Determination of neutralizing epitopes in variable domains I and IV of the major outer-membrane protein from *Chlamydia trachomatis* serovar K

Anne Villeneuve, Laurent Brossay, Gilles Paradis and Jacques Hébert

Author for correspondence: Anne Villeneuve. Fax: +1 418 654 2765

*Chlamydia trachomatis* is a leading cause of sexually transmitted diseases and a number of strategies have been developed to produce vaccines to prevent its transmission. The purpose of this study was to map the neutralizing epitopes of *C. trachomatis* major outer-membrane protein (MOMP) serovar K by using anti-MOMP antibodies and synthetic peptides. Seven anti-MOMP monoclonal antibodies and three polyclonal antisera were produced and characterized. Their fine specificity was defined by direct binding assay on 15 peptides of 10 amino acid residues, overlapping by five residues, corresponding to the four variable domains (VDI-VDIV: residues 64-85, 139-160, 224-237 and 287-319) of MOMP serovar K. Our data confirmed that a neutralizing epitope is found in VDIV, defined by peptides K12 and K13. This epitope is $^{296}$TTLNPTIAG$^{304}$, which has never been reported as a neutralizing epitope of serovar K. Another neutralizing epitope, defined by peptide K2, has been identified in VDI. This epitope is in the same position as $^{71}$VAGLEK$^{76}$, a peptide with neutralizing activity found in serovar A, but they are not identical because antibodies against peptide K2 do not bind to this epitope. No neutralizing epitope was found in the two other variable domains (VDII and III). In summary, two neutralizing sites, one in variable domain I and one in variable domain IV, were identified in serovar K.

**Keywords:** *Chlamydia trachomatis*, neutralizing epitopes, vaccines, outer-membrane protein
et al., 1988; Conlan et al., 1988, 1989; Peterson et al., 1991; Stephens et al., 1988; Su et al., 1990b; Zhong & Brunhn, 1990, 1991). Also, MOMP seems to play an important role in the establishment of a protective immunity because it contains antigenic determinants that elicit serovar-specific neutralizing antibodies. Protective immunity to ocular reinfection with C. trachomatis in experimentally infected humans and primates is serovar specific (Grayston et al., 1971; Jawetz et al., 1965; Wang et al., 1967). Furthermore, resistance to ocular reinfection correlates with the presence of serovar-specific tear antibodies, suggesting that chlamydial antigens implicated in the serovar-specific immunity are target antigens for the development of a vaccine (Murray et al., 1973; Nichols et al., 1973). Recent studies using peptide mapping led to the determination of a serovar A-specific neutralizing epitope in VDI of the major outer membrane protein, \( 71VAGLEK \), which was shown to induce a neutralizing response in mice injected colinearly with a species-conserved T cell epitope (Baeh et al., 1988; Su & Caldwell, 1992; Su et al., 1990a).

Determination of B- and T-cell epitopes for all 15 serovars of C. trachomatis is essential to make a judicious choice of the components of a synthetic vaccine. To define more fully the neutralizing epitopes of the MOMP of serovar K, we produced both anti-MOMP monoclonal antibodies (mAbs) and polyclonal Abs and characterized them by direct binding assay on synthetic peptides encompassing the four variable domains by binding inhibition studies and by assessment of their in vitro neutralizing activity. We confirmed the presence of neutralizing epitopes in variable domain IV, one containing nine uncharged residue j.

Production of mAbs and rabbit polyclonal antisera. For the mAbs, the Ab-producing cells were obtained from the fusion of spleen cells from BALB/c mice immunized with EBs or with purified MOMP from the serovar K and myeloma cell lines (SP2/O-Ag14) in the presence of polyethylene glycol as previously described (Brossay et al., 1994). The Ab-producing hybridomas were selected by direct binding assay using EBs or MOMP (0.5 \( \mu \)g per well) coated microtitre plates. Antibody isotypes were determined with a commercially available kit. Seven anti-MOMP mAbs were obtained and purified on Protein G columns. Rabbit polyclonal antiserum were produced after immunization with whole C. trachomatis serovar K, pooled serovars A, B and C, or purified MOMP from serovar K. Rabbits were injected subcutaneously with 200 \( \mu \)g of C. trachomatis or purified MOMP in complete Freund’s adjuvant on day 0, followed by 200 \( \mu \)g given intramuscularly in incomplete Freund’s adjuvant on days 21, 42 and 63.

