Effect of Polycations, Polyanions, and Neuraminidase on the Infectivity of Trachoma-Inclusion Conjunctivitis and Lymphogranuloma Venereum Organisms in HeLa Cells: Sialic Acid Residues as Possible Receptors for Trachoma-Inclusion Conjunctivitis

CHO-CHOU KUO, SAN-PIN WANG, AND J. THOMAS GRAYSTON

Department of Pathobiology and Department of Epidemiology and International Health, School of Public Health and Community Medicine, University of Washington, Seattle, Washington 98195

Received for publication 22 December 1972

The infectivity of trachoma-inclusion conjunctivitis (TRIC) organisms (TW-5) was enhanced by pretreatment of HeLa cell monolayers before inoculation with diethylaminoethyl (DEAE)-dextran (30 μg/ml) and poly-l-lysine (10 μg/ml) and inhibited by dextran sulphate (250 μg/ml), fetuin (4%), ovomucoid (5%), N-acetyl neuraminic acid (0.5%), and Cholera vibrio neuraminidase (100 U/ml). The infectivity of lymphogranuloma venereum organisms (434) was not affected by DEAE-dextran, fetuin, and neuraminidase, was slightly inhibited by poly-l-lysine, and was inhibited by dextran-sulphate, ovomucoid, and N-acetyl neuraminic acid. The study suggested that sialic acid residues on the cell surface may be specific receptors for TRIC organisms. The receptors for TRIC organisms (TW-5 and TW-3) could be specifically blocked with inactivated (56 C for 30 min) TRIC organisms at the ratio of one live to 100 inactivated TRIC organisms, but not by inactivated lymphogranuloma venereum (434) or influenza virus (A/2/Jap 305).

Diethylaminoethyl (DEAE)-dextran has been reported to enhance the infectivity of psittacosis (5) and trachoma-inclusion conjunctivitis (TRIC) (10) organisms in cell culture. We have tested the effect of DEAE-dextran in HeLa 229 cell culture with TRIC and lymphogranuloma venereum (LVG) strains of all serological types (8). These strains included 10 TRIC and 3 LGV types as classified by a micro-immunofluorescence test (18). It was found that all the TRIC, but none of the LGV, strains tested were enhanced. Since we found that the effect of DEAE-dextran was on the cell surface rather than on the organisms (8), we concentrated our efforts to determine the mechanism of DEAE-dextran action on studying the relation between changes of cell surface and infectivity. Among those substances which have been most commonly used for this purpose are polycations, polyanions (9, 11, 13, 15) and neuraminidase (1, 14, 21). This report is concerned with the effect of pretreatment of HeLa cells with these substances on infectivity of chlamydiae organisms (psittacosis, LGV, and TRIC organisms).

MATERIALS AND METHODS

Chlamydiae organisms. Chlamydial strains used were: G-17 E53 (TRIC-A), TW-5 E9 and E9 H28 (TRIC-B), TW-3 E24 and E16 H29 (TRIC-C), UW-5 E10 (TRIC-E), 440 E6 (LGV-I), 434 E10 and E10 H4 (LGV-II), 404 E10 (LGV-III), and meningopneumoniae Cal-10 E76 (a psittacosis strain). E-number indicates the passage level in eggs and H-number indicates the passage level in HeLa cells. The serological types are indicated in the parenthesis. The origin and source of these strains have been reported (8).

Preparation of inocula. Chlamydiae were propagated in the yolk sac of chicken embryos (16) or in HeLa 229 cell culture (7). Infected yolk sac membranes were homogenized, suspended in sucrose-phosphate-glutamate (SPG) solution (16) to 40% (wt/vol), and frozen at −70 C. The infectivity titer of the inocula was determined by titration in eggs (16). Inocula prepared from cell culture were also preserved in SPG and frozen at −70 C. The elementary body
particle count of the cell culture preparations were estimated by using an electron microscope (17).

