The role of lipopolysaccharide in the exposure of protective antigenic sites on the major outer membrane protein of *Chlamydia trachomatis*

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(Received 11 September 1991; revised 12 February 1992; accepted 28 February 1992)

A species-specific monoclonal IgM antibody (mAb) 9BF8 directed against the major outer membrane protein (MOMP) of *Chlamydia trachomatis* neutralized several chlamydial serovars in a complement-independent manner. The presence of Mg2+ ions negated the neutralization in serovars F, L1 and L2, but not in serovars A, B, E, D and K. The ability of monovalent Fab-fragments of this mAb to neutralize chlamydial infectivity in a Mg-independent manner suggested that conformational alterations on the chlamydial surface induced by the cation hindered the IgM but allowed the smaller Fab fragment access to its epitope. In order to determine the chlamydial component that binds Mg, elementary bodies (EB) of serovars E and L1 were treated with EDTA at pHs 8 and 9. The infectivity of the treated EB and the amount of released LPS were determined. Only after EDTA treatment at pH 9, as the LPS release increased, did the binding of the mAb on the chlamydial surface become Mg-independent. The infectivity of the EB was almost completely lost after such a treatment. These results suggest that the chlamydial LPS has the potential to modulate the exposure of antigenic sites on the MOMP, when it is cross-linked by Mg2+. They further imply that serovars protected by Mg and those that are not differ in the surface topology of one particular MOMP epitope, but are antigenically very similar. This difference might be of considerable importance *in vivo.*

Introduction

*Chlamydia trachomatis* is an obligately parasitic bacterium causing a variety of human diseases. Many of the pathogenic properties of this micro-organism appear to be related to its surface. Ligands for cell attachment and entry, as well as targets for immune attack, and antigens for potential vaccination must reside on the outer cell wall. The major outer membrane protein (MOMP) and the LPS are among the major constituents of the outer membrane. These major antigens have been shown to be in very close contact (Birkelund *et al.*, 1988), but little is known about their spatial arrangement on the chlamydial cell surface and their possible interactions. Both antigens have been considered relevant for vaccine production. Immunization with recombinant LPS in animal models was not protective (Taylor & Prendergast, 1987). Antibodies against the MOMP have been shown to protect *in vitro* and *in vivo* (Caldwell & Perry, 1984; Peeling *et al.*, 1984; Zhang *et al.*, 1987). One of these studies emphasized that the protective capability of the antibodies depended upon the immunoaccessibility of the target MOMP epitopes on the surface of chlamydiae (Zhang *et al.*, 1987). In this paper, we report on the complement-independent neutralizing activity of a monoclonal antibody (mAb) directed against the MOMP, and on the potential of Mg2+ ions to induce conformational changes on the surface of the elementary bodies (EB) interfering with the protective action of the antibody. We further show that the Mg-independent action of the antibody correlates with maximal release of LPS from the chlamydial outer membrane. Part of the data reported here were first presented by Vretou *et al.* (1991).

Methods

Growth and purification of chlamydial strains. The *C. trachomatis* strains used in this study were A (G-17), B (TW-5), C (TW-3), D (UW-3), E (UW-5), F (UW-6), I (UW-12), K (UW-31), L1 (440), L2 (434), and L3 (404) and the *C. psittaci* meningopneumonitis strain (Mn). They were provided by the American Type Culture Collection (Rockville, MD) and the Centers for Disease Control (Atlanta, GA). Chlamydiae were grown in monolayers of cyclohexime-treated McCoy cells as described before (Vretou *et al.*, 1989) and EBs were purified by centrifugation twice through 30% (w/v) sucrose. The final pellet was resuspended in either HSC buffer (HEPES/sucrose/cation;
Vretou et al., 1989) or in PBS (0.14 M NaCl, 0.026 M KCl, 1.44 mm KH₂PO₄, 8 mm Na₂HPO₄) containing 0.2 M-sucrose (S-PBS). It contained 3–5 mg protein ml⁻¹ determined with the BioRad assay kit using Standard I.

