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

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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

INTRODUCTION

Chlamydia trachomatis, an obligately intracellular bacterium, is a leading cause of sexually transmitted diseases in industrialized countries and is also a major cause of preventable blindness in underdeveloped countries (Schachter, 1983). It is responsible for a wide spectrum of human diseases, such as inclusion conjunctivitis, trachoma, lymphogranuloma venereum and genital tract infections that can evolve to chronic salpingitidis and sterility (Grayston & Wang, 1975). Based on pathogenicity, antigenicity and nucleic acid composition, human pathogen strains of *C. trachomatis* have been subdivided into 15 serovars (L1, L2, L3, A through K, and Ba). By their antigenic properties, these serovars were grouped into three complexes: the B complex (B, Ba, D, E, L1 and L2), the intermediate complex (F, G, K and L3) and the C complex (A, C, H, I and J) (Grayston & Wang, 1975; Stephens *et al.*, 1982).

All serovars of *C. trachomatis* bear a major outer-membrane protein (MOMP) with an approximate molecular mass of 40 kDa that accounts for 60%, by weight, of the chlamydial outer-membrane proteins (Caldwell *et al.*, 1981). The MOMP genes encode a highly conserved protein structure that contains four evenly spaced domains with sequences varying among the different serovars. These variable domains (VDs) have precise locations in the MOMP: VDI, residues 64–85; VDII, residues 139–160; VDIII, residues 224–237; and VDIV, residues 288–319 (Baehr *et al.*, 1988; Stephens *et al.*, 1986, 1987). Some neutralizing epitopes in VDIV have been previously reported. Among them, a species-specific epitope of nine uncharged residues, ^{296}TTLNPTIAG^{304}, has been identified in some *C. trachomatis* serovars (Baehr

Abbreviations: EB, elementary body; mAb, monoclonal antibody; MOMP, major outer-membrane protein; VD, variable domain.

et al., 1988; Conlan *et al.*, 1988, 1989; Peterson *et al.*, 1991; Stephens *et al.*, 1988; Su *et al.*, 1990b; Zhong & Brunham, 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, ^{71}VAGLEK^{76}, which was shown to induce a neutralizing response in mice injected colinearly with a species-conserved T cell epitope (Baehr *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 residues conserved among all 15 serovars. This epitope has already been reported for some serovars; however, its properties as a neutralizing epitope have never been demonstrated in the serovar K. We also identified a new neutralizing epitope located in the VDI of MOMP serovar K. A synthetic peptide corresponding to this epitope was able to inhibit the binding of anti-MOMP mAbs to MOMP serovar K and to inhibit neutralizing activity of mAb. This peptide also induced, upon immunization, the production of an antibody response with neutralizing activity comparable to that of anti-MOMP mAb, supporting its specificity.

METHODS

Organisms. The strains of *C. trachomatis* used in this study were A (G-17/OT), B (TW-S/OT), Ba (AP-2/OT), C (TW-3/OT), D (UW-3/Cx), E (UW-5/Cx), F (UW-55/Ur), G (UW-57/Cx), H (UW-4/Cx), I (UW-12/Ur), J (UW-36/Cx), K (UW-31/Cx), L1 (LLCM 440), L2 (LLCM 434) and L3 (LLCM 404); they were obtained from the Laboratoire de Santé Publique du Québec (LSPQ, Qc, Canada). They were propagated in Syrian hamster kidney cells (HaK, obtained from the American Type Culture Collection, Rockville, MD, USA), and elementary bodies (EBs) were purified by centrifugation on renografin density gradients (Caldwell *et al.*, 1981).

Purification of the MOMP. MOMP of *C. trachomatis* serovar K was purified as previously reported (Caldwell *et al.*, 1981) and its purity was evaluated by SDS-PAGE in 10% (w/v) polyacrylamide gels under reducing conditions.

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 μg 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 antisera 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 μg of *C. trachomatis* or purified MOMP in complete Freund's adjuvant on day 0, followed by 200 μg given intramuscularly in incomplete Freund's adjuvant on days 21, 42 and 63.