Cell culture and medium. HeLa 229 cells were cultured in Eagle minimum essential medium with 10% inactivated fetal calf serum (MEM) without antibiotics (7).

Method of infectivity assay in cell culture. The method of Furness et al. (4) was used. A 1-ml amount of MEM, containing 5 x 10^5 HeLa cells which gave a confluent monolayer at 24 h, was inoculated in a Leighton tube with a window size of 12 by 40 mm and a cover slip of 10 by 22 mm. These one-day-old monolayers were used for the infectivity assay. The medium in a Leighton tube was removed and replaced by 0.1 ml of inoculum diluted in SPG. An inoculum containing 10^6 to 10^8 50% egg-lethal dose (ELD_50) (0.1 to 1% infected yolk sac material) was found to give an optimal inclusion count (50 to 400 inclusions per 30 microscopic fields at x400 magnification). After 2.5 h of absorption at room temperature with intermittent agitation, the inoculum was removed, and the monolayer was rinsed once with 1 ml of MEM. The tube was then fed with 1 ml of MEM and incubated at 35 C for TRIC and 37 C for LGV and psittacosis strains. After 3 days of incubation, the cover slip was removed, stained with Giemsa, and examined with a microscope at x400 magnification. A square micrometer (7 by 7 mm) was placed in the ocular. An inclusion count was made in 30 fields. The result was expressed by an average count per 30 fields from 3 or 4 cover slips. Each test was run at least twice.

Pretreatment of cells. The following substances were used for pretreatment of cells before inoculation: MgCl_2, DEAE-dextran (2 x 10^4 mol wt) and dextran-sulphate (dextran-S0_4) (5 x 10^4 mol wt) (Pharmacia, Uppsala, Sweden), poly-l-lysine (poly-lys) (139,000 mol wt) (Sigma Chemical Co., St. Louis, Mo.), fetuin, ovomucoid, N-acetyl-neuraminic acid (NANA), and Cholera vibrio neuraminidase (Calbiochem, San Diego, Calif.). All the substances except neuraminidase were dissolved in Hanks balanced salt solution (BSS) at pH 7.2 to 7.4 and sterilized by autoclaving (MgCl_2, DEAE-dextran, dextran-SO_4, and poly-lys) or by filtration (fetuin, ovomucoid, and NANA). Pretreatment of cells was done by removing the medium and rinsing the monolayer twice with 1 ml of BSS (control) or BSS containing various concentrations of the test substances. The tube was inoculated immediately after the second rinsing.

Pretreatment of cells with neuraminidase. Neuraminidase (100 U/ml) was prepared in 0.02 M tris(hydroxymethyl)aminomethane (Tris)-maleic acid buffer in BSS, pH 6.2, containing 0.1% CaCl_2 and 0.05% bovine plasma albumin. The preparation was sterilized by filtration. The medium was removed, the monolayer was rinsed twice with Tris buffer, and 1 ml of either neuraminidase or Tris buffer control was added to each tube. After 1 h of incubation at 37 C, neuraminidase or buffer was removed. The monolayer was rinsed twice with BSS and inoculated.

Inhibiting cell culture infection with inactivated organisms. Chlamydiae (TW-5, TW-3, and 434) were propagated in cell culture and influenza (A/H1N1 73) in the allantoic sac of chicken embryos. Cell culture preparations were adjusted to contain at least 1.4 x 10^10 particles per ml. Influenza virus was pelleted from the infected allantoic fluid by centrifugation at 30,000 x g for 2 h and concentrated 10-fold in 0.1 M phosphate buffer saline (PBS). The preparation contained 25,600 hemagglutination units per ml. Chlamydiae and influenza were inactivated in a 56 C water bath for 30 min. The inhibition experiments were run as follows. An inoculum containing 1.4 x 10^10 live particles per ml (a multiplicity of infection of 20 particles per cell) was mixed with an equal volume of various concentrations of inactivated chlamydiae or influenza. A 0.2-ml amount of the mixture was inoculated onto a cell monolayer which had been rinsed twice with BSS. After 2.5 h of absorption, the inoculum was removed and the cell monolayer was rinsed and fed with MEM and incubated for 3 days at 35 C. Inclusion counts were done as described above.

