Differential glycosaminoglycan binding of *Chlamydia trachomatis* OmcB protein from serovars E and LGV

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We recently showed that OmcB protein from *Chlamydia trachomatis* serovar LGV1 functions as an adhesin. In this study, we produced *Escherichia coli* expressing OmcB from serovar E and compared this OmcB to OmcB from serovar LGV1. Infectivity inhibition assays carried out with serovars LGV1 and E of *C. trachomatis* in the presence of recombinant OmcB showed considerable (~60%) inhibition of infectivity. In the presence of heparan sulphate, there was significant inhibition (68%) of adherence of *E. coli* expressing OmcB from serovar LGV1 only. In a further experiment, recombinant OmcB from serovar LGV1 showed minimal binding to glycosaminoglycan (GAG)-deficient cells, whilst to the same cells, recombinant OmcB from serovar E showed binding equal to that to the wild-type cells. Our experiments strongly suggest that OmcB from serovar E, in contrast to that from serovar LGV1, is not binding to host cells through a GAG-dependent mechanism.

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INTRODUCTION

Despite many years of study, there is still controversy surrounding the interaction of *Chlamydia trachomatis* with the human host and whether glycosaminoglycans (GAGs) are involved as chlamydial adhesins or host receptors (Zhang & Stephens, 1992; Chen & Stephens, 1994; Stephens *et al.*, 2000; Taraktchoglou *et al.*, 2001; Fadel & Eley, 2004). One of a small possible number of adhesins for *C. trachomatis* is the 60 kDa OmcB protein, which has been reported to bind GAGs such as heparan sulphate (Stephens *et al.*, 2001; Fadel & Eley, 2007). It has also been known for a while that two *C. trachomatis* biovariants, trachoma (such as serovar E) and lymphogranuloma venereum (LGV), differ in their heparin-inhibitable interaction with mammalian cells (Chen & Stephens, 1997; Davis & Wyrick, 1997). In previous studies, we confirmed that serovar LGV1 is significantly more dependent on a heparan sulphate-related mechanism of infectivity than is serovar E (Taraktchoglou *et al.*, 2001; Fadel & Eley, 2004). As the above work on OmcB binding to GAGs was established with serovars LGV1 and LGV2 (Stephens *et al.*, 2001; Fadel & Eley, 2007) and with known differences in binding of GAGs by the two biovariants, we produced *Escherichia coli* expressing OmcB from E and LGV1 serovars and compared the two in adherence assays with and without heparan sulphate and using GAG-deficient cells.

METHODS

Cloning of the *omcB* gene and expression of OmcB protein. All methodology was as described in Fadel & Eley (2007) except for the following modifications. Template DNA for the PCR was obtained from *C. trachomatis* serovar E (E/UW-5/CX), which was a gift from Deborah Dean (University of California at San Francisco). The primers used for PCR amplification were synthetic oligonucleotides (MWG Biotech) that were designed based on the DNA sequences deposited in GenBank (X54389) to amplify the entire *C. trachomatis omcB* gene (forward, AAT ACC ATG GAC AAA CTC ATC AGA CGA; reverse, CAA TGT AAG CTT ATA GAT GTG TGT ATT CTC TGT). The expressed protein was identified by a Western blot using rabbit anti-OmcB serum which was raised against recombinant OmcB and by mass spectrometry coupled with microcapillary liquid chromatography analysis as described by Fadel & Eley (2007). As in the above study, the protein was purified by chelate affinity chromatography using the Qiaexpress purification method (Qiagen) according to the manufacturer's instructions. The final concentration of the purified protein was estimated by the method of Bradford (1976).

Infectivity inhibition assays using recombinant OmcB.

Confluent Hec-1B cell monolayers were prepared in a 24-well TC tray, washed using pre-warmed PBS and subsequently infected with an inoculum of 5×10^5 i.f.u. of *C. trachomatis* serovars LGV1 or E. Inocula were supplemented with serial dilutions of the recombinant OmcB protein solution ($50\text{--}150 \mu\text{g ml}^{-1}$) from serovar E. Cells were incubated for 1 h at 37 °C and then washed with pre-warmed PBS to remove non-adherent bacteria. One millilitre of *C. trachomatis* growth medium was added to each well. After incubation for 48 h at 37 °C in 5% CO₂, the infected monolayers were fixed and stained by direct immunofluorescence microscopy using a *C. trachomatis* culture confirmation test (Trinity Biotech).

