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Binding of the Glycan of the Major Outer Membrane Protein of *Chlamydia trachomatis* to HeLa Cells

ALBERTINA F. SWANSON AND CHO-CHOU KUO*

Department of Pathobiology, University of Washington, Seattle, Washington 98195

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Recent studies have shown that the major outer membrane protein (MOMP) of *Chlamydia trachomatis* is glycosylated. The glycan of the MOMP of *C. trachomatis* serovar L_2 was separated from the glycoprotein with *N*-glycanase, reduced with tritiated $NaBH_4$, and tested for its ability to interact with HeLa cells. The [^{3}H]glycan was shown to attach readily to HeLa cells at 25 or 37°C. This process was slower at 4°C. Competition for possibly similar receptor sites on HeLa cells between the glycan and a sugar, an aminosaccharide, or elementary bodies (EBs) was then studied. D-Galactose, D-mannose, or *N*-acetylglucosamine was shown to reduce the attachment of the glycan to HeLa cells at concentrations of 0.1 to 0.5 M. Sedoheptulose, D-fructose, or sialic acid did not inhibit the binding of glycan to HeLa cells. The presence of at least 100 native or UV-inactivated EBs per HeLa cell interfered with the glycan's ability to bind to HeLa cells. Heat-inactivated EBs did not compete with the glycan for binding. In the reverse situation, nonradiolabeled glycan prevented the EBs from infecting and forming inclusions in HeLa cells. Incubation of [^{3}H]glycan with rabbit immune serum prepared against antigens of whole EB and the MOMP inhibited attachment. In contrast, incubation of glycan with mouse monoclonal antibodies against the protein portion of the MOMP or the chlamydial lipopolysaccharide did not inhibit attachment. These results suggest that the glycan portion of the MOMP is involved in the attachment process of *C. trachomatis* organisms to HeLa cells.

The obligate intracellular bacterium *Chlamydia trachomatis* contains a cysteine-rich major outer membrane protein (MOMP) with a molecular mass of 40,000 Da (19). Besides regulating the exchange of nutrients and metabolites for the intracellular form of the developmental cycle, i.e., reticulate body (1), the MOMP is the principal structural protein exposed on the surface of the infectious elementary body (EB) (2). Interaction between *C. trachomatis* and the host cell has been partly attributed to the MOMP (24). This has been demonstrated by neutralizing the infectivity of chlamydia with antibodies that react with the serological determinants on the MOMP (5, 18). Eukaryotic cell components have also been shown to bind to the MOMP (24, 26).

We recently reported that an N-linked carbohydrate is associated with the structure of the MOMP (27). Isolated MOMP bound specifically to concanavalin A, wheat germ agglutinin, and *Dolichos biflorus* agglutinin in the lectin binding assay (27). These lectins recognize α-D-mannose, D-*N*-acetylglucosamine, sialic acid, and α-*N*-acetylgalactosamine. Determination of the glycan structure of the MOMP has been hampered by the difficulty in obtaining sufficient amounts of purified carbohydrate. The significance of the MOMP as a glycoprotein needs to be addressed. The information available on glycoproteins in prokaryotes is limited. However, the functional aspects in eukaryotes have been studied extensively (20, 21). One of the several functions of the glycan moieties of glycoproteins in eukaryotes is cell adhesion. We examined the interaction of the radiolabeled glycan of the MOMP with HeLa cells and its role in the attachment process of chlamydial organisms to mammalian host cells.

* Corresponding author. Mailing address: Department of Pathobiology, SC-38, University of Washington, Seattle, WA 98195. Phone: (206) 543-8689. Fax: (206) 543-3873.

MATERIALS AND METHODS

Organisms. EB preparations of *C. trachomatis* L_2/434/Bu were grown in HeLa 229 cells and were purified by Hypaque gradient centrifugation (Hypaque-76; Winthrop Laboratories, Sterling Drug Inc., New York, N.Y.) (15). The number of EB particles was enumerated after direct fluorescent-antibody staining with a species-specific monoclonal antibody (MAb) against the MOMP (Syva Co., San Jose, Calif.) (12).