Neuraminidase assay. HeLa grown TW-5 organisms (7) at a concentration of 10^8 particles per ml were used as enzyme preparation. Intact and sonically treated, as well as Triton-X 100-treated organisms were tested. Sonic treatment was done for 30 min at a minium intensity with a Biosonic III sonicator (Bronwill Scientific, Rochester, N.Y.) with a microtip. Detergent treatment was done by first pelleting the organisms by high-speed centrifugation (30,000 x g for 30 min) and dissolving the pellet in 20% of Triton-X for 24 h. The Triton-X was removed by dialysis. The substrates were neuramnbose lactose, fetuin (Calbiochem), and alpha-glycoprotein (purified from human serum, kindly supplied by Sen-Itiro Hakomori). Acetic acid-sodium acetate buffers containing 0.1% CaCl_2 and 0.05% bovine plasma albumin, at pH 4.2, 4.6, 5.0, 5.4, and 5.8, were used. A mixture of 0.05 ml of buffer, 0.05 ml of an aqueous solution of substrate (neuraminose lactose, 2 mg/ml; alpha-glycoprotein, 1 mg/ml; or fetuin, 2.4%), and 0.1 ml of an aqueous solution of TW-5 (10^8 particles) was incubated at 37 C for 3 h. The reaction was stopped by adding 0.1 ml of 0.2 M sodium periodate, and the free sialic acid was then determined by the thiobarbituric acid method of Warren (19). A positive control using C. vibrio neuraminidase was included in every test.

RESULTS

Effect of cations, polycations, and polyanions on TW-5 strain. HeLa cells were pretreated with Mg^{2+}, DEAE-dextran, poly-lys, and dextran-SO_4 at various concentrations. Tubes were inoculated with a 1% suspension of TW-5-infected yolk sac (10^8 ELD_50). The results are presented in Fig. 1. No significant effect was observed with Mg^{2+} at concentrations up to 50 mM. Enchancement of infectivity was seen with polycations, DEAE-dextran and poly-lys. The greatest enhancement was obtained at 30 mg of DEAE-dextran per ml (11-fold increase) and at 10 mg of poly-lys per ml (5-fold increase). The size of the inclusions was larger when the cells were treated. An inhibitory effect was seen with the polyanion, dextran-SO_4. At 250 mg of dex-
of Poly-lys egg at completely removed solutions. After the strains in was mer inclusion were treated with BSS. The inclusion counts in the cells rinsed twice before inoculation with the substances at various concentrations as indicated in the figure. Inclusion counts were the average of 2 to 3 quadruplicate tests.

Figure 1. Dose-response relations between infectivity of TW-5 and Mg, DEAE-dextran, poly-lys, and dextran-SO₄ in HeLa cell culture. Cells were washed twice before inoculation with the substances at various concentrations as indicated in the figure. Inclusion counts were the average of 2 to 3 quadruplicate tests.

Dose-response relations between infectivity of TW-5 and Mg, DEAE-dextran, poly-lys, and dextran-SO₄ in HeLa cell culture. Cells were washed twice before inoculation with the substances at various concentrations as indicated in the figure. Inclusion counts were the average of 2 to 3 quadruplicate tests.

Effect of pretreatment of cells with neuraminidase inhibitors on TW-5 and 434 strains. Table 2 presents the results of inclusion counts with TW-5 and 434 in cells pretreated with fetuin (1 and 4%), ovomucoid (1 and 5%), and NANA (0.1 and 0.5%). The infectivity of TW-5 was consistently inhibited by fetuin, ovomucoid, and NANA, while 434 was inhibited by ovomucoid and NANA but not by fetuin.

