Is lipopolysaccharide a factor in infectivity of *Chlamydia trachomatis*?

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Lipopolysaccharide (LPS) is a major surface component of *Chlamydia trachomatis*, as with all Gram-negative bacteria. The effect of *C. trachomatis* LPS on *C. trachomatis* infectivity of human epithelial cells was investigated. *C. trachomatis* LPS and *C. trachomatis* LPS antibody significantly reduced infectivity, mostly in a dose-dependent manner. As the structure of LPS in *C. trachomatis* is simple and consists only of lipid A and 3-deoxy-D-manno-octulosonic acid (Kdo), we investigated whether lipid A or Kdo was inhibitory to chlamydial infectivity. Polymyxin B, as a lipid A inhibitor, and Kdo considerably reduced *C. trachomatis* infectivity. With all the LPS inhibitors used, there was greater inhibition against serovar E than serovar LGV. These results suggest a role for LPS in chlamydial infectivity. Elucidation of how LPS acts in infectivity and identification of host-cell receptors would help in understanding pathogenicity.

INTRODUCTION

Several mechanisms by which *Chlamydia trachomatis* attaches to and infects host cells have been described. Proposed ligands include the major outer-membrane protein (MOMP) (Su *et al.*, 1990; Kuo *et al.*, 1996), heat-shock protein 70 (Raulston *et al.*, 1993) and glycosaminoglycans (GAGs) (Zhang & Stephens, 1992). For the last there is some debate as to whether the GAG is on *C. trachomatis* or is a host molecule (Taraktchoglou *et al.*, 2001; Fadel & Eley, 2004). However, recent work has shown that interaction of *C. trachomatis* with mammalian cells may be independent of host-cell heparan sulphate GAGs (Stephens *et al.*, 2006). There has also been interest in the entry of *C. trachomatis* into host cells involving cholesterol-rich membrane domains or lipid 'rafts' which appear to be serovar-dependent (Norkin *et al.*, 2001; Stuart *et al.*, 2003). Use of a chlamydial glycolipid exo-antigen vaccine (GLXA; unrelated to lipopolysaccharide) in a vaccine has shown some protection against genital chlamydial infection in a mouse model (Whittum-Hudson *et al.*, 2001), although in other experimental conditions, it enhances infection (Vora & Stuart, 2003). However, little evidence has been reported on whether *C. trachomatis* lipopolysaccharide (LPS) might be implicated in infectivity. This is surprising, as LPS is a major surface component of *C. trachomatis* (Brade, 1999) and has been widely used as the target molecule in laboratory diagnosis by enzyme immunoassay (EIA) (Jones *et al.*, 1984; Chernesky *et al.*, 2001). The present study investigated

whether *C. trachomatis* LPS might be involved in infectivity towards human epithelial cells.

METHODS

Cell lines and *C. trachomatis* serovars. The Hec-1B cell line (endometrial carcinoma) was obtained from the American Type Culture Collection (ATCC) and maintained according to the supplier's instructions. Serovar LGV1 was kindly provided by M. Ward (University of Southampton), and *C. trachomatis* reference strain E/UW-5/CX was a kind gift of D. Dean (Children's Hospital, Oakland Research Institute). Chlamydiae were grown in semiconfluent McCoy cells for 48–72 h in maintenance medium [minimum essential medium Eagle (EMEM) supplemented with cycloheximide (2 µg ml⁻¹)]. The cell suspension containing elementary bodies (EBs) was harvested, and sonicated twice for 10 s at 15 µm amplitude. Cell debris was removed by centrifugation at 500 g for 15 min, and the remaining suspension was further purified by centrifugation at 30 000 g for 1 h at 4 °C. The resulting crude EB pellet was resuspended in 8 ml PBS, sonicated as described above, layered over 30 % urografin (Schering) and centrifuged at 30 000 g. Urografin is commonly used to purify EBs in a discontinuous density gradient. The final pellet was resuspended in sucrose phosphate buffer (SPG; 5 mM glutamine, 0.2 M sucrose, 0.2 M phosphate buffer, pH 7.4) and stored at –70 °C for further use.

