Neutralization of *Chlamydia trachomatis* in Cell Culture

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Neutralization of *Chlamydia trachomatis* was assayed by the decrease in inclusion-forming units in baby hamster kidney cells grown in culture. Five percent fresh guinea pig sera increased neutralization titers of rabbit antisera 100- to 1,000-fold but had no effect when normal rabbit sera were tested. Neutralization of a type A or B trachoma isolate was strain specific. Neutralization by human eye secretions and sera also was demonstrated when guinea pig sera were included in the test. All of the six human sera tested showed strain specificity against types A or B, in agreement with typing by the fluorescent antibody technique.

A neutralization test for *Chlamydia trachomatis* has been sought in a number of laboratories as a means of detecting the serological response of vaccinated laboratory animals (2, 3, 24, 30) and of humans with suspected or confirmed infections (1) with this intracellular parasite. Neutralization of trachoma strains by human eye secretions has been reported (21), using infectivity for the owl monkey eye as a test system. Graham and Layton (11) found that the neutralization test in cell culture was strain specific and therefore could be a means of serotyping *C. trachomatis* isolates, as has been demonstrated with *C. psittaci* strains of ovine origin (27). Neutralization tests could also be a useful technique in isolation and identification of type- or strain-specific antigens.

Neutralization tests with *C. trachomatis* strains have been of limited value because of low titers or undetectable activity in sera of vaccinated animals or trachoma patients (1, 2, 24). Antisera directed against the immunoglobinulin component of immune sera enhanced neutralization of *C. trachomatis*; however, antisera against immunoglobulin also significantly enhanced inactivation when normal sera were tested in place of immune sera (3, 30). In this paper, a sensitive, complement-mediated neutralization test for *C. trachomatis* in cell culture is reported. Human sera and eye secretions, containing antitrachoma antibody, and sera from immunized rabbits were active in this neutralization test.

**MATERIALS AND METHODS**

*C. trachomatis* strains. Cloned trachoma strains HAR-13 (TRIC/A/ET/HAR-13/OT) and HAR-36 (TRIC/B/SAU/HAR-36/OT) were used throughout these studies.

**Cell cultures.** A BHK-21 cell line (28) which grows in suspension was received from W. A. Blyth and has been previously described (4). Cells were grown in suspension in flasks agitated at 200 rpm on a shaker at 35 C. Subcultures were tested routinely for mycoplasma.

Eagle minimal essential medium prepared in Earle balanced salt solution with 0.22% NaHCO₃ was obtained from Microbiological Associates. This medium was supplemented with 10% fetal bovine serum (Microbiological Associates), 10% tryptose phosphate broth (Difco), 0.3% glucose, and double concentrations of Eagle minimal essential medium vitamin mixture and nonessential amino acids. Cells were subcultured in medium containing 50 µg of streptomycin per ml.

For cover slip cultures, BHK-21 cells were irradiated with 5,000 R from a cobalt-60 source and washed once, and 1 x 10⁴ cells were placed on an 8-mm diameter glass cover slip in a flat-bottom tube with a rubber stopper. Cells covered greater than 80% of the cover slip area and were used 24 to 48 h after irradiation.

**Preparation of rabbit antisera.** Strains HAR-13 and HAR-36, propagated in the yolk sac of embryonated eggs, were purified by centrifugation on Renografin density gradients (13) and stored at –80 C in phosphate buffer containing glutamate and sucrose (5). Yolk sac from uninfected eggs was subjected to the purification procedure for control immunogen. Antigen preparations were irradiated with 10⁴ R from a cobalt-60 source and had lost infectivity for the owl monkey eye.

Sera were obtained from rabbits before immunization. At each immunization time the total dose per rabbit was 10¹⁴ purified chlamydial particles, estimated by the procedure of Reeve and Taverne (25). Control rabbits received the residual yolk sac from uninfected eggs, after the purification procedure, in a dose derived from the same amount of yolk sac as 10¹⁴ chlamydial particles. Rabbits were injected at four sites subcutaneously and in each footpad with a chlamydial suspension mixed with an equal volume of...
complete Freund adjuvant (Difco) on two occasions 1 month apart. One week after the second immunization, rabbits were given an intravenous injection and 1 week later were bled (first bleeding). Three months later rabbits were given two additional intravenous injections 2 weeks apart and were bled 1 week after the final injection (second bleeding). Sera were stored at -20 C and, except where noted, samples were heated at 56 C for 30 min before use.

