were incubated as previously. After washing the beads were transferred into clean tubes and bound radioactivity was measured. The cpm value of two times the buffer blank is taken as the cut-off line.

Before incubating with the guinea pig immunoglobulin coated beads, specimens were variously pretreated as indicated: vigorous mixing (Whirlimixer[R]), sonication (Branson Sonifier[R]), heating at 56°C as well as the addition of chemicals [Triton X 100, polyethylene glycol (PEG), molecular weight 6 000, sodium dodecyl sulphate (SDS), Nonidet P 40 and sodium deoxycholate (DOC)].

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### Immunofluorescence

The microimmunofluorescence tests were performed as described by Treharne, Dines and Darougar (24). Chlamydial inclusions were stained with FITC conjugated antichlamydial rabbit (R 4) serum.

## RESULTS

### Hyperimmune Sera

After the complete immunization with egg-grown antigens the

resulting guinea pig and rabbit hyperimmune sera were tested by RIA using different cell-grown chlamydial serotype EB:s, L2 RB:s and control antigens.

As an example, figure 1 shows the RIA titers of rabbit (R 4) serum for representative antigens (L2/RB/McCoy, L2/EB/amnion) and control (McCoy) cells. The final antichlamydial (L2) antibody RIA titer of R 4 hyperimmune serum is close to $1x10^{-6}$. As can be seen R 4 serum has no antibodies against McCoy cells.

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The RIA and type-specific titers (as measured by MIF by John Treharne) for guinea pigs were high against different serotypes. The RIA titers against RB antigen were the highest (about 7 to 50 x $10^{-6}$), as could be expected (27). The titers for L2 were the same, regardless of the cells in which this antigen was grown.

The titer against McCoy cells was at least 1 000 times less and about the same as was seen in preimmunization serum.

## Pretreatment of Antigen Specimens

Purified L2/McCoy EB antigen in PBS was diluted in the diluent buffer (1 000 ng/ml) and various treatments were applied before the antigen (in 200 µl volume) was incubated with GP 3 immunoglobulin (0.5 µg/bead) coated beads. The results of subsequent antigen detection test are presented in table 1.

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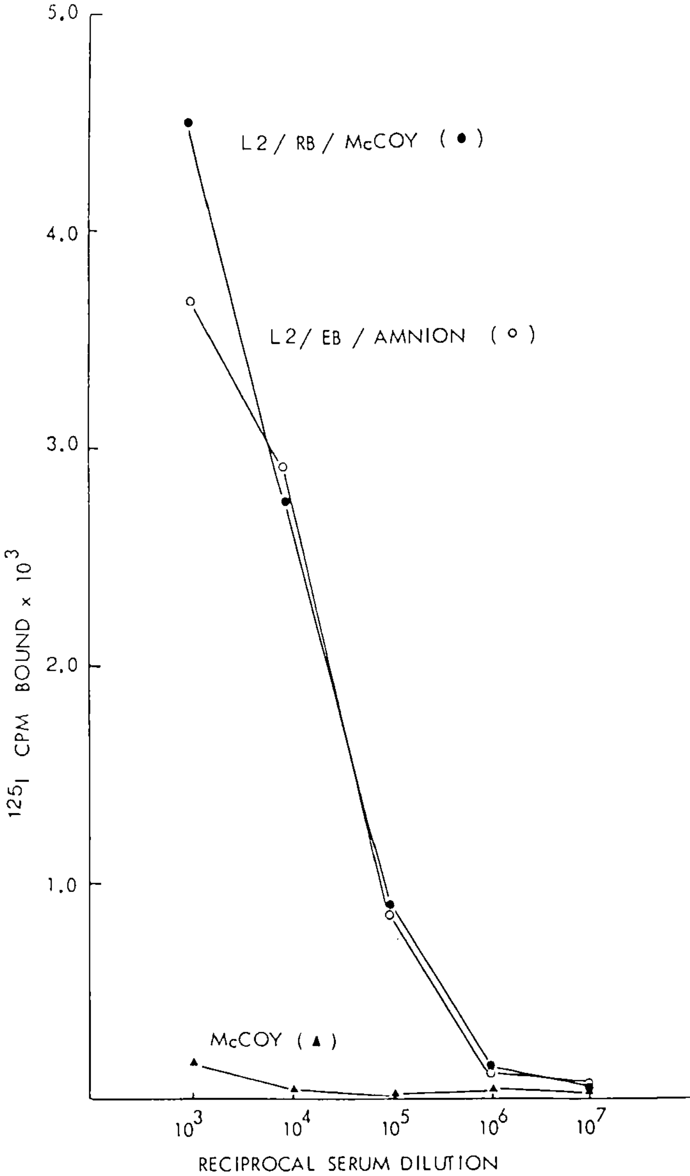