Effect of pretreatment of cell monolayers with neuraminidase on the susceptibility to some chlamydial strains. Four TRIC strains of different serologic types (A, B, C, and E), three LGV strains of different serologic types (I, II, and III), and psittacosis strain, meningopneumonitis Cal-10, were inoculated onto HeLa cells pretreated with neuraminidase or buffer in triplicate tests. Pretreatment of cells with neuraminidase inhibited infection by all of the TRIC strains, but not the LGV and psittacosis strains. The results are presented in Table 3.

Neuraminidase activity in TW-5 organisms. Since the above experiments suggested that sialic acid residues on the HeLa cell surface may be receptors for TRIC organisms, TW-5 organisms were tested for the presence of neuraminidase (sialidase). No neuraminidase was detected in the intact organism when neuraminidase was detected in the intact organism when neuraminidase was detected in the intact organism.

Effect of DEAE-dextran on neuraminidase-pretreated cells. HeLa cells were incubated with neuraminidase or buffer. After incubation, cells were rinsed twice with BSS or DEAE-dextran (30 µg/ml) and inoculated immediately with TW-5 or meningopneumonitis Cal-10. The results of two tests are presented in.

was observed with 434. There was no difference in the inclusion counts and the titer between the cells treated with DEAE-dextran and BSS. However, a slight inhibitory effect with poly-lys was observed. The inclusion counts in the cells treated with poly-lys were one-third of the BSS-treated controls at 10⁻³ and 10⁻⁴ dilutions.

Effect of polyanions on 434 strain. Since the infectivity of 434 was not enhanced by polycations, its response to polyanions was studied. Cells were treated with dextran-SO₄ at concentrations of 0, 1, 2.5, 5, 10, 15, 20, and 25 µg/ml and inoculated with a 0.1% suspension of 434 infected yolk sac (10⁶ ELD₅₀). Infectivity was inhibited. More than 93% inhibition was seen at concentrations of 10 to 25 µg/ml per ml the inclusion counts were about one-tenth that of the controls. The viability of the organism was not affected by dextran-SO₄. The egg infectivity titration of the organism treated with 250 µg of dextran-SO₄ per ml for 2.5 h at room temperature gave an ELD₅₀ of 10⁻³, as compared to 10⁻⁴ for the untreated control. Poly-lys was found to be toxic to HeLa cells at 20 µg/ml and dextran-SO₄ at 500 µg/ml. The enhancing effect of DEAE-dextran was not readily removed by rinsing with BSS but was completely removed by dextran-SO₄. When cells pretreated with 30 µg of DEAE-dextran per ml were rinsed with BSS 5, 15, and 30 min after the DEAE-dextran treatment, enhancement was 60, 80, and 80% of the unrinsed controls, respectively. When DEAE-dextran pretreated cells were rinsed with 25 µg of dextran-SO₄ per ml, enhancement by the former was completely nullified.

Infectivity titration of TW-5 and 434 strains in HeLa cells pretreated with polycations. HeLa cells were pretreated with 30 µg of DEAE-dextran per ml or 10 µg of poly-lys per ml and inoculated with 10-fold dilutions of TW-5 or 434 infected yolk sac, with 10% (wt/vol) suspensions being regarded as 10⁻¹. Controls were pretreated with BSS. The inclusion counts are shown in Table 1. Enhancement was observed with TW-5. Seven to 14-fold increases in inclusion counts were seen at 10⁻¹ and 10⁻² dilutions. The titers were 2 and 3 logs higher than controls in the DEAE-dextran- and poly-lys-treated cells, respectively. No enhancing effect
TABLE 1. Effect of polycations on the infectivity of TRIC (TW-5) and LGV (434) in cell culture

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment of cells</th>
<th>Dilution of infected yolk sac in inocula</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10^-2</td>
</tr>
<tr>
<td>TRIC</td>
<td>BSS</td>
<td>13*</td>
</tr>
<tr>
<td>TW-5</td>
<td>DEAE-dextran, 30 µg/ml</td>
<td>131</td>
</tr>
<tr>
<td></td>
<td>BSS</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Poly-L-lysine, 10 µg/ml</td>
<td>107</td>
</tr>
<tr>
<td>LGV</td>
<td>BSS</td>
<td>434</td>
</tr>
<tr>
<td>434</td>
<td>Poly-L-lysine</td>
<td>154</td>
</tr>
</tbody>
</table>

* Average number of inclusions per 30 fields.
* Inclusions found by scanning the entire cover slip.