**Fluorescent antibody.** Rabbit antisera against trachoma strains HAR-13 and HAR-36 were titrated by the indirect fluorescent antibody (FA) technique using fluorescein-labeled goat anti-rabbit serum. Human sera and eye secretions were titrated and typed by the FA technique using samples adsorbed with *C. trachomatis* types A, B, and C as previously described (17).

**Human eye secretions.** Human eye secretions were collected on Weck-Cel sponges (21) and stored undiluted at -20 C. Eye secretions were pooled from the following groups of humans: (i) children in Saudi Arabia whose eye secretion antibody had type A specificity; (ii) Arabian children with no detectable eye secretion antibody by FA at 1:2 dilution; and (iii) Americans similarly negative.

**Guinea pig sera.** Guinea pig sera (GPS), a gift from E. S. Murray, were obtained from herds of animals free of guinea pig inclusion conjunctivitis. Serum was pooled from several animals and stored at -80 C in small samples which were frozen and thawed only once. Complement was inactivated by heating GPS at 56 C for 30 min.

**Neutralization test.** When used in the neutralization test, strains HAR-13 and HAR-36 had been passaged three and ten times, respectively, in cell culture and were stored at -80 C in phosphate-buffered saline (29) containing 0.25 M sucrose and 0.1% bovine serum albumin in 0.5- to 1.0-ml samples, which were used only once. Rabbit, human, and guinea pig sera and human eye secretions were diluted 1:4 or 1:5 with tissue culture medium and filtered through sterile 0.45-μm pore size membrane filters (Millipore Corp.). The incubation mixture, in 0.8 ml of tissue culture medium, contained dilutions of antisera or eye secretions, 5% GPS, and between 2 x 10⁴ and 10⁵ inclusion-forming units of chlamydia. The mixture was incubated at 37 C for 30 min and subsequently 0.2 ml was placed on each of three cover slip cultures, from which the medium had been removed. Incubated cultures were centrifuged at 30 C at 780 x g for 30 min and incubated at 35 C. Two to 3 h after centrifugation, the medium was replaced with 0.5 ml of fresh medium and, after a total of 40 to 44 h of incubation, monolayers were fixed with methanol and inclusions were stained with iodine (10). Inclusions were counted in 40 fields at x320 and the geometric means of the inclusion counts of the triplicate monolayers were calculated.

Rabbit sera were tested in twofold serial dilutions and the neutralizing titer was expressed as the highest dilution which gave a 50% or greater decrease in inclusion-forming units. Sera obtained before immunization gave little or no neutralization at 1:10 dilution; therefore, neutralization by immune sera was compared to tubes with no sera. At least one preimmunization serum was included in all tests as a control. Neutralization by FA-positive human sera from trachomatous patients was compared to the inactivation given by four control human sera negative by FA. Neutralization by the FA-positive eye secretion pool was compared to the two FA-negative pools.

**RESULTS**

Incubation of the infectious chlamydia alone for 1 h at 37 C in culture medium resulted in 50 to 70% inactivation compared to samples stored at 4 C. During the 30-min incubation routinely used in the neutralization test, little or no heat inactivation of chlamydia was observed. Addition of 10% normal GPS to the test mixture resulted in significant nonspecific inactivation, whereas at a concentration of 5% no inactivation occurred. No damage to host cells was observed after the incubation mixture containing 5% GPS was centrifuged onto monolayers.

**Neutralization by immune rabbit sera.** Serum from a rabbit immunized with HAR-36 had an homologous neutralization titer of 1:16 or less when tested without additional serum or with heated 5% GPS in the incubation mixture (Table 1). Addition of 12.5% fresh normal rabbit sera or 5% fresh GPS to the test increased the titer to 1:64 and 1:8,000, respectively. Except where noted, 5% GPS was included in further neutralization tests.

