

## Cleavage of the N-Linked Oligosaccharide from the Surfaces of *Chlamydia* Species Affects Infectivity in the Mouse Model of Lung Infection

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**Previous studies determined that the *Chlamydia* glycan contains a high-mannose oligosaccharide, which is involved in attachment and infectivity of the organism, and that removal of the glycan decreases infectivity in vitro. The present study demonstrates that treatment of the organism with *N*-glycanase decreases or ablates infectivity in vivo.**

Chlamydiae are obligate intracellular bacteria whose uptake by nonphagocytic host cells is thought to be mediated by receptor-mediated endocytosis (12). Several mechanisms have been proposed by which chlamydiae attach and enter the host cells as well as ligands that mediate attachment. These ligands include glycosaminoglycans (GAGs) (13), the major outer membrane protein (MOMP) (10), and heat shock protein 70 (7). Our studies have focused on the role that the high-mannose oligosaccharide glycan plays in attachment and infectivity (4). Collectively, these studies demonstrated that *Chlamydia trachomatis* MOMP is glycosylated (11) and that the glycan contains an N-linked high-mannose oligosaccharide (4). Hapten inhibition assays using structurally defined oligosaccharides showed that attachment and infectivity of *C. trachomatis*, *Chlamydia pneumoniae*, and *Chlamydia psittaci* in HeLa cells were inhibited by high-mannose oligosaccharides and that inhibition of metabolically radiolabeled organisms was affected at both 4°C (organisms attach, but are not internalized) and 37°C (organisms attach and are internalized), suggesting that the glycan moiety mediates attachment and infectivity of chlamydiae (4). In a recent study, this role of the glycan was further confirmed by demonstrating that treatment of organisms with *N*-glycanase, which cleaves the chlamydial glycan from the protein backbone, significantly decreased the infectivity of *C. pneumoniae*, *C. trachomatis*, and *C. psittaci* for both HL (human line) cells and endothelial cells, while treatment with *O*-glycanase, which removes O-linked but not N-linked glycan, had no effect (2). The current study investigates whether removal of the surface-exposed glycan from chlamydial organisms affects its infectivity in a mouse model of lung infection.

*C. pneumoniae* AR-39 and *C. trachomatis* UW-5 (E/UW-5/Cx) and L<sub>2</sub>(L<sub>2</sub>/434/Bu) were grown in HL cells and HeLa cells, respectively. Chlamydial organisms purified by density gradient centrifugation were incubated with *N*-glycanase (PNGase F) (Sigma, St. Louis, MO) in 5 mM potassium phosphate buffer, pH 7.5, for 3 h at room temperature (2). Subsequently, organisms were pelleted, washed once, and resuspended in the chla-

mydial transport medium sucrose-phosphate-glutamic acid. Positive controls were organisms incubated in buffer alone. To rule out any effects of residual glycanase in the inoculum on the host cell, HL cells were pretreated with 3 U of *N*-glycanase (a concentration significantly higher than the expected dilution factor) for 1 h and subsequently analyzed for cell viability and susceptibility to *C. pneumoniae* infection. No differences were observed between treated and untreated cells in cell viability or inclusion counts.