FIGURE 1. Titration of rabbit (R 4) hyperimmune serum by radioimmunoassay (RIA). Immunization was done intracutaneously with purified egg grown genital and lymphogranuloma venereum (LGV) Chlamydia trachomatis strains. The antigens in RIA were LGV (L 2) reticulate bodies (RB) grown in McCoy cells ( ● ) as well as L 2 elementary bodies (EB) grown in amnion cells ( ○ ). McCoy cell lysate ( ▲ ) was used as control antigen.

TABLE 1.

Effect of Various Pretreatments on the Chlamydial Antigen Detection; Purified L2 (434-B) Antigen Grown in McCoy or Amnion Cells (1 000 ng/ml) was treated as indicated and thereafter processed for Antigen Detection by RIA (see Materials and Methods). The Results are the Average of four Separate Determinations.

| Pretreatment | Antigen grown in | | |
|---|---|---|---|
| | McCoy | Amnion | BB[2] |
| Agitation 0.5 min | 644[1] | 774[1] | 200[1] |
| Sonication 2.5 min | 1 764 | 2 942 | 207 |
| Triton X 100 0.2% | 492 | 688 | 187 |
| Triton X 100 0.2%,sonic. 2.5 min | 2 755 | 3 373 | 241 |
| Heating +56°C, 30 min | 517 | 717 | 207 |
| Heating +56°C, 30 min ,sonic. 2.5 min | 2 225 | 3 578 | 214 |
| SDS 0.1% | 457 | 449 | 377 |
| SDS 0.1%, sonic. 2.5 min | 537 | 503 | 394 |

1) cpm
2) buffer blank

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The addition of Triton X 100 (0.2%) and SDS (0.1%) as well as heating (56°C,30 min) resulted in counts bound comparable with vigorous agitation only. Sonication (2.5 min, 20kHz, 20W) alone increased counts bound 3-fold. When various sonication times were compared, it was found that counts bound with 2.5 and 5 min sonication were higher than those obtained with 1 and 10 min sonication. A sonication of 2.5 min was therefore chosen for all later experiments.

The treatment of antigen with SDS, PEG (4%, mw 6 000) (17), Nonidet P 40 (0.5%) (15) or DOC (0.1%) (12) alone or combined with

sonication gave lower bound counts than treatment with Triton X 100 plus sonication. Heating of the antigen at 56°C for 30 min combined with sonication also increased counts bound when compared with either treatment separately. Since the heating may destroy important type-specific antigens (16) the addition of Triton X 100 followed by sonication was chosen as the final pretreatment of antigens.

## Sensitivity of Antigen Detection

L2/EB antigen grown in McCoy cells was purified and was measured by RIA after sonication (2.5 min) in the presence of Triton X 100 (0.2%). The antigen detection curve is presented in figure 2.

The RIA test is able to detect about 10 ng chlamydial antigenic protein/ml. This figure was approximately the same for L2 antigen grown in other hosts (amnion cells and eggs, data not shown). Purified McCoy cell grown genital serotypes were also tested and the detection limit for them in RIA test was 10-20 ng/ml (data not shown).