Infectivity inhibition assays. Inocula of *C. trachomatis* serovars LGV and E were adjusted so that not more than ~50 inclusions were present in each field at ×400 magnification [$\sim 5 \times 10^5$ inclusion forming units (i.f.u.)] in 100 µl PBS. The inocula were mixed with dilutions of *C. trachomatis* LPS, *C. trachomatis* LPS antibody, polymyxin B (PmB) or synthetic 3-deoxy-D-manno-octulosonic acid (Kdo), and used to infect confluent Hec-1B cell monolayers in 24-well tissue culture (TC) trays, which we have used in previous infectivity studies (Taraktchoglou *et al.*, 2001). *C. trachomatis* LGV LPS was extracted by the method of Nurminen *et al.* (1985) and quantified

Abbreviations: EB, elementary body; GAG, glycosaminoglycan; Kdo, 3-deoxy-D-manno-octulosonic acid; PmB, polymyxin B; TC, tissue culture.

using the Limulus amoebocyte lysate (LAL) test (Cambrex Biosciences). Fig. 1 shows the purity of the extracted LPS after electrophoresis through a 14% acrylamide gel and staining with the Bio-Rad Silver Stain kit. Re mutant LPS from *Salmonella enterica* 595 (Sigma) was used as a control for *C. trachomatis* LPS. *C. trachomatis* LPS mAb was obtained from Biogenesis and shows activity against all *C. trachomatis* serovars. Isotype IgG2A (Sigma) was used as a control for *C. trachomatis* LPS antibody. Both PmB and synthetic Kdo were obtained from Sigma, and PmB nonapeptide (Sigma) and D-glucuronic acid (Sigma) were used as their controls, respectively. The cell monolayers were incubated for 1 h at 37 °C in 5% CO₂, and then washed with PBS three times before adding 1 ml EMEM TC fluid supplemented with cycloheximide to each well. After incubation at 37 °C in 5% CO₂ for 48 h, infected monolayers were fixed, stained, and counted by direct immunofluorescence microscopy using the *C. trachomatis* culture confirmation test (Trinity Biotech). The mean number of inclusion bodies for 25 fields ($\times 400$ magnification) was determined, and compared to the control to calculate the percentage infectivity.

Examination of the binding of LPS to Hec-1B cells using immunohistochemistry. Semiconfluent Hec-1B cell monolayers in 24-well TC trays were incubated with *C. trachomatis* LPS for 2 h, washed twice with PBS then incubated with *C. trachomatis* LPS horseradish peroxidase (HRP)-conjugated antibody for 1 h, and the reaction was developed using 4-chloro-1-naphthol. The reaction was viewed with a light microscope at $\times 1000$ magnification. For comparison, the reaction was carried out using *Escherichia coli* LPS mAb (Biogenesis). The reaction performed in the absence of *C. trachomatis* LPS was used as a negative control. A positive control included Hec-1B cells infected with serovar LGV1, incubated directly with *C. trachomatis* LPS antibody for 1 h at room temperature, and developed using the same method.

RESULTS AND DISCUSSION

Effect of chlamydial LPS on infectivity

LPS from serovar LGV inhibited the infectivity of *C. trachomatis* in a dose-dependent manner (Fig. 2). At all concentrations of LPS used, there was greater inhibition against serovar E than with serovar LGV, such that at 10 $\mu\text{g ml}^{-1}$

Fig. 1. LPS extracted from *C. trachomatis* LGV1 (lane 1) and a control *E. coli* O55:B5 LPS (lane 2) electrophoresed through a 14% acrylamide gel and stained with silver stain.

Fig. 2. Infectivity inhibition assay using *C. trachomatis* LGV LPS. The assay was performed with *C. trachomatis* serovar LGV or serovar E using Hec-1B cells at 37 °C. Re mutant LPS was used as a control. The figure represents the mean of six experiments, and error bars represent SEM. Results were statistically analysed using one-way-paired ANOVA. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; NS, non-significant. To compare inhibitory effects on serovars E and LGV, an unpaired *t* test was used. Using 10 $\mu\text{g ml}^{-1}$ LPS, the two-tailed *P* value was <0.0001 .

ml^{-1} there was 33 and 48% inhibition with serovars LGV and E, respectively ($P<0.0001$). As a control, we used a Re mutant LPS of *S. enterica*, which more closely resembled the overall composition of *C. trachomatis* LPS. Although *S. enterica* LPS is closely related to *C. trachomatis* LPS, the latter has a family-specific epitope (a 2–8 linkage in the core trisaccharide Kdo) which results in a 100-fold decrease in endotoxicity (Kosma, 1999) and is presumably responsible for the difference in infectivity inhibition. It is appreciated that LPS from only one *C. trachomatis* serovar was tested; however, it is widely believed that the structure of LPS from different serovars is in fact very similar (Heine *et al.*, 2003). *C. trachomatis* LPS was not toxic to Hec-1B cells at a concentration of 10 $\mu\text{g ml}^{-1}$.