Direct binding assays. The specificity of the mAbs (1 \( \mu \)g ml\(^{-1}\)) was tested by direct binding assay on a large panel of solid-phase-fixed synthetic peptides (peptides named K1 to K15). Briefly, microtitre plates were coated overnight at 4°C with 100 \( \mu \)l synthetic peptide (50 \( \mu \)g ml\(^{-1}\)), purified MOMP (5 \( \mu \)g ml\(^{-1}\)), or formalin-killed EBs (5 \( \mu \)g ml\(^{-1}\)) in carbonate buffer (pH 9.6) and in phosphate-buffered saline (PBS, pH 7.4), respectively. Monoclonal or polyclonal antibodies, diluted in PBS plus 0.5% bovine serum albumin (BSA) were then added. After washes, an anti-mouse IgG-peroxidase conjugate was added and the reaction developed as reported. Absorbance at 405 nm was then measured.

Peptide synthesis. Fifteen peptides of 10 amino acid residues, overlapping by five residues, corresponding to the four variable domains (VDI to VDIV) were synthesized by the method of Geysen and co-workers using the recommendations supplied in the commercially available epitope mapping kit (Geysen et al., 1984, 1987). Peptides were conjugated to BSA by the EDC procedure using recommendations supplied by the manufacturer (Pierce Chemical Co.).

In vitro neutralization of chlamydial infectivity. Neutralization assays were done without centrifugation, as described by Caldwell et al. (1981) and Su et al. (1990b). Briefly, purified EBs were diluted in 0.25 M sucrose/10 mM sodium phosphate/5 mM L-glutamic acid (SPG, pH 7.2) to give a final concentration of 4 \( \times 10^7 \) inclusion-forming-units (i.f.u.) ml\(^{-1}\). The bacteria were then mixed with an equal volume of mAbs serially diluted in SPG plus 0.5% BSA. The mixture was incubated at 37°C for 60 min, and 200 \( \mu \)l of the appropriate C. trachomatis/antibody mixture dilution was inoculated in triplicate onto confluent HAK cell monolayers grown in 24-well plates (Costar). After 2 h incubation, the inocula were removed and the monolayers washed with Hanks’ balanced salts solution. Monolayers were fed with 500 \( \mu \)l of Eagle’s minimal essential medium supplemented with 10% foetal calf serum containing 0.5 \( \mu \)g cycloheximide ml\(^{-1}\) and incubated at 37°C for 48 h. The monolayers were fixed with methanol, and chlamydial inclusions were identified by indirect fluorescent antibody staining using a genus-specific mAb to chlamydial LPS. The total number of inclusions per well were counted. The calculations for determining percentage reduction of i.f.u. by mAbs were done as described by Sabet et al. (1984). A > 50% reduction from control i.f.u. in infectivity was considered positive for neutralization.

Induction of anti-C. trachomatis Ab response by K2 and K12 synthetic peptides. Groups of four BALB/c mice were injected with 50 \( \mu \)g of K2, K12 or VAGLEK synthetic peptides coupled to KLH and emulsified 1:1 in complete Freund’s adjuvant.
Three booster injections of 50 µg of the same preparation in incomplete Freund’s adjuvant were given biweekly. Mice were bled 1 d before each injection and 14 d after the last one. The sera were then tested in ELISA for anti-C. trachomatis activity and to MOMP and corresponding synthetic peptides by direct binding assay. The ability of the synthetic peptide to induce an anti-K2 or anti-K12 response with a neutralizing activity was also assessed as described above.