Antisera. Following our method described previously (23), three murine MAbs were prepared: a *C. trachomatis* serovar L_2-specific MAb, 155-35, immunoglobulin G1 (IgG1) (18); a *C. trachomatis* species-specific MAb, KK-12, IgG2a (27); and a chlamydia genus-specific MAb, CF-2, IgG2a (27). The three MAbs have been tested by immunoblotting against chlamydiae. The serovar-specific MAb 155-35 has reacted only to the MOMP of serovar L_2. The species-specific MAb KK-12 has reacted with the MOMP of all known human *C. trachomatis* serovars. The genus-specific MAb CF-2 is reactive against the lipopolysaccharide. Both the species- and serovar-specific MAbs reacted accordingly to the EBs of respective serovars in the microimmunofluorescent-antibody test (29). The concentration of MAbs was standardized to contain 1 mg of IgG per ml. The rabbit immune sera were prepared by immunization of New Zealand White rabbits with whole EBs, isolated MOMP (6), or affinity-purified 155-kDa protein (3) of serovar L_2 as previously described (4). Anti-L_2 MOMP antiserum was obtained from H. D. Caldwell at the National Institutes of Health, Rocky Mountain Laboratories, Hamilton, Mont. Pre- and postimmunization sera were obtained for testing. The antibody titers determined by indirect fluorescent-antibody staining of L_2 inclusions in HeLa cells were 1:4,000 for anti-L_2 whole EB antiserum, 1:2,000 for anti-L_2 MOMP antiserum, and 1:>16,000 for anti-L_2 155-kDa antiserum. Antibody titers against L_2 were negative for all preimmune sera.

SDS-PAGE and immunoblotting. Sodium dodecyl sulfate

polyacrylamide gel electrophoresis (SDS-PAGE) was run by the method of Laemmli (16). For immunoblotting, the procedure of Towbin et al. (28) was followed.

Determination of carbohydrate and protein. The presence of carbohydrate was determined by the orcinol-sulfuric acid method (10). Proteins were quantitated by the bicinchoninic acid protein assay protocol (Pierce, Rockford, Ill.) (22).

Preparation of 40-kDa glycoprotein. Whole-cell lysates of EBs of L_2 serovar were separated on an SDS–12.5% polyacrylamide gel. The 40-kDa glycoprotein was excised, electroeluted from the gel as described previously (27), and stored at −20°C. The electroeluted material from 60 gels (3.2 mg) was pooled and concentrated by centrifuging at 5,000 × *g* at 4°C in an Ultrapure filter unit with an exclusion factor of a molecular weight of 10,000 (Millipore, Bedford, Mass.). The isolated glycoprotein was run on an SDS–12.5% polyacrylamide gel and transferred onto nitrocellulose. The 40-kDa glycoprotein was visualized by Aurodye and was confirmed to be MOMP by immunoblot reaction against MAb KK-12.

Delipidation. The isolated 40-kDa glycoprotein was voided of lipid contamination by using the following procedure described by Finne and Krusius (9). Briefly, 8 volumes of methanol was added to the 40-kDa glycoprotein and mixed. Four volumes of chloroform was then added. The mixture stood at 25°C for 30 min and was then centrifuged at 15,000 × *g* for 15 min. The pellet was resuspended in water-methanol-chloroform (3:8:4) and centrifuged again. The pellet contained the glycoprotein.

Separation of glycan from 40-kDa glycoprotein. The carbohydrate used in this study was released from the glycoprotein by incubation for 48 h at 37°C with 0.3 U of *N*-glycanase (Genzyme Corp., Boston, Mass.) by the Genzyme protocol (11). Prior to use, *N*-glycanase was dialyzed against 0.2 M sodium phosphate buffer (pH 8.6) to remove glycerol which would interfere with the sodium borohydride assay. The enzymatic reaction was halted by boiling for 5 min or by adding SDS-PAGE sample buffer. Following the addition of 3 volumes of ice-cold 95% ethanol, the mixture was centrifuged at 5,000 × *g* for 10 min. The supernatant was saved, and the pellet was washed with 2 to 3 ml of 75% ethanol and centrifuged again. The pellet was quantitated for protein and evaluated for the absence of carbohydrate by the orcinol-sulfuric acid procedure. The supernatants that contained the glycan were combined, and the liquid was evaporated with a stream of nitrogen.