## Detection of Chlamydial Antigen in Cell Culture by RIA, Sensitivity Comparison with Infectivity and Staining

Inocula of L2 were diluted tenfold and inoculated simultaneously into three irradiated McCoy cell tubes (with 1.0 ml medium). The

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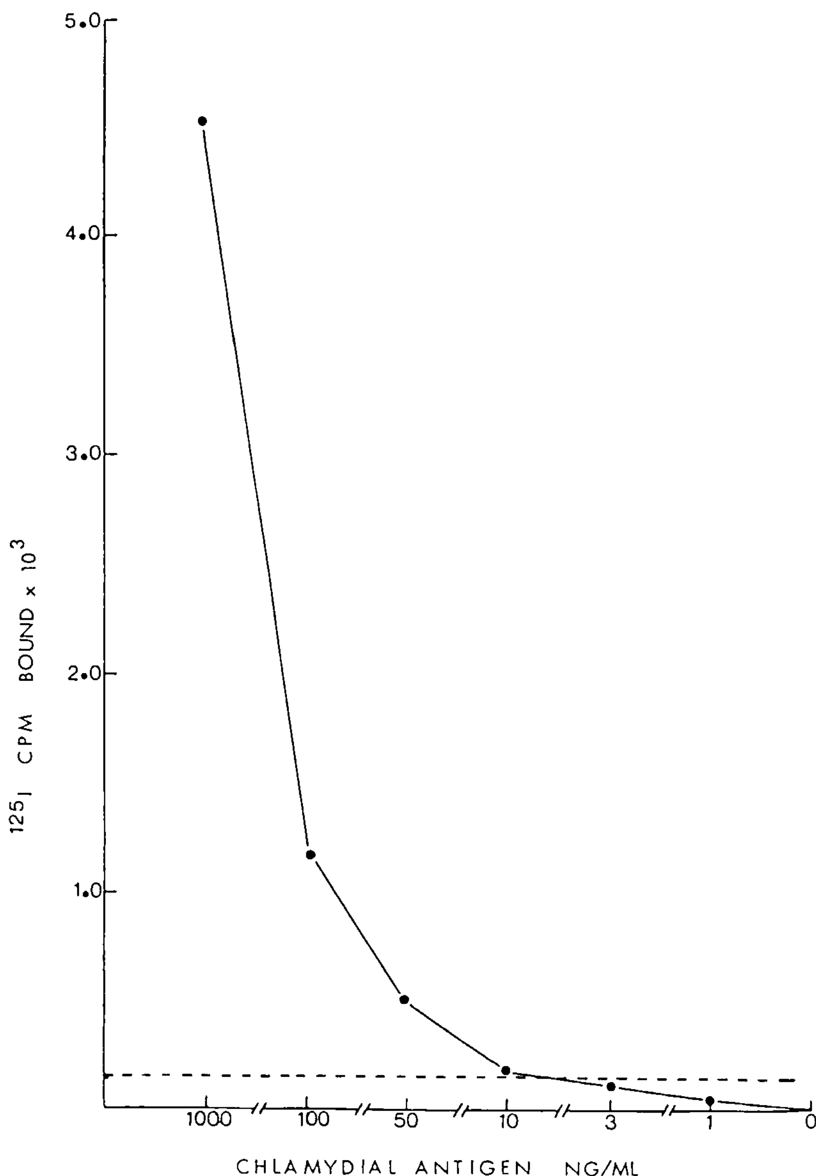


FIGURE 2. Sensitivity of the indirect RIA for the detection of purified C. trachomatis (L 2) antigen. A 200 μl volume of antigen dilution was incubated with captive antichlamydial guinea pig immunoglobulins adsorbed on polystyrene beads. Rabbit antichlamydial immunoglobulins were used as the secondary antibodies and $^{125}$I-labelled sheep antirabbit immunoglobulin as the indicator antibodies. Less than 10 ng/ml of antigen could be detected, the spotted line shows the cut-off line of the RIA.

tubes were processed further as follows: immediately after the inoculation the first tube was stored at -70°C and was later tested for antigen by RIA. The other two tubes were centrifuged and incubated for 72 hours. The cells in the second tube were then fixed with methanol and stained with iodine to determine the number of inclusions produced. The cells in the third tube were detached by agitation with glass beads in a Whirlimixer$^R$ for 30 seconds and equal amounts of medium (0.5 ml) were processed separately: one portion was inoculated into new tubes (second passage) and the other was tested for antigen. The results of this experiment are presented in table 2.