RESULTS

Characterization of anti-MOMP mAbs

Seven anti-MOMP mAbs were obtained following immunization with purified MOMP of serovar K and their binding activity on the 15 serovars and their neutralizing activity were assessed. Five mAbs (9F12, DP10, 5C2, 2D7 and 11A12) neutralized the serovar K in a dose-dependent manner in a complement-independent neutralization assay (Fig. 1). The profiles of reactivity of these mAbs were then analysed by immunoblotting; four are shown in Fig. 2. Anti-MOMP mAbs DP10 and 5C2 reacted with the MOMP of all 15 C. trachomatis serovars, although 5C2 showed by far the greatest reactivity with C. complex serovars by immunoblotting (Fig. 2). Direct-binding ELISA, however, confirmed the species-specific nature of this mAb (data not shown). MAb 9F12 was specific to the MOMP of serovar K, and mAb 2D7 was subspecies-specific, reacting with serovars C, H, J, K, L3 (Fig. 2).

Specificity of anti-MOMP Ab by peptide mapping

To determine further the binding site of our mAbs, 15 peptides of 10 amino acid residues, overlapping by five residues, corresponding to the four VDs of MOMP serovar K (VD1–VDIV: residues 64–85, 139–160, 224–237 and 288–319) were synthesized. To characterize more fully the immune response against C. trachomatis serovar K, the specificity of three rabbit polyclonal antisera was also analysed by peptide mapping. Anti-MOMP mAb DP10 recognized, by direct binding ELISA assay, the peptide LDVTTLNPTI (designated K12) and, to a lesser extent, the peptide LNPTITGKGA (designated K13) (Fig. 3). These epitopes are located in the VDIV of the MOMP. This portion of the molecule has been described as a species-conserved region flanked with serovar-variable regions (Baehr et al., 1988; Stephens et al., 1988). Further analysis with overlapping synthetic peptides of five residues covering this region of the MOMP permitted us to restrict the reactivity of DP10 mAb to the peptide LNPTI. On the other hand, mAb 5C2 reacted with peptide K12 only when conjugated with BSA, and not with peptide K13. Moreover, overlapping peptides of five residues covering the K12–K13 region did not react with this mAb, suggesting that the anti-MOMP mAb 5C2 epitope overlaps the anti-MOMP mAb DP10 epitope (Fig. 3).

Subspecies-specific mAbs 11A12 and 2D7 reacted with the peptide LQNDPTTNVA (designated K3) and, to a lesser extent, with the peptide SDVEGLQNDP (designated K2), whereas anti-MOMP K-specific mAb 9F12 reacted only with peptide K2 (Fig. 3). Peptides K2 and K3 are located in VDI. Finally, serovar K-specific mAbs 3F6 and 4A1 reacted with peptide VEFPLDITAG (designated K9), located in the VDIII of the MOMP.

The profile of reactivity of the three rabbit polyclonal antisera was comparable to those of the seven anti-MOMP mAbs, except peptide TKTQYSKFNT (designated K5), which was recognized, along with peptides K2, K12 and K13, only by rabbit polyclonal antiserum obtained after immunization with whole C. trachomatis serovar K. Following immunization with pooled serovars A, B and C, peptides K12, K13 and, to a lesser extent, peptides K2 and K14 were recognized; rabbit polyclonal antiserum obtained after immunization with purified MOMP of serovar K bound to peptide K3, K12 and K13 (Fig. 3).