Radiolabeling of glycan. The glycan preparation was reduced with 0.2 ml of sodium borate (pH 9.8) and 1.2 Ci of tritiated sodium borohydrate ($NaB_3{}^3H_4$) per mmol for 3 h at 30°C (8). The reaction was terminated by adding 100 μl of glacial acetic acid. The sample was desalted by gel filtration on a Bio-Gel P-2 column (Bio-Rad, Richmond, Calif.). The radioactive contaminants were removed by paper chromatography that was run on Whatman 3MM filter paper in butanol-ethanol-distilled water (4:1:1) for 48 h. Verification that there was no contamination of protein from glycanase or the deglycanated protein was done with ninhydrin. The glycan was recovered by eluting with water from 0 to +5 cm from the origin of the paper chromatograph. The glycan-water mixture was concentrated to 0.5 ml. This was diluted 1:10 in 0.1 M phosphate buffer (pH 7.0) and used in the adhesion assays. Samples of glycanase only, sodium borate only, and deglycanated protein were radiolabeled. Sodium borate only was used as the negative control, having less than 100 counts per 10 ml.

Preparation of HeLa cell monolayers. Cells were cultured in Eagle's minimum essential media (MEM) with 10% fetal calf serum. One milliliter of 2×10^5 cells was pipetted into a 1-dram (4-ml) shell vial with a rubber stopper. Vials were incubated at 35°C overnight until the monolayer was confluent on the bottom of the glass vial; the monolayer was then used for adhesion assays.

Adhesion assay. Adherence of the glycan to HeLa cells was examined by the method described previously for radiolabeled EBs (13). Before addition of reactants onto HeLa cell monolayers, MEM was removed and washed twice with balanced salt solution (BSS). A total volume of 100 μl of the tritiated glycan mixture was inoculated into a shell vial containing a monolayer of HeLa cells. One set of vials was incubated at 4°C, another was incubated at room temperature (22 to 26°C), and a third was incubated at 37°C. All assays were performed in duplicate and repeated. At specific time intervals (5, 15, 30, 60, and 120 min), the inoculum from two vials from each temperature group was removed. The cell monolayer in the vial was washed thrice with phosphate-buffered saline (PBS). One milliliter of NCS tissue solubilizer (Amersham, Arlington Heights, Ill.) was added to each vial. Parafilm-covered vials were left at room temperature overnight. On the following day, the contents in each vial were dissolved in 10 ml of scintillation fluid (Aqueous Counting Scintillant, Amersham) and counted on a scintillation counter (LS-5800 series; Liquid Scintillation Systems, Beckman Instruments, Inc., Palo Alto, Calif.). The background counts were determined by measuring the adherence of the labeled glycan to uninoculated vials. The appearance of the cells in the vials were observed microscopically before the beginning and at the final step of the assay to ensure the confluency of cell monolayers.

Competition experiments. In these experiments, either sugar or EB and [^{3}H]glycan were added together to make a final volume of 1 ml. From that mixture, 0.1 ml was inoculated into the vial of HeLa cells and adsorbed for 30 min at 4°C. The solution was removed, and the cells were washed. The cell-associated radioactivity was counted as described above and compared with that in vials that had only PBS and glycan added to the HeLa cell monolayer. In the experiments with antibody, the same procedure was followed, except glycan and antisera were incubated for 1 h at room temperature prior to being inoculated into the vial.

Agents for inhibition assays. D-Galactose, *N*-acetylglucosamine, D-mannose, D-fructose, and sedoheptulose were used at 0.5, 0.1, and 0.02 M concentrations. Sialic acid was tested at 0.0325, 0.0065, and 0.0013 M. All were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Treatment of the EBs. (i) For heat inactivation, the EBs were incubated at 56°C in a water bath for 30 min. (ii) For UV irradiation, EBs in a small petri dish were irradiated with a germicidal lamp (Westinghouse G30T8) at a distance of 13 cm for 15 min.