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The infectivity of L2 serotype increased during one single passage about a thousandfold. The amount of antigen detectable by RIA increased similarly. When the sensitivity of iodine staining and RIA antigen detection were compared, it can be seen that at the first passage the dilution $10^{-5}$ was iodine negative, whereas it expressed cpm value above the control tubes and also produced 750 new inclusions. It can be calculated that RIA test is sensitive enough to detect between 1 and 10 inclusions in cell culture.

The other experiment was carried out to determine if a diagnosis could be made from infected cell cultures by RIA antigen detection earlier than by staining of the cells, i.e. during 15-20 hours postinfection.

TABLE 2.

Detection of Chlamydial (L2) Antigen in Cell Culture by RIA; Comparison of Infectivity with the Sensitivity. See Text for Experimental Details. Combined Results of Two Separate Experiments.

| In inoculum | | 1st passage | | 2nd passage |
|---|---|---|---|---|
| dilution | cpm[1] | No inclusions[2] | cpm[3] | No inclusions[2] |
| $10^1$ | 923 | 10 000 | 5 383 | pos[4] |
| $10^2$ | 325 | 1 024 | 4 819 | pos[4] |
| $10^3$ | 303 | 108 | 2 822 | pos[4] |
| $10^4$ | 320 | 8 | 926 | 10 000 |
| $10^5$ | 270 | neg | 354 | 750 |
| $10^6$ | 361 | neg | 276 | neg |
| 0 (BB) | 287 | neg | 270 | neg |

1) As determined directly from inoculated tubes,/0.5 ml
2) As determined after 72 hours on incubation for the whole coverglass (1.0 ml) by iodine staining
3) As determined from tubes incubated for 72 hours,/0.5 ml
4) Cells abundant with inclusions and partly toxic; the amount of inclusions undeterminable
BB buffer blank; uninfected cell culture tested (0.5 ml)

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Irradiated McCoy cells ($10^5$) were infected with a standard inoculum to produce about $10^4$ inclusions; this represents to the multiplicity of infection (MOI) 0.1 IFU/cell.

Following inoculation and centrifugation for 1 h each tube (except one transferred to -70°C immediately after the inoculation) was washed two times with growth medium to remove nonattached EB:s. Fresh medium (1.0 ml) was added and the tubes were incubated at 35°C. At certain intervals the incubation was stopped and two cell

TABLE 3.

Detection of CT (L2) Antigen from Cell Culture; Comparison of Diagnosis by Staining (Iodine and Immunofluorescence), RIA and Second Passage in Relation to Time. The Average of Three Separate Experiments is presented.

| | 1st passage No inclusions[2] | | | 2nd passage No inclusions[2,4] |
|---|---|---|---|---|
| Time, hours[1] | Iodine | IF | RIA, cpm[3] | Iodine |
| 0 | neg | neg | 2 815 | 1 340 |
| 1 | neg | neg | 1 096 | 148 |
| 2 | neg | neg | 1 519 | 96 |
| 4 | neg | neg | 1 527 | 70 |
| 6 | neg | neg | 1 579 | 34 |
| 12 | neg | neg | 1 149 | 3 |
| 24 | neg | pos | 2 948 | 8 |
| 36 | 1 250 | pos | 4 185 | 19 000 |
| 48 | 10 000 | pos | 4 138 | 158 000 |
| control[5] | neg | neg | 245 | neg |

1) $10^5$ irradiated McCoy cells infected with $10^4$ IFU of L2 (434-B), centrifuged 15 000xg for 1 h at 35°C; MOI 0.1 IFU/cell; hours postinfection
2) As determined after 72 hours incubation for the whole coverglass (1.0 ml)
3) As determined from tubes incubated for 72 hours/0.5 ml
4) As produced from 0.5 ml of medium from the first passage
5) Non-infected McCoy cells

monolayers were fixed with methanol and stained with iodine and IF, respectively. A third tube incubated for the same time stored at -70°C and used for a new passage and antigen detection by RIA as described previously. The results of this experiment are presented in table 3. Table 3 shows that following centrifugation for 1 h and washing, the counts bound in the RIA antigen detection test were diminished to one third.