Abbreviation: GAG, glycosaminoglycan.

Adherence assay to examine the binding of recombinant OmcB to eukaryotic cells. The method used was similar to that of Thornley *et al.* (1996). *E. coli* cells containing the *omcB*-pET28 plasmid were induced to produce OmcB protein and harvested by centrifugation, washed twice with PBS (pH 7.4) and resuspended in an appropriate amount of Eagle's minimal essential medium. One millilitre of this bacterial suspension (OD_{600} 0.14) was inoculated onto Hec-1B cell monolayers (approx. 1×10^6 cells were seeded per well) and incubated for 90 min at 37 °C with 5% CO_2 . After incubation, non-adherent bacteria were removed by washing three times with PBS. The cell layer and adherent bacteria were then fixed with a 90% methanol/acetic acid solution (3:1) for 5 min. After washing with distilled water, the remaining cells were stained with 10% Giemsa for 20 min, mounted and viewed by light microscopy at a magnification of $\times 100$. Twenty-five fields were counted and the mean number of adherent bacteria was then calculated. Negative controls included BL21 cells, and BL21 containing pET28 vector (with no insert), in addition to BL21 expressing a periplasmic protein, ComH. ComH has a similar molecular mass and pI to OmcB, which means that the use of ComH as a negative control limits the possibility of OmcB adhering via non-specific electrostatic effects. The effect of heparan sulphate at 0.5 mg ml^{-1} on OmcB binding was examined using the above adherence assay and controls.

Examination of binding of *E. coli* producing OmcB to GAG-deficient cells. The degree of adherence was compared for GAG-deficient (pgsA-745 and pgsD-677) and wild-type (CHO-K1) cells to evaluate the importance of host cell GAGs for OmcB binding. Cell monolayers were grown to semi-confluence in 24-well tissue culture trays on sterile cover slips. The production of OmcB was induced from *E. coli* containing the *omcB*-pET28 plasmid. The bacterial suspension (OD_{600} 0.14) was inoculated on to the cell monolayers and incubated for 90 min at 37 °C with 5% CO_2 and the adherence assay was completed as described above, using the same controls.

Statistical analysis. Statistical analysis of the results was carried out using either an unpaired *t*-test or a one-way paired analysis of variance test using Instat (Graph Pad software).

RESULTS AND DISCUSSION

Cloning of the *omcB* gene and expression of OmcB protein

DNA sequence analysis of selected *omcB* constructs obtained from *E. coli* transformants confirmed the correct

Fig. 1. Identification of the recombinant OmcB protein from serovar E using anti-OmcB serum in a Western blot. *E. coli* was grown to mid-exponential phase in LB medium at 37 °C. Expression was induced by addition of 1 mM IPTG followed by incubation at 37 °C for 1 h and then at room temperature overnight. Lanes: M, low range molecular mass marker; 1, induced culture containing the vector with no insert; 2, uninduced culture; 3 and 4, induced cultures; 5, *C. trachomatis* EBs as a positive control.

orientation and complete nucleotide content of the *omcB* inserts. The recombinant OmcB protein was identified using anti-OmcB serum in a Western blot reaction (Fig. 1).

Infectivity inhibition assays using recombinant OmcB

We recently showed, using recombinant OmcB from serovar LGV1, that OmcB is a surface-exposed protein that functions as a chlamydial adhesin (Fadel & Eley, 2007) and in infectivity inhibition assays with Hec-1B cells resulted in 60% inhibition of infectivity. Moreover, using anti-OmcB serum, previously shown to reduce chlamydial infectivity (Fadel & Eley, 2007), binding of recombinant OmcB to Hec-1B cells was shown to be specific. The same experiments performed with recombinant OmcB from serovar E gave almost identical results to those with OmcB

Fig. 2. Infectivity inhibition assay of *C. trachomatis* in the presence of the recombinant OmcB protein. The assay was performed using Hec-1B cells. The calculated means of inhibition were compared with those of two controls, one in which no OmcB protein was added and the other containing $100 \text{ µg ComH protein ml}^{-1}$. Error bars represent the standard error. Results of six experiments were statistically analysed using a one-way paired analysis of variance test. NS, Not significant; ***, $P \leq 0.001$.