Inhibition of EBs' infectivity of HeLa cells with glycan. A confluent monolayer of HeLa cells was attained by following the same procedure as described above for preparation of HeLa cell monolayers, except each culture vial contained a round coverslip. The monolayer was washed twice with BSS. One hundred microliters of L_2 organisms and 100 μl of nonradiolabeled glycan were inoculated into each vial and adsorbed onto the cells for 1 h at 25°C. Two control inocula were used: (i) EBs and PBS and (ii) EBs and ethanol-precipitated deglycanated protein. After incubation, the inoculum was removed and washed with BSS, and 1 ml of MEM was added to each vial. To rule out that glycan was not toxic to chlamydial organisms, the glycan was added

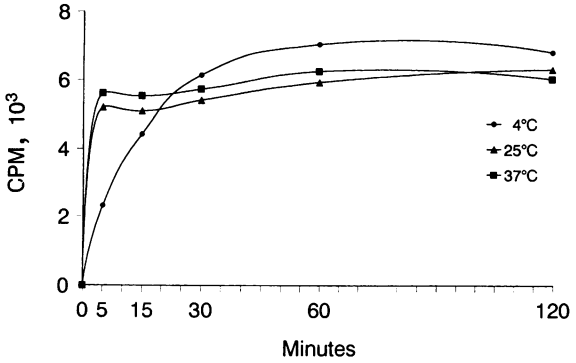

FIG. 1. Attachment of the [^{3}H]glycan of L_2 serovar of *C. trachomatis* to HeLa 229 cells at three different temperatures monitored for 120 min.

with MEM after the adsorption of EBs onto cell monolayers. The infected HeLa cells were incubated for 72 h at 35°C. After the MEM was removed, the coverslips were fixed with methanol and stained by a direct fluorescent-antibody method with the genus-specific MAb CF-2. Inclusions in triplicate coverslips were counted in 30 fields at a 400× magnification under a fluorescence microscope.

RESULTS

Isolation and radiolabeling of glycan. The glycan portion of the SDS-PAGE-electroeluted 40-kDa GP of L_2 organisms was released from the protein by treatment with *N*-glycanase and separated by precipitating the protein with ethanol. The extraction procedure recovered 1.22 mg of protein, which was 98.6% of the initial amount. When run on SDS-PAGE, the recovered protein pellet moved at a different mobility than the pretreated glycoprotein, but retained its immunogenicity against MAb KK-12 by immunoblot as shown before (27). By the orcinol-sulfuric acid method that verifies the presence of hexoses, the optical density at 505 nm for the supernatant containing the glycan was 0.009 compared with 0.400 for 0.125 mg of mannose as a standard. The glycan was reduced with $NaB_3{}^3H_4$ and was removed from the other radioactive components by gel filtration followed by paper chromatography. Protein contamination was not detected when the paper chromatograph containing the glycan was sprayed with ninhydrin reagent.

Adhesion of [^{3}H]glycan to HeLa cells. The attachment of glycan to HeLa cells was monitored over a 2-h period, and incubation was at 4°C, room temperature, or 37°C. Figure 1 shows that the adherence of glycan to HeLa cells was rapid at room temperature or 37°C. The HeLa cell-associated radioactive counts reached a maximum at 5 min and remained constant throughout the incubation period. At 4°C, the binding of glycan to HeLa cells increased gradually and plateaued after 30 min. The fraction of adsorbed glycan was 2.73% of the total count in the inoculum. From these experiments, incubation at 4°C and for 30 min were found to be optimal conditions for studying the initial phase of glycan-cell interaction and were used in subsequent experiments.

Effect of sugars on binding of glycan to HeLa cells. Mannose, galactose, and *N*-acetylglucosamine inhibited the binding of glycan to HeLa cells (Table 1). The maximum inhibition observed was an average of 73% with mannose, 85% with galactose, and 79% with *N*-acetylglucosamine. The inhibition was dose dependent. Fructose and sedoheptulose did not hinder the binding of glycan to HeLa cells. In contrast, 0.0325 M sialic acid enhanced glycan adhesion by up to 94%; the two lower concentrations had no effect.

TABLE 1. Effects of sugars on binding of chlamydial glycan to HeLa cells[a]

Ligand	% Inhibition[b] at following concn (M) of ligand		
	0.02	0.1	0.5
Aldose			
Mannose	20, 25	69, 54	75, 71
Galactose	14, 13	89, 81	86, 80
N-Acetylglucosamine	39, 22	65, 72	71, 87
Ketose			
Sedoheptulose	−5, 8	−13, 4	−1, −14
Fructose	11, −18	−31, 4	−10, −7

[a] Absorption at 4°C for 30 min.
[b] Percent inhibition was calculated from controls without ligand that had radioactivity counts from 2,000 to 2,600 cpm. Results from two tests are shown for each ligand.