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Following one hour centrifugation and washing only one tenth of infectivity in repassage was restored. The infectivity was further diminished to approximately 1% of the original during the first twelve hours of incubation. Thereafter the infectivity rose three fold from 12 to 24 hours, but the subsequent increase of infectivity up to 36 and 48 hours was exponential reflecting the maturation of new EB:s.

The amount of antigen detectable by RIA was lowest at one hour immediately after centrifugation and washing, but one hour later there was a 50% increase in cpm value in RIA perhaps indicating the initial synthesis of chlamydial antigens. This synthesis seemed to remain at fairly constant level up to 12 hours and increased almost three fold at 24 hours postinfection. The amount of chlamydial antigens detectable by RIA continued to increase during the next 12 hours and the RIA counts bound reached a plateau at 36 hours postinfection. RIA was able to detect chlamydial antigen during the whole developmental cycle, at least when a relatively high MOI e.g. 0.1 IFU/cell is applied. When the amount of antigen detectable by RIA is compared with the formation of inclusions, it is of interest to note that iodine staining demonstrated inclusions only 36 hours and IF 24 hours postinfection.

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Effect of Temperature

The McCoy cells in a Roux bottle were infected with L2 inoculum

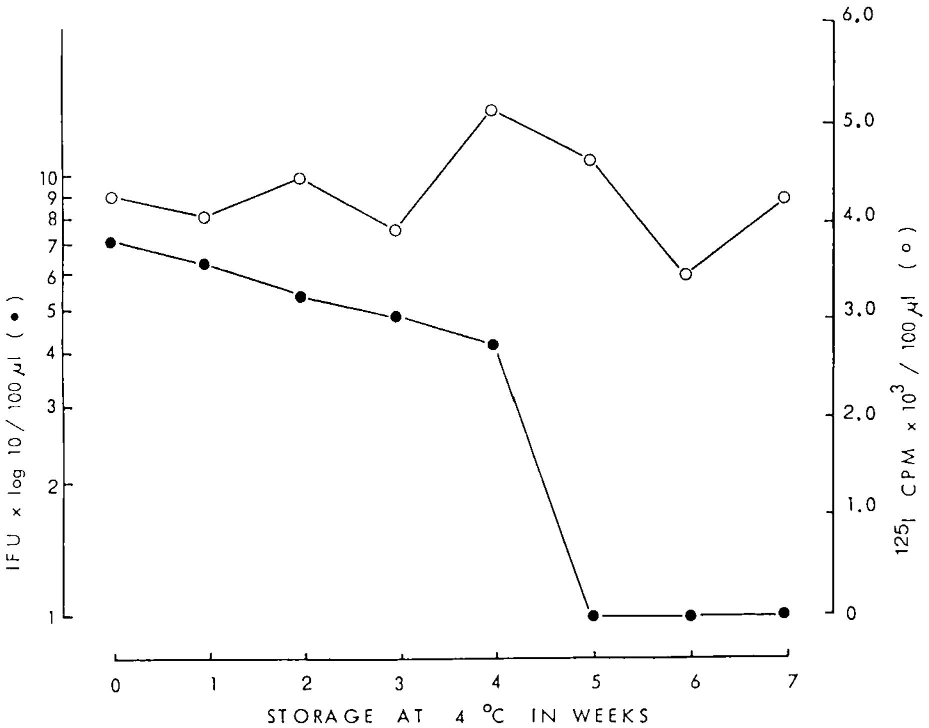


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FIGURE 3. Preservation of chlamydial infectivity and antigen detection during the storage at 4°C. McCoy cells in a Roux bottle were infected with C. trachomatis (L 2). The bottle was stored at 4°C and two specimens (100 µl) were taken weekly 1) for isolation in McCoy cell tubes, the inclusions produced were calculated (inclusion forming units, IFU, ● ) 2) for testing by RIA as described in methods, the bound radioactivity was indicated as cpm values ( o ).

(5 IFU/cell). After 72 hours the cells were detached and the bottle culture held at 4°C. Samples were taken weekly and tested for infectivity in McCoy cell tubes and for antigen detection by RIA. The results are presented in the figure 3.