Effect of anti-*C. trachomatis* antibody on infectivity

Similar findings were obtained with a commercial *C. trachomatis* LPS antibody (Fig. 3). However, for serovar E at anti-LPS concentrations between 12.5 and 75 $\mu\text{g ml}^{-1}$, there appeared to be more of a plateau than dose-dependent effect. At a concentration of 100 $\mu\text{g ml}^{-1}$, the LPS antibody inhibited infectivity of serovars LGV and E by 36 and 54% respectively. We used an isotype IgG2A antibody as a control. The isotype showed no inhibition of infectivity at the highest antibody concentration used (100 $\mu\text{g ml}^{-1}$), confirming that the inhibitory effect of anti-LPS antibody was not due to a non-specific reaction.

Fig. 3. Infectivity inhibition assay using *C. trachomatis* LPS antibody. The assay was performed with *C. trachomatis* serovar LGV or serovar E using Hec-1B cells at 37 °C. Isotype IgG2A was used as a control. The figure represents the mean of six experiments, and error bars represent SEM. Results were statistically analysed using one-way-paired ANOVA. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; NS, non-significant.

Effect of PmB and Kdo on infectivity

PmB is often used as an LPS inhibitor as it specifically binds to lipid A (Morrison & Jacobs, 1976). However, as it also has some antibacterial activity, its use in infectivity experiments could be misleading. Nevertheless, as the drug acts almost exclusively on extracellular pathogens, an antibiotic effect on *C. trachomatis*, which is obligately intracellular, seems unlikely (Redecke *et al.*, 1998). Moreover, we used PmB nonapeptide as a control. This cationic cyclic peptide is derived from PmB. Although it has extremely low antimicrobial activity, it is still capable, like PmB, of binding to LPS (Tsubery *et al.*, 2002). At 100 $\mu\text{g ml}^{-1}$, PmB showed inhibition of infectivity of serovars LGV and E by 49 and 65 %, respectively (Fig. 4), which again is suggestive of LPS, especially lipid A, involvement. Similarly, PmB nonapeptide showed a comparable inhibitory effect, confirming that inhibition by PmB was due to LPS binding, and not due to antibacterial activity. As the structure of LPS in *C. trachomatis* is simple and consists only of lipid A and Kdo, we next investigated whether Kdo was inhibitory. Unfortunately, Kdo from *C. trachomatis* was unavailable, so instead we used synthetic Kdo. Interestingly, at 100 $\mu\text{g ml}^{-1}$ Kdo, there was inhibition of serovars LGV and E by 39 and 57 %, respectively (Fig. 5). As a control, we used another synthetic carbohydrate, D-glucuronic acid, which like Kdo is negatively charged and is also found in chlamydial LPS; this showed no inhibitory effect and confirmed the specificity of Kdo.

Fig. 4. Infectivity inhibition assay using PmB. The assay was performed with *C. trachomatis* serovar LGV or serovar E using Hec-1B cells at 37 °C. PmB nonapeptide was used as a control. The figure represents the mean of three experiments, and error bars represent SEM. Results were statistically analysed using one-way-paired ANOVA. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; NS, non-significant.

Immunohistochemistry

Another approach to investigate the role of LPS in chlamydial adherence was by immunohistochemistry using a specific *C. trachomatis* LPS HRP-conjugated antibody. As shown in Fig. 6, incubation of Hec-1B cells with chlamydial LPS followed by *C. trachomatis* LPS antibody showed a

Fig. 5. Infectivity inhibition assay using synthetic Kdo. The assay was performed with *C. trachomatis* serovar LGV or serovar E using Hec-1B cells at 37 °C. D-Glucuronic was used as a control. The figure represents the mean of three experiments, and error bars represent SEM. Results were statistically analysed using one-way-paired ANOVA. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; NS, non-significant.

Fig. 6. Examination of the binding of *C. trachomatis* LPS to Hec-1B cells using immunohistochemistry. Cells were incubated with chlamydial LPS for 2 h, washed, then incubated with *C. trachomatis* HRP-conjugated antibody for 1 h, and the reaction was developed and immediately viewed with a light microscope ($\times 1000$). (a) Negative control in which *C. trachomatis* LPS was omitted. (b) Hec-1B cells incubated with *C. trachomatis* LPS, followed by *C. trachomatis* antibody. (c) Positive control, in which Hec-1B cells infected with serovar LGV1 were incubated with *C. trachomatis* antibody. Bar, 10 μ m.

positive reaction in the form of a deep-purple colour distributed evenly at the edges of the cells. Hec-1B cells infected with LGV1 serovar and then incubated with *C. trachomatis* LPS antibody showed that most of the cells displayed dark staining. This was used as a positive control. The reaction carried out in the absence of LPS was negative.