Eight-week-old Swiss Webster mice (Charles River Laboratories, Boston, MA) were mildly sedated by intraperitoneal injection of a mixture of ketamine and xylazine and inoculated intranasally with  $1 \times 10^7$  inclusion forming units (IFU) of glycanase-treated or untreated chlamydial organisms. Mice were fed a chow diet and water ad libitum throughout the study. Protocols were approved by the University of Washington Institutional Animal Care and Use Committee. At 3 and 7 days postinfection (p.i.), necropsies were performed. Lungs were dissected intact, immediately placed in sterile glass vials, and placed on ice prior to freezing at  $-70^{\circ}\text{C}$ . Subsequently, lung tissue was thawed and homogenized with a sterile pestle and mortar in sucrose-phosphate-glutamic acid medium to make a 10% (wt/vol) solution. Homogenates were centrifuged at  $500 \times g$  for 5 min to sediment tissue debris. The supernatant was aspirated for inoculation of human epithelial cells (HL cells for *C. pneumoniae* and HeLa cells for *C. trachomatis*) as described previously (5). Briefly, cell monolayers in shell vials were inoculated with 100  $\mu\text{l}$  of the homogenate in triplicate. Following incubation for 3 days at 37°C, one coverslip was stained with either TT-401 (specific for *C. pneumoniae*) or CF-2 (genus specific, used for *C. trachomatis*) conjugated to fluorescein isothiocyanate. The remaining two unstained vials were harvested and passaged onto HL or HeLa cells for confirmation of positive or negative lung cultures. Infected cells were incubated at 37°C for 2 days for *C. trachomatis* and for 3 days for *C. pneumoniae*. Infectivity was quantified by counting inclusions following fluorescent antibody staining and expressed as IFU per ml. Lung burden was calculated by determining the IFU/g of lung. Statistical analyses were done by a  $\chi^2$  or a Student's *t* test. Changes in the molecular mass of the MOMP following *N*-glycanase treatment of organisms was

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TABLE 1. Inhibition of chlamydial infectivity in Swiss Webster mice by pretreatment of organisms with *N*-glycanase

Strain	Inhibition of chlamydial infectivity with (+) and without (-) glycanase treatment							
	Inhibition at 3 days p.i.				Inhibition at 7 days p.i.			
	No. of mice positive/no. of mice tested		IFU/g of lung (% decrease) <sup>a</sup>		No. of mice positive/no. of mice tested		IFU/g of lung (% decrease) <sup>a</sup>	
	-	+	-	+	-	+	-	+
<i>C. pneumoniae</i> AR-39	6/6	0/6 <sup>b</sup>	$2.8 \times 10^5$	0 (100)	6/6	1/7 <sup>b</sup>	$1.1 \times 10^5$	$1.2 \times 10^4$ (91)
<i>C. trachomatis</i> E/UW-5/Cx	6/6	6/6	$1.4 \times 10^6$	$8.4 \times 10^{5b}$ (40)	6/6	6/6	$9.9 \times 10^3$	$1.0 \times 10^{3b}$ (90)
<i>C. trachomatis</i> L <sub>2</sub> /434/Bu	6/6	6/6	$1.1 \times 10^6$	$4.6 \times 10^{2b}$ (99)	6/7	0/7	$2.6 \times 10^5$	0 (100)

<sup>a</sup> Data are the mean titers in lung. Percent decrease is relative to the IFU/g of lung in the control.

<sup>b</sup>  $P < 0.05$ .

analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting (for *C. trachomatis*) using KK-12, a species-specific monoclonal antibody that recognizes the MOMP.

*N*-Glycanase treatment inhibited the infectivity of *C. pneumoniae* AR39 and *C. trachomatis* E/UW-5/Cx and L<sub>2</sub>/434/Bu as measured by either the inability to culture the organism from the lung or a reduced lung burden of organisms (Table 1). However, strain differences were observed in the degree of the reduction. Specifically, *N*-glycanase treatment of *C. pneumoniae* or *C. trachomatis* serovar L<sub>2</sub>/434/Bu greatly reduced the ability of the organism to establish lung infection since the organism was cultured from the lungs of only 1 of 12 mice infected with *C. pneumoniae* (0/6 at 3 days p.i. and 1/6 at 7 days p.i.) and 2 of 13 mice infected with *C. trachomatis* L<sub>2</sub>/434/Bu (2/6 at 3 days p.i. and 0/7 at 7 days p.i.); in comparison, in mice infected with untreated organism, the organism was cultured from lungs of all *C. pneumoniae*-infected mice ( $n = 12$ ) and from 12 of 13 *C. trachomatis* L<sub>2</sub>/434/Bu-infected mice. In contrast, lungs of all mice infected with glycanase-treated *C. trachomatis* E/UW-5/Cx were culture positive ( $n = 12$ ). However, the lung burden was significantly reduced by 40% ( $8.4 \times 10^5 \pm 4.3 \times 10^5$  versus  $1.4 \times 10^6 \pm 5.5 \times 10^5$  IFU/g of lung) and 90% ( $1.04 \times 10^3 \pm 6.1 \times 10^2$  versus  $9.9 \times 10^3 \pm 7.7 \times 10^3$  IFU/g of lung) at 3 and 7 days p.i., respectively, in mice infected with *N*-glycanase-treated organisms in comparison to mice infected with untreated organisms. Analysis of protein profiles by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed a subtle change in the MOMP mobility of *N*-glycanase-treated organisms in comparison to untreated organisms (data not shown), consistent with earlier studies showing a change in mobility after treatment of gel-purified *C. trachomatis* MOMP with *N*-glycanase (11).