Monoclonal antibodies. The production and characterization of mAbs against C. trachomatis has been described elsewhere (Vretou et al., 1992). mAb 9BF8, an IgM, has been determined to be a species-specific antibody by immunofluorescence, immunodot and immunoblotting techniques. mAbs OPG10 and 1BC6, both IgG3, are genus-specific anti-LPS antibodies. mAbs 171 and 169, both IgG1, are directed against the 57 kDa chlamydial heat shock protein (Morrison et al., 1989) and the 60 kDa OMP 2 protein, respectively (E. Vretou, in preparation). The antibodies were grown in ascites in pristane-primed Balb/c mice and purified according to Pouletty et al. (1988). The purity of the preparation was judged by SDS-PAGE in 12.5%-5% (w/v) polyacrylamide gels. SDS-PAGE and Western blots were done as described by Goswami et al. (1990). Fab fragments were obtained by trypsinization of the IgM mAb under reducing conditions (Bidlack & Mabie, 1986). After reduction with cysteine, the IgM was digested with trypsin in the ratio 1:50 (trypsin: IgM) at 37°C for 4 h. The reaction was stopped by trypsin inhibitor followed by alkylation with iodoacetamide. The reaction mixture was separated on a Superoxep FPLC column (Pharmacia). Fractions containing the Fab fragments were pooled and checked for purity on SDS-PAGE under reducing and non-reducing conditions (Bidlack & Mabie, 1986).

In vitro neutralization assay. The assays were performed in 96-well plates (Costar). Chlamydial suspensions were adjusted with S-PBS to give 50–100 inclusions per field (160 x). They were incubated with continuous shaking with 1 vol. purified mAb 9BF8, an unrelated mAb, or purified Fab fragments at 37°C for 45 min. Ca²⁺, Mg²⁺ and HEPES were sometimes included, depending upon the experiment. A 100 μl sample of the mixtures was used to infect triplicate 24 h McCoy cell monolayers (5 x 10⁴ cells per well). After 60 min at 37°C, the inoculum was removed, the cells washed once with PBS and 200 μl of cycloheximide-containing medium was added. The plates were incubated at 37°C for 48 h. After fixation with methanol, inclusion-containing cells were visualized with mAb OPG10 (ascites fluid diluted 1:1000 in PBS, 37°C, 30 min) and an FITC-conjugated anti-mouse rabbit serum (1:20 dilution in PBS, 37°C, 15 min, Dakopatts). The microtiter plates were inverted and viewed with a Zeiss-Axiophot microscope. Five fields in each well were read in the test and controls, and an average was taken from the 15 fields. The results are expressed as a percentage of the inclusion-forming units (IFU) relative to control wells ([IFU control-IFU test]/IFU control) x 100.

Antibody absorption/recovery assay. Samples (100 μl) of mAb 9BF8 (0.160 μg mAb 9BF8 ml⁻¹ in PBS, 1% (w/v) BSA, 0.1% (w/v) Tween 20 (PBS/BSA/T)) were incubated with increasing amounts of purified EB from serovar E, L1 and the C. psittaci meningonemotis strain Mn as control for 30 min at 37°C in the presence or absence of 1 mm MgCl₂. After centrifugation at 25000 g for 10 min the amount of mAb recovered in the supernatant was titrated in a capture-ELISA assay with rabbit serum against mouse immunoglobulins (Dakopatts) as the immobilized antibody (Nunc plates, 10 μg ml⁻¹ in 0.05 M-carbonate/bicarbonate buffer, pH 9-6) and peroxidase-conjugated anti-mouse rabbit serum (Dakopatts, 1:1000 dilution in PBS/BSA/T) as the detecting antibody. Colour was developed with o-phenylenediamine (OPD, Sigma) and read at 492 nm. The recovery is calculated as A₄₉₂ of 9BF8 incubated with Mn (control) – A₄₉₂ of 9BF8 incubated with E or L1.