TABLE 2. Effect of neuraminidase inhibitors on the infectivity of TRIC (TW-5) and LGV (434) in cell culture

<table>
<thead>
<tr>
<th>Neuraminidase inhibitors</th>
<th>Concentration (%)</th>
<th>Inclusion counts</th>
<th>TRIC (TW-5)</th>
<th>LGV (434)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetuin</td>
<td>0</td>
<td>157*</td>
<td>215</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>21</td>
<td>299</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>7</td>
<td>241</td>
<td></td>
</tr>
<tr>
<td>Ovomucoid</td>
<td>0</td>
<td>33</td>
<td>168</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>7</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>N-acetyl</td>
<td>0</td>
<td>86</td>
<td>172</td>
<td></td>
</tr>
<tr>
<td>Neuraminic acid</td>
<td>0.1</td>
<td>27</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>2</td>
<td>27</td>
<td></td>
</tr>
</tbody>
</table>

* Average number of inclusions per 30 fields.

Table 4. The infectivity of TW-5 was enhanced by DEAE-dextran and inhibited by neuraminidase. DEAE-dextran appeared to partially reverse the inhibitory effect of neuraminidase. The infectivity of Cal-10 was enhanced by DEAE-dextran and not inhibited by neuraminidase. The enhancing effect of DEAE-dextran with the Cal-10 was apparent even in the neuraminidase-pretreated cells.

**Inhibition of infection with inactivated organisms.** Live and inactivated TW-5 organisms were mixed at the ratios of 1:1, 1:10, and 1:100, to determine the effective dosage of inactivated organisms to inhibit HeLa cell infection. It was found that infection of TW-5 was effectively inhibited at a ratio of 1:100. In a similar experiment, no inhibition was observed with 434 at a ratio of 1:100 or 1:1,000. Cross inhibi-

**DISCUSSION**

HeLa cell infection with TW-5 was enhanced by polycations but inhibited by polyanions. Since the cell surface is negatively charged due to the presence of sialic acid residues on the surface of the plasma membrane (1), it is tempting to assume that TRIC organisms are...
negatively charged; hence, adsorption onto the cells would be enhanced by pretreatment of cell monolayer with DEAE-dextran or poly-lys and inhibited by dextran-SO₄. Failure of the Mg ion to enhance the infectivity may be due to its low molecular size (9, 12).

If TRIC organisms are nonspecifically adsorbed onto the cell only by the interaction of electric charge, an enhancing effect might have been observed when the cell surface was rendered less negative by removing sialic acid residues with neuraminidase. However, TRIC infection was inhibited by removing sialic acid residues from the cell surface by neuraminidase, suggesting that the sialic acid residues may serve as receptors. The inhibitory effects observed with neuraminidase inhibitors (fetuin, ovomucoid, and NANA) may be due to competition by the inhibitors for the combining sites of TRIC organisms. Since LGV-434 does not require sialic acid receptors, the inhibition by ovomucoid and NANA could be due to other factors. The presence of specific receptors on the cell surface for TRIC organisms is strongly suggested by the inhibition of infection with inactivated TRIC organisms, but not by inactivated LGV and influenza.

Since sialic acid receptors appear to be necessary for adsorption of TRIC organisms, the main action of DEAE-dextran may well be to enhance the adsorption of TRIC to the receptors on the HeLa cell surface. Alternative explanations such as stimulation of nonspecific uptake of foreign protein (11, 13) seem less attractive because of failure of DEAE-dextran to enhance LGV infection. Also DEAE-dextran action was associated with the sialic acid receptors since only slight enhancement was seen after neuraminidase treatment (probably due to residual receptors).