In this study, all the findings suggest that *C. trachomatis* LPS plays a role in infectivity towards epithelial cells, although some of our data support the idea that infectivity is complex and likely involves more than one moiety. That

C. trachomatis LPS might be playing a role in infectivity was demonstrated using LPS, *C. trachomatis* LPS antibody, PmB and Kdo as inhibitors. To the best of our knowledge, this is the first report of experiments with PmB nonapeptide and D-glucuronic acid. Immunohistochemistry experiments also suggested that *C. trachomatis* LPS binds to Hec-1B cells. However, the nature of the receptor to which LPS binds remains unclear. An approach using heparin affinity chromatography showed that LPS did not bind to heparin, indicating that the host receptor is unlikely to belong to the GAGs (data not shown). Nevertheless, different results have been shown by other workers (Su *et al.*, 1990; Byrne *et al.*, 1993) in *C. trachomatis* LPS antibody experiments, and although their *C. trachomatis* LPS antibody showed little inhibitory effect, there were several key differences in methodology. The serovar tested was B, the cells used were HaK (Syrian hamster kidney), and the inoculum size was significantly larger. All these differences make it extremely difficult to compare findings. We also realize that the Kdo we used in our experiments was synthetic and that inhibition by Kdo could have been due, at least in part, to its negative charge; however, these results do suggest that further work should be initiated.

We should also make it clear that even though we used a standard method for LPS quantification, it is known that *C. trachomatis* LPS has very low endotoxic activity (Kosma, 1999), and therefore the actual amounts of LPS used in our experiments are likely to have been higher than those measured.

Also of note in our experiments was the fact that unlike many other inhibition studies with *C. trachomatis*, especially those using GAG inhibitors, in which serovar LGV is almost always inhibited to a greater degree than serovar E (Taraktchoglou *et al.*, 2001; Fadel & Eley, 2004), the converse was true when LPS was the target for infectivity. It may suggest that this possible target of infectivity is more important for serovar E than serovar LGV.

LPS, or in some cases lipooligosaccharide (LOS), has been reported to be important in bacterial adherence (Jacques, 1996), and more specifically in attachment of several bacterial pathogens of the genital and respiratory tracts, including *Neisseria gonorrhoeae* (Harvey *et al.*, 2000), *Haemophilus ducreyi* (Alfa & DeGagne, 1997) and *Actinobacillus pleuropneumoniae* (Belanger *et al.*, 1990). For the last organism, further evidence shows that its adherence to porcine tracheal cells is inhibited by *A. pleuropneumoniae* LPS antibody (Paradis *et al.*, 1999). The same workers have shown that the active component of its LPS in adherence is more likely to be Kdo than lipid A (Paradis *et al.*, 1994). Our data also show that Kdo from *C. trachomatis* LPS may play a significant role in *C. trachomatis* infectivity that is therefore not unique.

Although it is premature to consider if lipid 'rafts' might be related in any way to the role of LPS in infectivity, recent evidence suggests that *A. pleuropneumoniae* LPS binds to the phospholipid phosphatidylethanolamine (PE) of the

host (Jeannotte *et al.*, 2003). This is of interest, as back in 1991, *C. trachomatis* was found to bind PE as well as asialo-GM1 and asialo-GM2, and at that time these molecules were considered to be putative host-cell receptors (Krivan *et al.*, 1991).

Several studies have reported that chlamydial LPS is present on the surface of infected cells (Campbell *et al.*, 1994; Wyrick *et al.*, 1994), albeit in low quantities; therefore, it may be that *C. trachomatis*, like *A. pleuropneumoniae*, uses LPS as an adhesin. However, whether surface exposure of LPS is greater on chlamydial reticulate bodies (RBs) than on EBs (Collett *et al.*, 1989) remains an important question in determining whether LPS is more likely to be implicated in infectivity mechanisms other than adherence. Further work is therefore warranted to investigate the role of *C. trachomatis* LPS in infectivity, including the use of chlamydial lipid A and Kdo, despite limitations caused by difficulties in their extraction.

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