Inhibition of glycan binding to HeLa cells with EBs. To determine whether EBs inhibit glycan binding, 2.3×10^4 to 2.3×10^7 EB particles were incubated with glycan and HeLa cells. As shown in Table 2, an average of greater than 70% inhibition was noted with 2.3×10^6 native EBs. The inhibition was dependent on the EB concentration. UV-inactivated EBs also inhibited the binding of glycan to HeLa cells. However, a higher concentration of UV-inactivated EBs was needed to obtain the same percent inhibition. The ligand on EBs competing with glycan for binding sites seems to be heat sensitive, because 2.3×10^4 heat-inactivated EBs produced an average of only 45% inhibition compared with 78% with native EBs and 79% with UV-treated EBs. It has been shown that heat-inactivated EBs attach to HeLa cells less effectively (13).

Blocking of attachment of glycan to HeLa cells with antibody. Rabbit polyclonal antisera as well as three chlamydia-specific MAbs were examined for their abilities to inhibit attachment of the glycan to HeLa cells (Table 3). At the highest concentration, the rabbit immune sera against the whole EB and the MOMP inhibited attachment compared with their preimmune sera. The antiserum against the 155-kDa protein had no effect. The three chlamydia-specific MAbs, including one anti-lipopolysaccharide genus-specific one anti-MOMP species specific, and one anti-MOMP serovar specific, did not affect glycan adherence to HeLa cells.

Inhibition of infection of HeLa cells by EBs with glycan and deglycanated protein. The effect of nonradiolabeled glycan on the infectivity of EBs was measured (Table 4). Three

TABLE 2. Inhibition of binding of chlamydial glycan to HeLa cells by EBs

EB[a]	% Inhibition at following dilution of EBs[b]			
	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Native	74, 81	72, 81	53, 45	15, 6
UV inactivated	80, 77	44, 43	49, 37	35, 23
Heat inactivated[c]	49, 41	30, 40	28, 34	23, 23

[a] Glycan and EBs were of the same serovar, L_2.
[b] $10^0 = 2.3 \times 10^9$ EB particles per ml; 0.1 ml of each dilution from 10^0 was inoculated per vial. Results of two tests are shown.
[c] 56°C for 30 min.

TABLE 3. Effects of antibody on adsorption of the *C. trachomatis* serovar L_2 glycan onto HeLa cells

Antibody	% Inhibition[a] at following antibody dilution		
	10^{-1}	10^{-3}	10^{-5}
Rabbit serum[b]			
Anti-EB preimmune	34	37	40
Anti-EB immune	75	62	48
Anti-MOMP preimmune	25	23	4
Anti-MOMP immune	57	25	7
Anti-155-kDa protein preimmune	12	0	0
Anti-155-kDa protein immune	8	0	4
MAb[c]			
Anti-MOMP serovar specific	1	0	0
Anti-MOMP species specific	14	9	14
Anti-lipopolysaccharide genus specific	14	2	3

[a] Averages of two experiments; 100% adsorption was equal to the radioactivity count of the controls without antibody.
[b] Immunized against whole EB (titer, 1:4,000), isolated MOMP (titer, 1:2,000), or affinity-purified 155-kDa protein of the serovar L_2 (titer, 1:>16,000); titer of 0 for all preimmune sera.
[c] Antibodies used were 155-35 (L_2 specific), KK-12 (species specific), and CF-2 (genus specific); immunoglobulin concentration was 1 mg/ml.

concentrations (undiluted and 1:5 and 1:25 dilutions in PBS) of glycan were tested. One hundred microliters of glycan was mixed with an equal volume of EB suspension containing 10^5 EB particles (predetermined to obtain an optimal inclusion count) and inoculated per culture vial. Inclusion counts were reduced by greater than 50% (74 and 75% in repeated tests) with undiluted glycan. For antibody neutralization of chlamydial infectivity, a reduction in inclusion counts of greater than 50% has been considered significant (18). The glycan was not inhibitory to chlamydial growth. When the glycan was incorporated into the media, the inclusion counts differed by 7 and 12% from the culture that did not contain the glycan. The protein fraction of the MOMP was also shown to inhibit inclusion formation by 78, 60, and 17% at 24.0, 4.8, and 0.96 μg, respectively.

DISCUSSION

In this set of experiments, we studied the interaction between the glycan of the MOMP of *C. trachomatis* serovar L_2 and HeLa cells through competition and adsorption assays.