This study revealed some interesting biological differences between TRIC and LGV organisms. The finding that the infectivity of TRIC, but not of LGV, is inhibited by neuraminidase and inactivated homologous organisms, may be useful for the differentiation of TRIC and LGV organisms in addition to the method of infectivity enhancement with DEAE-dextran which we reported previously (8). It appears that there are three groups of Chlamydia: TRIC, LGV, and psittacosis, with respect to reaction to DEAE-dextran and neuraminidase. The infectivity of TRIC organisms was enhanced by DEAE-dextran and inhibited by neuraminidase; LGV was not influenced by either; and psittacosis (one strain tested) was enhanced by DEAE-dextran but was not affected by neuraminidase.

In spite of all efforts we failed to detect neuraminidase activity in TRIC organisms. It has been known that TRIC organisms are not readily released from cells to start the second cycle of infection in new cells (2, 6). The influenza virus neuraminidase is a specific enzyme which hydrolyzes terminal sialic acid from the specific virus receptors on the cell surface and allows the virus particles to elute (20). If the analogy could be applied it is conceivable that TRIC organisms are not readily released from the host cells because of the absence of neuraminidase.

Fris (3) has reported a study on the mechanism of uptake of radioisotope-labeled C. psittaci (meningopneumonitis strain) by L cells. His study showed that entry of the parasite was accomplished by an act of phagocytosis by the host which was dependent on temperature and host energy metabolism, and that adsorption was not a significant factor in the process of uptake since attachment of C. psittaci to L cells was nearly imperceptible at 0 C. Although our infectivity assay based on inclusion count was a combined result of attachment, entry, development, and maturation of the TRIC-LGV organisms, only the stage of attachment and entry

### Table 4. Infectivity of TW-5 and Cal-10 strains in HeLa cells pretreated with DEAE-dextran or neuraminidase, or both

<table>
<thead>
<tr>
<th>Strains</th>
<th>Test no.</th>
<th>Pretreatment of cell monolayer</th>
<th>Buffer</th>
<th>DEAE-dextran (30 μg/ml)</th>
<th>Neuraminidase (100 U/ml)</th>
<th>Neuraminidase + DEAE-dextran</th>
</tr>
</thead>
<tbody>
<tr>
<td>TW-5</td>
<td>1</td>
<td>57*</td>
<td>181</td>
<td>7</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>46</td>
<td>115</td>
<td>7</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Cal-10</td>
<td>1</td>
<td>12</td>
<td>156</td>
<td>10</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>18</td>
<td>207</td>
<td>21</td>
<td>179</td>
<td></td>
</tr>
</tbody>
</table>

* Cell monolayer was treated first with neuraminidase and then with DEAE-dextran.

** Average number of inclusions per 30 fields.

### Table 5. Cross-inhibition tests of HeLa cell infection by inactivated organisms at a ratio of 1:100 live to inactivated organisms

<table>
<thead>
<tr>
<th>Infecting strains</th>
<th>Inactivated strains*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TW-5</td>
</tr>
<tr>
<td>TRIC/B/TW-5</td>
<td>2</td>
</tr>
<tr>
<td>TRIC/C/TW-3</td>
<td>1</td>
</tr>
<tr>
<td>LGV/II/434</td>
<td>72</td>
</tr>
</tbody>
</table>

* Inactivated at 56 C for 30 min.

** Average number of inclusions per 30 fields from 2 to 4 triplicate tests.
SIALIC ACID AS RECEPTOR FOR TRIC

was likely to have been affected by pretreatment of the host cell. Our study was not designed to determine specifically the effect of the various treatments of the host cell on attachment of the organism to the cell versus entry into the cell after attachment.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grant no. 5-R01-EY-00219 from the National Eye Institute.

The technical assistance of Vicki McCartney is gratefully acknowledged.

LITERATURE CITED