As is evident from the figure, L2 strain loses its infectivity when kept at 4°C, at a rate of about 90% per week. RIA antigen

detection values are stable over at least 7 weeks, by which time all infectivity has disappeared.

### Detection of Various Chlamydial Serotypes

An experiment was performed to detect various serotypes in cell culture. Irradiated McCoy cells in duplicate tubes were inoculated with prototype strains. The 'genital' serotypes were C to K. In addition 'ocular' serotypes A (SA-1) and B (TW-5) were included. Types A and B were not used for immunization. After 72 hours incubation the cells in one tube were fixed and stained to determine the number of inclusions. The other tube was processed further for antigen detection by RIA. The test detected all chlamydial genital and ocular serotypes. The sensitivity limit of the assay was less than 10 inclusions.

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## DISCUSSION

There is a need for a simple and sensitive test to demonstrate Chlamydia trachomatis in clinical (e.g. urogenital) specimens. In order to develop such a test we applied the solid-phase indirect RIA to detect chlamydial antigen in cell culture.

This technique required high-titered chlamydial antisera. For immunization we used egg-grown genital C. trachomatis. It is possible

to purify these EB antigens efficiently in Renografin$^R$ gradients (21). Using intact EBs as antigen we were able to produce reactive antisera in guinea pigs and rabbits, tested by micro-IF (24) and RIA (22) (Fig.1.). It is evident that the antibodies produced with present immunization are directed against type specific and group specific antigens. The type specific heat labile antigens (16) may be of less importance in antigen detection since the heating of the antigenic specimens results only in a slight decrease in sensitivity (table 1).

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Group specific antigens are located on or just below the surface of the EB (5). These antigens have been analyzed after applying sonication (12) and various chemical treatments. For example Caldwell, Kuo and Kenny (3) used the nonionic detergent Triton X 100 to solubilize EBs. We found that it was necessary to pretreat the antigen with combined sonication and chemical treatment (Table 1), Triton X 100 in a concentration of 0.2% being optimal.

The other chemical treatments (SDS, Nonidet P40, PEG 6 000) alone or combined with sonication gave lower cpm values than Triton X 100. An advantage of using PEG 6 000 would be shortening of the incubation periods as reported by Salonen and Vaheri (17). SDS apparently made the antigen undetectable by our RIA although Caldwell, Kromhout and Schachter (2) reported that the major component (MP 39.5) of chlamydial outer membrane complex (COMC) maintained at least some of its native antigenic properties after treatment with SDS.

Chlamydial antigen in cell culture can be detected during the whole developmental cycle (Table 3). The RIA test demonstrates chlamydial antigen at least as early as IF staining, i.e. about 24 hour post infection. IF staining has been reported to demonstrate chlamydial infection at an early stage in cell culture (23). Since the RIA method is easily automated and is less laborious than IF microscopy it could be used routinely to demonstrate chlamydial infection in cell culture.

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The viability of chlamydiae is not a prerequisite for our RIA test, since chlamydial antigen in specimens stored at 4°C for long periods is also detectable (Fig.3). This would be of importance if it becomes possible to test clinical specimens which are often subjected to transport and storage delays.

The sensitivity of our RIA test for the direct demonstration of chlamydial antigen is approximately 10 nanograms of protein (Fig.2). This amount of chlamydial protein is equivalent to $10^4$ purified EB:s (10) and corresponds well with the sensitivity level of our test which detects approximately $10^4$ IFU (Table 2). We also found that our test could detect five to ten chlamydial inclusions (Fig.4). Wang, Kuo and Grayston (26) estimated that one inclusion contains about 100 EBs. When comparing the sensitivity level of our RIA test between purified EBs and inclusions, it can be assumed that our test also detects other excreted or membrane bound antigenic material present in chlamydial inclusions in cell culture.

The most important modification for this kind of immunologic antigen detection will be the demonstration of chlamydial antigen directly in clinical specimens. The circumstances in clinical specimens differ essentially from those in cell culture, e.g. by containing secreted antibodies. Therefore, the application of chlamydial antigen detection test in urogenital or eye specimens requires further developments; these are in progress in our laboratory.

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