The results of the competition assays between the sugar or aminosaccharide and the glycan are consistent with those from a previous study using hapten inhibition tests to prevent lectin binding to the MOMP (27). Mannose and galactose had inhibited concanavalin A and *Dolichos biflorus* agglutinin, respectively, while sialic acid did not inhibit wheat germ agglutinin. Likewise, in this study mannose, galactose, and *N*-acetylglucosamine prevented glycan from binding to HeLa cells, while sialic acid was not inhibitory but enhanced binding. In contrast to trachoma organisms, sialic acid has been found not to be necessary for attachment for lymphogranuloma venereum (14). The three ligands (mannose, galactose, and *N*-acetylglucosamine) that did inhibit the binding of the glycan are aldoses which are monosaccharides in which the double bond is located at the first carbon. For the two ketoses tested in which the double bond was at a different position, no inhibition of glycan binding was observed. This finding supports the notion that the ligand-receptor interaction is mediated by the specific glycan structure.

TABLE 4. Inhibition of inclusion formation of the L_2 serovar of *C. trachomatis* in HeLa cells by chlamydial glycan

Test	Inclusion counts[a] (% inhibition)			
	Control	Glycan (dilution)		
		1:1	1:5	1:25
1	434	114 (74)	261 (40)	466 (0)
2	394	99 (75)	288 (27)	399 (0)

[a] Average of number of inclusions per 30 fields, counted in triplicate coverslips.

A competitive nature existed between glycan and EBs. The glycan interfered with the infectivity of the EBs into HeLa cells. Alternatively, EBs interfered with the glycan attachment to HeLa cells. The interference was not due to the binding of glycan to EBs (data not shown). These findings indicate that the carbohydrate is involved in the attachment process of chlamydial organisms to HeLa cells. However, because the glycan's conformation may not be the same as that of the intact glycoprotein, the interaction between the MOMP in the EB and the HeLa cells may differ from the interaction between the glycan and the HeLa cells.

From these experiments, it was determined that the glycan and the protein portion of the MOMP recognize different receptor sites on the HeLa cells. The serovar- and species-specific MAbs against *C. trachomatis* failed to inhibit the glycan from attaching to HeLa cells. These MAbs react only with the protein portion of the MOMP after the MOMP has been treated with glycanase or periodate (27). The finding that rabbit immune sera against whole EB antigens and the MOMP inhibited glycan binding suggests that rabbit immune serum contains antibodies with carbohydrate specificity against chlamydiae.

The function of the protein portion of the MOMP in attachment has been evaluated by Su et al. (25). They reported that trypsin treatment of *C. trachomatis* EBs, which cleaves the MOMP, reduces attachment to HeLa cells. They were not able to inhibit chlamydial infectivity by synthetic peptides, although MAbs recognizing these epitopes neutralize cell culture infectivity (24). These findings led them to suggest that the binding of the MOMP to host cell receptors is mediated by electrostatic interactions contributed by charged hydrophilic residues of the MOMP molecule (24). Inhibition of chlamydial infectivity with the deglycanated MOMP in this study implies that the protein portion is also involved in the attachment process.

The kinetic experiments of attachment of glycan to HeLa cells showed that the binding is temperature and time dependent. At room temperature and 37°C, the binding reached the maximum within 5 min and did not change through 120 min of observation. This suggests that the binding is avid and, once saturated, the receptor sites need to be regenerated before free glycan can attach again. The curve at 4°C was hyperbolic. A rapid increase in attachment of glycan was noted within 30 min, and attachment then reached the saturation point at 60 min. This finding indicates that the initial adherence of glycan to HeLa cell receptors is not temperature dependent. The amount of total glycan attached was equal at the three adsorption temperatures. Similar attachment kinetics have been observed with the

binding of *Streptococcus sanguis* to saliva on hydroxylapatite mediated by lectin-carbohydrate interaction (7) and insulin to rat white fat cells (17).

We have shown that the glycan of the MOMP may be involved in the attachment of *C. trachomatis* organisms to HeLa cells in a ligand-receptor type of interaction and that the binding is mediated by the specific glycan structure. Our study also shows that the use of a defined molecule consisting of specific ligands may facilitate elucidation of the mechanisms of attachment and entry of chlamydial organisms into mammalian host cells.

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