This report extends our previous *in vitro* studies demonstrating that chlamydial infectivity was significantly reduced or ablated following treatment that specifically cleaves glycans that are N-linked to the protein backbone by demonstrating that *N*-glycanase treatment significantly reduced lung burden or rendered the organism noninfectious in a mouse model of lung infection. Interestingly, the same patterns of differences in the reduction of infectivity observed *in vivo* strongly correlated with *in vitro* findings (2). Specifically, after infection of HL cells, the infectivity of *C. pneumoniae* and *C. trachomatis* L<sub>2</sub>/434/Bu was almost completely inhibited (97% reduction in infectivity). In contrast, infectivity of *C. trachomatis* E/UW-

5/Cx for HL cells was reduced by 52%. Remarkably, infection of mice with *N*-glycanase-treated *C. pneumoniae* or *C. trachomatis* L<sub>2</sub>/434/Bu resulted in an inability to culture the organisms from the lung or a 99% reduction in lung burden, respectively, at 3 days p.i. In contrast, although lungs of all mice infected with *N*-glycanase-treated *C. trachomatis* E/UW-5/Cx were culture positive, there was a 40% reduction in lung burden at 3 days p.i., which was similar to the reduction observed *in vitro* after a 3-day incubation. Such strain differences have also been reported with the binding of elementary bodies to GAGs (1, 8, 9). For example, the attachment of *C. trachomatis* E was not affected by GAG, while *C. trachomatis* L<sub>2</sub> and *C. pneumoniae* were affected (1).

Overall, these studies suggest that there are differences in the ligands involved in attachment, the exposure of ligands on the cell surfaces, or the host receptors used. Because high-mannose oligosaccharides competitively inhibit the attachment and infectivity of chlamydiae *in vitro*, recent reports have investigated the role of cell surface receptors on the host that bind mannose. In studies determining the susceptibility of mannose receptor-positive (MR<sup>+</sup>) and mannose receptor-negative (MR<sup>-</sup>) mouse macrophages, strain and species differences were also observed (3). Specifically, *C. trachomatis* E/UW-5/Cx and B/TW-5/OT had significantly higher infectivity for MR<sup>+</sup> cells, *C. trachomatis* L<sub>2</sub>/434/Bu had an increased but not significant difference in infectivity of MR<sup>+</sup> cells, and *C. pneumoniae* had significantly higher infectivity of MR<sup>-</sup> cells; this suggests that *C. trachomatis* can use the mannose receptor, while *C. pneumoniae* preferentially uses other receptors. Recently, *C. pneumoniae*, but not *C. trachomatis* E/UW-5/Cx, has been found to use the mannose 6-phosphate receptor for entry into endothelial cells (6). Collectively, these studies suggest that although the glycan is critical to attachment and infectivity, differences exist in the ligands, which affect binding specificities; e.g., the N-terminal mannose of the *C. pneumoniae* glycan may be phosphorylated, which would permit binding to the M6P receptor.

In conclusion, the cumulative evidence *in vitro* supports a role of the chlamydial glycan in attachment and infectivity of host cells. The present studies in a mouse model of lung infection indicate that with two strains from different species that are invasive, removal of the glycan reduces infectivity 99 to 100% and significantly reduces infection of the noninvasive genital strain. These findings suggest alternative targets for

designing therapeutic strategies for prevention of chlamydial infection.