EDTA treatment of EB. Samples (100 μl) of purified EB suspensions in S-PBS containing 2.5 mg ml⁻¹ were incubated with 10 mm EDTA at pH 8 or pH 9 for 30 min at 22°C. After centrifugation at 25000 g for 30 min, the pelleted EBs were washed once with S-PBS. Samples were used to determine their residual infectivity and neutralization by mAb 9BF8 or allowed to react with mAb 9BF8 in the absorption assay in the presence and absence of Mg²⁺ as described above. The amount of LPS released was measured in the supernatant with a capture-ELISA assay after treatment of the EB with proteinase K as described by Mearns et al. (1988). Briefly, polystyrene microtitre plates (Nunc) were coated overnight at 4°C with mAb OPG10 (10 μg ml⁻¹ in 0.05 M-carbonate/bicarbonate buffer, pH 9.6). After blocking for 1 h at 37°C with 5% (w/v) non-fat milk in PBS, the plates were incubated for 1 h at 37°C with the supernatants, or serial dilutions of digested EB in PBS/BSA, as standard, or PBS/BSA as controls. After several washes with PBS-T, the LPS captured by the first monoclonal was visualized by a second anti-LPS mAb labelled with peroxidase according to Nakane & Kawai, (1974, 1:100 in PBS/BSA). Colour was developed with o-phenylenediamine (OPD, Sigma) and read at 492 nm.

Results

In vitro neutralization of serovar L1 by a monoclonal antibody: interference by Mg²⁺

A species-specific IgM, named 9BF8, recognizing the fourth variable domain of the MOMP (not shown) was found to neutralize the infectivity of C. trachomatis serovars B, D, E, L1, L2, F, K and A but not of C, I and L3. Serovars Ba, G, H and J were not tested. The monoclonal antibody reacted with viable chlamydia in the dot–blot assay frequently used to identify immunoreactive antigens (Zhang et al., 1987; Peterson et al., 1988; not shown). Fluorescein-conjugated mAb 9BF8 bound to the cell surface of purified chlamydial EB in a direct immunofluorescence assay, suggesting that the relevant epitope is surface-exposed (not shown). Representative neutralization curves of the infectivity of the LGV serovar L1 and the trachoma serovar E by the purified 9BF8 IgM are shown in Fig. 1. mAb 9BF8 reduced the infectivity of E and L1 by 50% at protein concentrations of 43:5 and 7:2 μg ml⁻¹, respectively. When the neutralization assays were performed in a buffer containing divalent cations, such as HSC, mAb 9BF8 neutralized the infectivity of serovar E but not of serovar L1. This prompted us to study the effect of each component in HSC. Addition of Ca²⁺ and HEPES separately or together had no effect on the neutralizing activity of mAb 9BF8 (not shown). It was the presence of Mg²⁺ that completely prevented the inhibitory activity of mAb 9BF8 on serovar L1. The inhibition of the infectivity of E by the monoclonal antibody was only slightly affected by the presence of Mg²⁺ (Fig. 1). When the cation was absent from the preincubation mixture 9BF8-L1, but was added to the cells before or after the inoculation, 9BF8 reduced the infectivity of L1 by 60%.

Thus, the presence of Mg²⁺ directly affected the binding of the antibody to the EB of serovar L1. To support this suggestion, mAb 9BF8 was allowed to react with increasing amounts of both suspensions in the presence and
absence of Mg\(^{2+}\). After removal of the immune complex by sedimentation, the free antibody remaining in the supernatant was titrated with antiserum against mouse immunoglobulins. A substantial amount of free antibody was recovered when the reaction with L1 was done in the presence of Mg\(^{2+}\) (Fig. 2). No differences were observed in the amount of free antibody after absorption with E in the absence or presence of Mg\(^{2+}\).

When mAb 9BF8 was digested with trypsin and the monovalent Fab fragments were assessed for neutralization of serovar L1, the infectivity of L1 was reduced in the absence and presence of Mg\(^{2+}\), in contrast with the intact IgM molecule (Table 1). These data let us suggest that the presence of Mg\(^{2+}\) induced conformational changes at the chlamydial cell surface and altered the immunoaccessibility of this domain.

**Interference by Mg\(^{2+}\) in the neutralization of other serovars**

Complement-independent neutralization experiments with mAb 9BF8 in the presence of Mg\(^{2+}\) were performed with serovars B, D, L2, F, K, and A. The results are summarized in Table 2. In serovars L2 and F, the presence of Mg\(^{2+}\) negated the neutralizing activity of mAb 9BF8 almost completely, whereas the neutralization of serovar B, D and K and A was not significantly affected by the presence of Mg\(^{2+}\).
Release of LPS of serovars E and L1 by EDTA treatment

In order to determine the chlamydial compound interfering with the protective action of mAb 9BF8 in the presence of Mg\(^{2+}\) we examined the effect of EDTA at moderate alkaline pH (8.0 and 9.0) on the neutralizing activity of the mAb and on the release of chlamydial LPS. The residual infectivity of the pelleted EB was determined by counting inclusions, while the relative amount of released LPS was measured in the supernatant using a capture-ELISA assay. The experiment was repeated three times, means and standard deviations are shown.