In a typical test, homologous neutralization of HAR-13 and HAR-36 by immune sera in the presence of 5% GPS gave a sharp and reproducible end point when percentage of neutralization was plotted against serum dilution (Fig. 1). Routinely, the degree of neutralization decreased from greater than 90% to less than 20% in two or three tubes of a series of twofold dilutions. When six rabbit antisera were titrated on two separate occasions, four sera had unchanged titers upon retesting whereas two sera showed only a twofold change in titer. The standard deviation of replicate inclusion counts

| Table 1. Neutralization of strain HAR-36 with additions to incubation mixture |
|------------------|------------------|------------------|
| Rabbit antiserum | Additions to incubation mixture | Reciprocal of dilution giving 50% or greater neutralization |
| Unheated         | None             | 16               |
| Heated           | None             | <8               |
| Heated           | 12.5% unheated normal rabbit serum | 64               |
| Heated           | 5.0% unheated GPS  | 8,000            |
| Heated           | 5.0% heated GPS   | 16               |

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anti-HAR-36 sera from the first bleeding were tested, strain specificity also was apparent although homologous titers were not as high as when the immunogen was HAR-13. After two intravenous injections and a second bleeding, sera demonstrated increased homologous titers with the exception of rabbit 2, which was bled 2 weeks after the last immunization instead of after the 1 week as was done with other rabbits. Two of the sera, from rabbits 1 and 4, showed less strain specificity than at the first bleeding; however, specificity was retained with all sera. In the absence of GPS, neutralization titers were 1:10 or less when both HAR-13 and HAR-36 were tested.

FA titers of these unadsorbed sera from immunized rabbits demonstrated no strain specificity and there was no apparent relationship between FA titers and neutralizing activity. For example, sera from rabbit 2 (first bleeding) had the highest neutralizing titer, yet the FA titer was no higher than that of sera obtained at the same time from rabbit 1. Also, the titer of sera from rabbits immunized with HAR-36 gave an 8- to 16-fold increase in neutralizing titer between the first and second bleedings. There was no comparable increase in FA titer.

Neutralization by human sera and eye secretions. All six sera from trachomatous patients neutralized either HAR-13 or HAR-36 and five of these sera had an eightfold or greater difference in titer when neutralization of the two strains was compared (Table 3). A twofold

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**Table 2. Neutralizing and fluorescent antibody titers of sera from rabbits following injection with trachoma strains HAR-13 and HAR-36**

<table>
<thead>
<tr>
<th>Rabbits immunized with:</th>
<th>Reciprocal of serum dilution giving 50% or greater neutralization</th>
<th>Reciprocal of FA titer against strains HAR-13 and HAR-36a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st bleeding</td>
<td>2nd bleeding</td>
</tr>
<tr>
<td><strong>Strain HAR-13</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R 1</td>
<td>800</td>
<td>20</td>
</tr>
<tr>
<td>R 2</td>
<td>8,000</td>
<td>50</td>
</tr>
<tr>
<td><strong>Strain HAR-36</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R 3</td>
<td>40</td>
<td>200</td>
</tr>
<tr>
<td>R 4</td>
<td>&lt;10</td>
<td>200</td>
</tr>
<tr>
<td><strong>Normal yolk sac</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R 5</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>R 6</td>
<td>10</td>
<td>20</td>
</tr>
</tbody>
</table>

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a Sera were tested in the FA technique at fourfold dilutions and titers against HAR-13 and HAR-36 were the same.

b Bled 2 weeks after last injection.
difference in titer was observed with one serum (7312), which had a low neutralizing titer against both strains. In all cases, the higher titer against one strain agreed with the strain-specificity determined by the FA technique. Although neutralization in the absence of GPS could be detected at a 1:10 dilution with some sera, in all cases the degree of neutralization was much greater when GPS was included in the test. The pool of antibody-positive eye secretions had a titer of 1:40 in the presence of 5% fresh GPS when compared to either pool of control eye secretions incubated under identical conditions (Table 3). The FA-positive eye secretions, at a 1:10 dilution, had no neutralizing activity when tested without GPS or with inactivated GPS.

**DISCUSSION**

The neutralization test in cell culture has not been routinely used to study the serological response to *C. trachomatis* antigens due to the low titers of neutralizing antibody in immune sera. In the present studies, the addition of GPS to the incubation mixture resulted in a reproducible test which is sufficiently sensitive to be useful in antigenic studies of trachoma strains. Five percent GPS, either in the presence or absence of normal rabbit or human sera, caused no inactivation of chlamydia; therefore, it is a specific enhancement. The non-specificity observed in the enhancement of neutralization by anti-immunoglobulin (3, 30) is avoided in the present test.