Inhibition of neutralization activity by synthetic peptides defining the binding-epitope of mAbs

To establish the functional value of the defined anti-MOMP mAb epitopes, synthetic peptides were used as competitive antigens in the neutralization assay. The results showed that 0.2 µg ml⁻¹ of anti-MOMP mAb 9F12 was required to neutralize 50% of chlamydial infectivity when incubated alone or with unrelated peptide, whereas 2.5 µg ml⁻¹ of the mAb (12.5 times more) was necessary when incubated with the synthetic peptide-defining epitope of mAb (peptide K2) (data not shown). Similarly, the neutralization titre of DP10 varied from 0.8 µg ml⁻¹ when incubated alone or with unrelated peptide to 40 µg ml⁻¹ (fivefold higher) when incubated with the corresponding synthetic peptide.
Induction of anti-C. trachomatis Ab response with neutralization activity upon immunization with synthetic peptides K2 and K12

The value of a synthetic peptide as a vaccine stems from the possibility of raising, upon immunization, an antibody response directed to a neutralizing epitope. BALB/c mice and rats were therefore immunized with peptides defining neutralizing epitopes, peptides K2 and K12, coupled to KLH. Mice and rats were bled 14 d after each immunization and 1 d before the next immunization; anti-C. trachomatis Ab response was measured by direct binding assay. The preimmune sera of all animals studied did not contain anti-C. trachomatis Ab at a serum dilution of 1:10 (data not shown), whereas those receiving peptides K2 or K12 developed a significant anti-MOMP response after four injections (Table 1). In addition, significant antibody response to whole bacteria with neutralizing activity was...
neutralizing anti-MOMP mAbs and epitope mapping

Table 1. Ability of peptides K2 and K12 to induce an antibody response

<table>
<thead>
<tr>
<th>Mouse or rat antiserum</th>
<th>ELISA Ig antibody titre against serovar K*</th>
<th>Neutralizing titre†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse anti-K2</td>
<td>3200 ND</td>
<td>400 1600 640</td>
</tr>
<tr>
<td>Rat anti-K2</td>
<td>1600 ND</td>
<td>800 1600 ND</td>
</tr>
<tr>
<td>Mouse anti-K12</td>
<td>ND</td>
<td>800 400 800 160</td>
</tr>
<tr>
<td>Rat anti-K12</td>
<td>ND</td>
<td>200 200 400 ND</td>
</tr>
</tbody>
</table>

ND, Not determined.

*ELISA titres are expressed as the reciprocal of pooled serum dilutions giving an absorbance reading (405 nm) of 0.3. Absorbance values of preimmune or normal sera were 0-15 or less.

†Neutralizing titres are expressed as the reciprocal of pooled serum dilutions that neutralized 50% of chlamydial infectivity. Preimmune or normal sera neutralized titres of less than 20.

‡Plates were coated with peptides K2 or K12 (50 μg ml⁻¹), or with MOMP K (5 μg ml⁻¹).

measured in the serum of mice immunized with either peptide K2 or peptide K12, supporting the specificity of these peptides (Table 1).

DISCUSSION

The MOMP of C. trachomatis has been extensively studied and is known to contain several neutralizing epitopes (Lucero & Kuo, 1985; Peeling et al., 1984; Peterson et al., 1991; Stephens et al., 1988; Zhang et al., 1987, 1989). These have been defined as linear epitopes that reside within variable domains of the protein, although their primary sequences may vary between different serovars (Baehr et al., 1988; Stephens et al., 1986, 1987; Yuan et al., 1989). They represent, therefore, the target of choice for the development of a vaccine. Antigenic variability among the variable domains of the 15 serovars represents the major challenge for development of a synthetic vaccine and implies a good definition of B- and T-cell epitopes of the serovar of choice. Therefore, the aim of this work was the mapping of neutralizing epitopes of serovar K, one of the serovars responsible for genital tract infections, by using a panel of anti-MOMP mAbs and overlapping synthetic peptides (peptides named K1 to K15) corresponding to the four variable domains of the MOMP (VDI to VDIV: residues 64–85, 139–160, 224–237 and 288–319).