The effect of EDTA at pH 8 and pH 9 on the infectivity of E and L1 and on the release of their LPS are presented in Table 4. In both serovars, the loss of infectivity paralleled the release of LPS. Only 2.9% of the chlamydiae from serovar L1 and 3.9% from serovar E were infectious after treatment with EDTA at pH 9. EDTA treatment at pH 7-2 did not significantly affect the infectivity or the release of LPS in either serovar (not shown). A considerable amount of LPS was released into the supernatant in both serovars at pH 9.0. It was 5.7 times the amount of the control (EB at pH 7-2) in serovar L1 and 4.5 times in serovar E. By comparison, the corresponding data at pH 8.0 were 3 to 6 times the control in serovar L1 and 3 to 5 in serovar E.

SDS-PAGE of the supernatant after EDTA treatment at pH 9.0 of serovar E revealed two major protein bands with molecular masses of 60 and 45 kDa, respectively (Fig. 4). Probing with specific mAbs against the 60 kDa chlamydial heat-shock protein (Bavoil et al., 1990;
Fig. 4. Profile of the proteins released in the supernatant after EDTA treatment at pH 9.0 of serovar E. Frozen EBs were thawed, pelleted and the pellets washed once with PBS. The washed EBs were treated with EDTA at pH 7.0 and pH 9.0 and centrifuged. The supernatants were subjected to SDS-PAGE and stained with Coomassie brilliant blue. Lanes: 1, EBs from serovar E; 2, supernatant of the pelleted thawed EBs; 3, PBS wash; 4, supernatant at pH 7.0; 5, supernatant after EDTA treatment at pH 7.0; 6, supernatant at pH 9.0; 7, supernatant after EDTA treatment at pH 9.0. Molecular mass markers are on the left side.

Morrison et al., 1989) and the 60 kDa cysteine-rich OMP 2 protein identified the released 60 kDa protein band as the chlamydial stress protein (not shown).

**LPS release and Mg-independent binding of mAb 9BF8 to L1**

Previous electron microscopy experiments with EB from a C. psittaci strain showed that the cell envelopes retained their rigid round shape after EDTA treatment at a pH 10 (Narita et al., 1976). Purified EB from serovar L1 were therefore incubated at pH 8 and pH 9 in the presence and absence of 10 mM-EDTA, pelleted, and allowed to bind mAb 9BF8 in the presence and absence of Mg2+. The amount of LPS released in the supernatant was quantified as above. After the reaction of the EDTA-treated chlamydia with the antibody and removal of the immune complex by sedimentation, the amount of free antibody was quantified in the supernatant as described above. The results are illustrated in Fig. 5. At pH 8, a substantial amount of mAb 9BF8 bound to serovar L1 in the absence of the cation, while almost 100% of the antibody was recovered from the supernatant in the presence of Mg2+. Raising the pH to 9 in the absence of EDTA did not substantially change the two parameters, i.e., recovery of antibody and release of LPS. Chlamydiae treated with EDTA at pH 8 showed a 32% decrease in the amount of antibody recovered in the supernatant, suggesting that some of the mAb bound to the surface of L1 in the presence of Mg2+. This result corroborated the data obtained in the neutralization assay of EDTA-treated EB at the same pH (Table 3). After EDTA-treatment at pH 9, antibody binding at the L1 surface became Mg-independent, in that the mAb recovered in the presence and in the absence of Mg2+ were equal. For the sake of clarity the amount of LPS released after this treatment was accorded the value of 100% (Fig. 5). These results, together with the neutralization data obtained with the monovalent Fab fragments, lead to the suggestion that in certain chlamydial serovars, cross-linking of LPS by Mg ions resulted in steric hindrance of antibody binding to the MOMP and therefore lack of neutralization of chlamydial infectivity.