The neutralization test reported here, which required fresh sera, is presumably complement mediated. Guinea pig inclusion conjunctivitis agent, a *Chlamydia psittaci* strain, was neutralized by sera from guinea pigs immunized with live or formalin-killed guinea pig inclusion conjunctivitis agent in the presence of fresh normal guinea pig sera but was not neutralized when the sera were heated to destroy complement activity (19). Complement-mediated neutralization has been reported for many gram-negative bacteria (20); however, gram-positive organisms are refractory to complement-dependent neutralization (20). The chlamydia are similar to gram-negative bacteria in the morphological characteristics of their cell walls (9) and in their susceptibility to complement-mediated neutralization.

Because of the strain specificity shown by the present neutralization test, the complement-fixing chlamydial group antigen is apparently not the major antigenic determinant in the neutralization of *C. trachomatis* strains. In intact viable chlamydia the group antigen may be inaccessible to antibody and the neutralization test may be useful in the isolation and identification of the outermost cell wall antigens. The results of Graham and Layton (11) suggested that their neutralization test, which was performed without complement, measured antibodies differing from those reacting with the complement-fixing chlamydial group antigen. Both the group antigen and type-specific antigens can be detected by the complement fixation test (6). Therefore, the antigens involved in the present neutralization test may be those measured in the complement fixation test using type-specific antigens. Alternatively, neutralization may involve antigens not detected by previously reported tests for antichlamydial antibodies.

Antibodies of the immunoglobulin (Ig)G and IgM classes were active in the complement-mediated neutralization of gram-negative bacteria, whereas IgA was reported to have slight or no bacteriocidal activity in this test (7, 26). Using the complement fixation test, Mogg et al. (18) found the IgG and IgM were present in sera of rabbits immunized with chlamydial antigens. In human sera, antichlamydial antibodies belong to the IgG, IgA, and IgM classes (15, 23). Antitrachoma antibodies in human eye secretions were predominantly in the IgG class (12), whereas the presence of IgA, IgE, and IgM also have been reported (15, 22). The present neutralization test may be a means of measuring IgG or IgM antibody; however, complement-mediated neutralization by IgA or IgE by means of the alternate pathway (8, 14) cannot be totally excluded at this time.

The general applicability of the neutraliza-

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**Table 3. Neutralization of *C. trachomatis* strains HAR-13 and HAR-36 by human sera and eye secretions**

<table>
<thead>
<tr>
<th>Serum</th>
<th>Reciprocal of serum dilution giving 50% or greater neutralization</th>
<th>Type determined by FA</th>
<th>Reciprocal of FA titer against homologous antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAR-13 (type A)</td>
<td>HAR-36 (type B)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7623</td>
<td>160 &lt;10</td>
<td>A</td>
<td>160</td>
</tr>
<tr>
<td>7150</td>
<td>640 20</td>
<td>A</td>
<td>640</td>
</tr>
<tr>
<td>7439</td>
<td>80 10</td>
<td>A</td>
<td>640</td>
</tr>
<tr>
<td>7447</td>
<td>80 &lt;10</td>
<td>A</td>
<td>320</td>
</tr>
<tr>
<td>7654</td>
<td>&lt;10 320</td>
<td>B</td>
<td>160</td>
</tr>
<tr>
<td>7312</td>
<td>&lt;10 10</td>
<td>B</td>
<td>160</td>
</tr>
</tbody>
</table>

* NT, Not tested. 

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tion test to the typing of chlamydial strains cannot be ascertained since only two strains were used in the present studies. The microim
muno-fluorescence test of Wang and Grayston has been used to establish 14 serotypes of chlamydia of ocular and venereal origin (16) and is a widely accepted method of typing chlamydial strains. The primary usefulness of the neutralization test may be as a tool in the isolation and identification of chlamydial anti
gens not measured by other immunological tests. This test also may be an aid in studying the pathogenicity of chlamydia and in evaluating
the host response to chlamydial infection.

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LITERATURE CITED