Fig. 3. Determination of the binding of recombinant OmcB to Hec-1B cells using an adherence assay. *E. coli* cells were incubated with Hec-1B cells for 90 min at 37 °C, followed by staining with 10% Giemsa. (a) *E. coli* cells induced to express OmcB protein, using 1 mM IPTG overnight at room temperature; (b) *E. coli* cells expressing ComH protein, induced under the same conditions.

from serovar LGV1, suggesting that the new recombinant OmcB protein was behaving similarly (Fig. 2).

Examination of the binding of recombinant OmcB to eukaryotic cells

Whilst *E. coli* producing OmcB from both serovars E (Fig. 3) and LGV1 bound to Hec-1B cells in the absence of heparan sulphate, in the presence of heparan sulphate, there was significant ($P \leq 0.001$) inhibition of *E. coli* producing OmcB from serovar LGV1 only (Fig. 4). This difference in binding was not due to different OmcB protein expression efficiencies, which were 1.4 mg ml^{-1} and 2.0 mg ml^{-1} for serovars LGV and E, respectively. In the same experiment, all negative controls showed only minimal binding to Hec-1B cells. We know from our previous study (Fadel & Eley, 2007) that OmcB was being expressed on the surface of *E. coli* as verified by a proteinase lysis method. In a further experiment, when we examined the binding of *E. coli* producing OmcB to GAG-deficient cells, whilst serovar LGV1 bound to CHO-K1 cells there was minimal binding ($P < 0.001$) to pgsA-745 and pgsD-677 cells (Fig. 5). In contrast, whilst serovar E also bound to CHO-K1 cells, similar binding took place with both GAG-deficient cell types. The *E. coli* (BL21) used as a negative control showed very little binding to all cells tested.

Both experiments strongly suggest that OmcB from serovar E is not binding to host cells through a GAG-dependent mechanism, in contrast to that from serovar LGV1.

It has recently been suggested that host cell surface heparan sulphate does not serve an essential role in chlamydial infectivity, as with a new set of different GAG-deficient cell lines, both serovars D and LGV2 appeared not to require host cell GAGs for infectivity (Stephens *et al.*, 2006). If this is true, it might be that a GAG-independent mechanism of binding of *C. trachomatis* to host cells, possibly via the OmcB protein, is particularly relevant to serovar E (which is more closely related to serovar D) as it clearly differed from serovar LGV1 in adherence experiments as described in this study. A possible explanation for this is that heparin dependence of infection can be conferred or reversed by a

Fig. 4. Effect of heparan sulphate (HS; 0.5 mg ml^{-1}) on the adherence of *E. coli* expressing OmcB from serovars LGV1 and E to Hec-1B cells. Negative controls included *E. coli* (BL21), *E. coli* with the vector only (pET28-BL21) and *E. coli* expressing ComH protein (pET28-BL21-ComH). Error bars represent the standard error. Results of three experiments were statistically analysed using the unpaired *t*-test. ***, $P \leq 0.001$.

Fig. 5. Examination of the binding of *E. coli* (BL21) and *E. coli* induced to produce OmcB protein from serovar LGV1 or serovar E to GAG-deficient cells (pgsA-745 and pgsD-677), in comparison to the wild-type CHO-K1. *E. coli* (BL21) was used as a control. Error bars represent the standard error. Results of three experiments were statistically analysed using a one-way paired analysis of variance test. ***, $P \leq 0.001$.

single amino acid alteration of OmcB as recently described by Moelleken & Hegemann (2008) using yeast display technology.

Our findings may be important as although there are known differences between serovars E and LGV in their response to GAGs (Chen & Stephens, 1994, 1997; Davis & Wyrick, 1997) and in the types of clinical presentations that result from their infection (Moulder, 1991; Davis & Wyrick, 1997), they confirm the work of Moelleken & Hegemann (2008), who suggested that OmcB might be responsible for these differences. Moreover, with differences in binding of *E. coli* producing OmcB from serovars E and LGV1, it is important that further work be performed on recombinant OmcB, possibly from a number of different serovars, to confirm these findings, which may well be a factor in differential binding among serovars E and LGV, an observation which has been made over many years and by many research groups.

It is equally important that further experiments are carried out with the new cell lines (Stephens *et al.*, 2006) and with different serovars to try to resolve the confusing situation regarding the nature of the interaction of *C. trachomatis* with mammalian cells, and in particular whether LGV serovars have a requirement for host cell GAG.

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