**Discussion**

In this report, we have studied the ability of Mg2+ to protect the infectivity of some chlamydial serovars from the neutralizing activity of a species-specific mAb directed against the MOMP. The ability of the Fab fragments of this mAb to neutralize chlamydial infectivity in a Mg-independent manner indicated steric hindrance and directed our attention to the chlamydial LPS. Protection of chlamydial infectivity by Mg2+ from complement-dependent neutralization by a species-specific mAb has been previously reported by
Peterson et al. (1988). In contrast to our results, the presence of Mg\(^{2+}\) did not influence the binding of the antibody to the surface of EB measured in the microimmunofluorescence assay. The authors suggested that the cation protected chlamydiae from antibody-complement attack in a similar way to the serum-resistance reported for Gram-negative bacteria with smooth LPS. The protection by Mg\(^{2+}\) was observed with serovars E and C. It is therefore very likely that the mechanisms underlying the two observations are intrinsically very different.

It is generally accepted that divalent cations play a major role in the stabilization of the outer membrane of Gram-negative bacteria by cross-linking the adjacent negatively charged LPS molecules (Hancock, 1991). Removal of the cations by chelation with EDTA results in enhanced permeabilization of the outer membrane and in destabilization and release of LPS (Leive, 1965). The effect of such treatments on the viability of the micro-organism depends upon the organism. While it is deleterious for Pseudomonas aeruginosa (Asbell & Eagon, 1966) it does not affect many strains of Escherichia coli (Marvin et al., 1989). Our data show that treatment of serovars E and L1 with EDTA resulted in a significant loss of their infectivity. Electron microscopy studies on the meningopneumonitis strain of C. psittaci by Manire and co-workers (Narita et al., 1976) showed intact rigid envelopes that had lost their internal contents and part of their cell surface components after EDTA treatment at pH 10. SDS-PAGE of the released protein–carbohydrate–lipid complex revealed two bands with molecular masses of approximately 17 and 13 kDa, respectively (Narita & Manire, 1976). Recently, the 18 kDa chlamydial histone analogue has been partially solubilized by a similar treatment (Hackstadt, 1991). The protein profile of the material released after EDTA treatment at pH 9-0 observed in this study resembled the protein pattern obtained after solubilization of chlamydial EB with Triton X-100 (Morrison et al., 1989). Although the role of the 60 kDa (or 57 kDa) heat shock protein in the pathogenesis of chlamydial disease has been well established, its function in the assembly of the chlamydial outer membrane is hypothetical (Bavoil et al., 1990). At present, the role of the 45 kDa protein on the surface of chlamydiae is not known.

Chlamydial LPS can be easily shed or liberated, another part remains tightly anchored in the lipid bilayer. Removal of this tightly anchored LPS would appear to have severe consequences for the infectivity of chlamydia. Several lines of evidence implicate smooth LPS in conferring hindrance of epitopes. The smooth Phase I LPS of Coxiella burnetti hinders the access of anti-phase II antibodies to a multitude of shared surface protein antigens. Phase II cells are therefore less promising vaccine candidates (Hackstadt, 1988). The core glycolipid region is usually not accessible to antibodies against the surface of wild strains in many Gram-negative bacteria (Luederitz et al., 1982). Our data are suggestive of a Mg\(^{2+}\)-mediated hindrance provoked by LPS molecules that are known to be deeply rough (Nurminen et al., 1985).

The most important implication of the data presented here is, however, the difference in the surface topology between the chlamydia serovars that are protected by Mg\(^{2+}\), such as serovars L1, L2 and F, and those that are not. The differences may occur in the topology, conformation or orientation either of the LPS molecules or of the MOMP epitope or of both. Complexing of Mg\(^{2+}\) ions with a proteinaceous chlamydial component can also not be excluded. In conclusion, differences in the surface topology of certain chlamydia serovars are responsible for their protection from antibody attack, possibly due to LPS cross-linking by physiological concentrations of Mg ions. The high degree of resistance to antibody-mediated neutralization may have important implications in vivo.

We thank Mrs Giorgia Conidou for growing and purifying the chlamydial stocks, Dr Leonidas Tzouvelekis for helpful discussions and Dr Subir Bose for invaluable help during the preparation of the manuscript.

References