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#### REFERENCES

1. **Davis, C. H., and P. B. Wyrick.** 1997. Differences in the association of *Chlamydia trachomatis* serovar E and serovar L<sub>2</sub> with epithelial cells *in vitro* may reflect biological differences *in vivo*. *Infect. Immun.* **65**:914–2924.
2. **Kuo, C.-C., A. Lee, and L. A. Campbell.** 2004. Cleavage of the N-linked oligosaccharide from the surfaces of *Chlamydia* species affects attachment and infectivity of the organisms in human epithelial and endothelial cells. *Infect. Immun.* **72**:6699–6701.
3. **Kuo, C.-C., M. Puolakkainen, T. M. Lin, M. Witte, and L. A. Campbell.** 2002. D-Mannose-receptor positive and negative mouse macrophages differ in their susceptibility to infection by *Chlamydia* species. *Microb. Pathog.* **32**:43–48.
4. **Kuo, C.-C., N. Takahashi, A. F. Swanson, Y. Ozeki, and S.-I. Hakomori.** 1996. An N-linked high-mannose type oligosaccharide, expressed at the major outer membrane protein of *Chlamydia trachomatis*, mediates attachment and infectivity of the microorganism to HeLa cells. *J. Clin. Investig.* **98**:2813–2818.
5. **Moazed, T. C., C.-C. Kuo, J. T. Grayston, and L. A. Campbell.** 1996. Murine models of *Chlamydia pneumoniae* infection and atherosclerosis. *J. Infect. Dis.* **175**:883–890.
6. **Puolakkainen, M., C.-C. Kuo, and L. A. Campbell.** 2005. Mannose 6-phosphate inhibits growth of *Chlamydia pneumoniae* in human arterial endothelial cells. *Infect. Immun.* **73**:4620–4625.
7. **Raulston, J. E., C. H. Davis, D. H. Schmiel, M. W. Morgan, and P. B. Wyrick.** 1993. Molecular characterization and outer membrane association of a *Chlamydia trachomatis* protein related to the hsp70 family of proteins. *J. Biol. Chem.* **268**:23139–23147.
8. **Rockey, D. D.** 2002. Chlamydial interaction with host cells: recent progress and remaining issues, p. 35–44. *In* J. Schachter et al. (ed.), *Chlamydial infections: proceedings of the Tenth International Symposium on Human Chlamydial Infections*, June 16–21, 2002, Antalya, Turkey. GRAFMAT Basim ve Reklam Sanayi Tic. Ltd., Sti. Antalya, Turkey.
9. **Stephens, R. S.** 1994. Cell biology of chlamydia infection, p. 377–386. *In* J. Orfila et al. (ed.), *Chlamydial infections: proceedings of the Eighth International Symposium on Human Chlamydial Infection*, June 19–24, 1994, Chantilly, France. Societa Editrice Esculapio, Bologna, Italy.
10. **Su, H., N. G. Watkins, Y. X. Zhang, and H. D. Caldwell.** 1990. *Chlamydia trachomatis*-host cell interactions: role of the chlamydial major outer membrane protein as an adhesin. *Infect. Immun.* **58**:1017–1025.
11. **Swanson, A. F., and C.-C. Kuo.** 1991. Evidence that the major outer membrane protein of *Chlamydia trachomatis* is glycosylated. *Infect. Immun.* **59**:2120–2124.
12. **Wyrick, P. B., J. Chong, C. H. Davis, S. T. Knight, M. D. Royal, A. S. Maslow, and C. R. Bagnell.** 1989. Entry of genital *Chlamydia trachomatis* into polarized human epithelial cells. *Infect. Immun.* **57**:2378–2389.
13. **Zhang, J. P., and R. S. Stephens.** 1992. Mechanism of *C. trachomatis* attachment to eukaryotic host cells. *Cell* **69**:861–869.

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