The in vitro neutralizing activity of each mAb was first assessed on serovar K in a complement-independent assay because chlamydial infections occur primarily on mucosa, where no active complement components could be found (McGhee & Mestecky, 1990). The specificity of mAbs was tested by direct binding assay on solid phase-fixed purified MOMP and synthetic peptides. Neutralizing epitopes within VDIV of several serovars, but not serovar K, have been previously described (Baehr et al., 1988; Peterson et al., 1991; Stephens et al., 1988; Su et al., 1990b; Zhong & Brunham, 1990, 1991). We report herein a neutralizing epitope within the VDIV of serovar K. Indeed, this epitope is defined by peptides K12 and K13 and is recognized by two species-specific mAbs (5C2 and DP10), neutralizing for serovar K. This epitope was further defined by the use of 13 peptides of five amino acid residues, overlapping by four residues and covering the K12–K13 sequence. Only one peptide, LNPTI, was recognized by mAb DP10: it is located in a species-specific epitope of nine uncharged residues, 26ṬTTLNPTIAG270, conserved among all 15 serovars. Anti-MOMP mAb 5C2, which cross-reacts with DP10 in a binding-inhibition radio-immunoassay (data not shown), was shown to react with peptide K12 only when conjugated to BSA. These data suggest that the 5C2 epitope differs from, but overlaps with, the DP10 epitope and that charged residues are implicated in the binding activity of mAb. Although direct-binding ELISA confirmed the species-specific nature of mAb 5C2, immunoblotting of these two mAbs showed slight differences in their reactivity profiles. We speculate that this discrepancy could be related to the unreactivity of 5C2 to peptide K13. Other investigators have reported both a subspecies-specific mAb and a species-specific mAb that recognized the same 16 amino acid peptide (Stephens et al., 1988). They proposed that this domain of the MOMP must include a series of overlapping antigenic determinants. Our results reporting one neutralizing epitope within the VDIV of serovar K and common to all other serovars are in the same line of thought.

Finally, we identified for the first time a neutralizing epitope corresponding to the synthetic peptide 68SDVEGLQNDP78, located in VDI of MOMP of serovar K and recognized by neutralizing mAbs 11A12, 2D7 and 9F12. This epitope is located in the same amino acid region as 71VAGLEK76, a serovar A-specific neutralizing epitope, which has been reported to induce a protective immune response in mice when injected concomitantly with a species-conserved T cell epitope (Baehr et al., 1988; Su & Caldwell, 1992; Su et al., 1990a). However, it differs from 71VAGLEK76 as these three mAbs do not react with serovar A. Also, Qu et al. (1993) recently reported the 71VAGLQNPDPT89 peptide in MOMP serovar C as a subspecies-specific epitope that is neutralizing for serovars C, I, J and L3. Rabbit antisera to serovar C MOMP were shown by Zhong & Brunham (1990) to recognize two overlapping peptides, 68SDVAGLQ73 and 74LQDPTTX78. Taken together, these results indicate that this region of the VDI of the MOMP is immunogenic and surface-exposed for the C- and C-related complex serovars. Negatively charged, divergent sequences in exposed MOMP VDs are implicated in the binding of C. trachomatis to host cells via electrostatic interactions, which is consistent with the surface exposure and charge properties of the MOMP VDs of all serovars, in spite of amino acid sequence variations. Our results permit us to define a neutralizing epitope within the VDIV of serovar K, which is immunologically different from other neutralizing epitopes located in the same region of other serovar(s). Such similarities
may have major implications in future studies on protective immunity. It could be of interest to study the immunogenic and neutralizing properties of this region of the VDI of the MOMP of B- and B-related complex serovars as we report herein, like others (Zhong & Brunham, 1990), such properties for the VDI of the MOMP of C- and C-related complex serovars.

In summary, we report two neutralizing epitopes on MOMP of serovar K, the first located within the VDIV and corresponding to those reported for other serovars and the other, although located in a region of VDI where neutralizing epitopes from other serovars have been identified, unique for serovar K. We provide evidence to support the functional and immunological properties of the corresponding peptides. These data suggest that these epitopes could be useful in vaccine development.

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