Direct binding assays. The specificity of the mAbs (1 μg 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 μl synthetic peptide (50 μg ml^{-1}), purified MOMP (5 μg ml^{-1}), or formalin-killed EBs (5 μg 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 Ig–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: residues 64–85, 139–160, 224–237 and 288–319) of MOMP serovar K 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 4×10^5 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 μl 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 μl of Eagle's minimal essential medium supplemented with 10% foetal calf serum containing 0·5 μg 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 $\geqslant 50\%$ 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 μg 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. Rats were injected and bled by the same procedure. 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 (VDI–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 antisera 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 antisera obtained after immunization with purified MOMP of serovar K bound to peptide K3, K12 and K13 (Fig. 3).

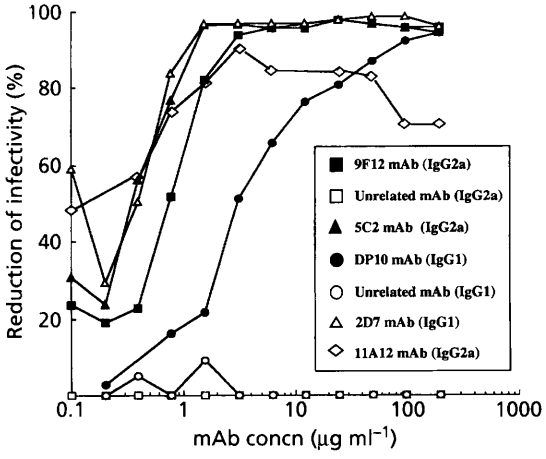

Fig. 1. Neutralization of chlamydial infectivity by anti-MOMP mAbs. *C. trachomatis* serovar K was used in this complement-independent assay with mAbs 9F12, DP10, 5C2, 2D7 and 11A12 or unrelated mAbs of the same isotype as control. Percentage reductions in infectivity were calculated as described in Methods. Each point represents the mean value of three experiments.

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 4·0 μg ml^{-1} (fivefold higher) when incubated with the corresponding synthetic peptide.

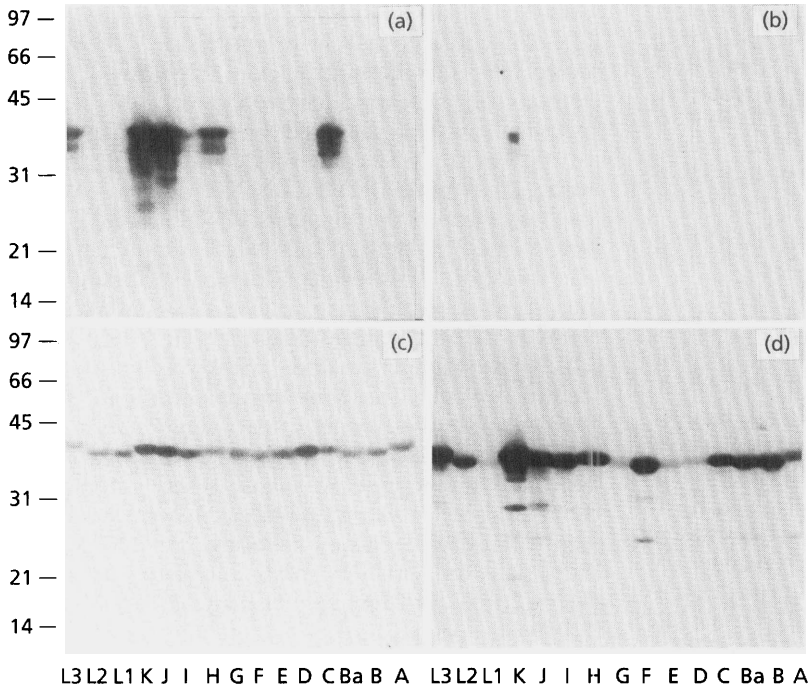

Fig. 2. Immunoblot analysis of anti-MOMP mAbs (a) 2D7, (b) 9F12, (c) DP10, and (d) 5C2 with proteins from purified EBs of 15 serovars of *C. trachomatis*. The positions of size markers (kDa) are shown on the left.

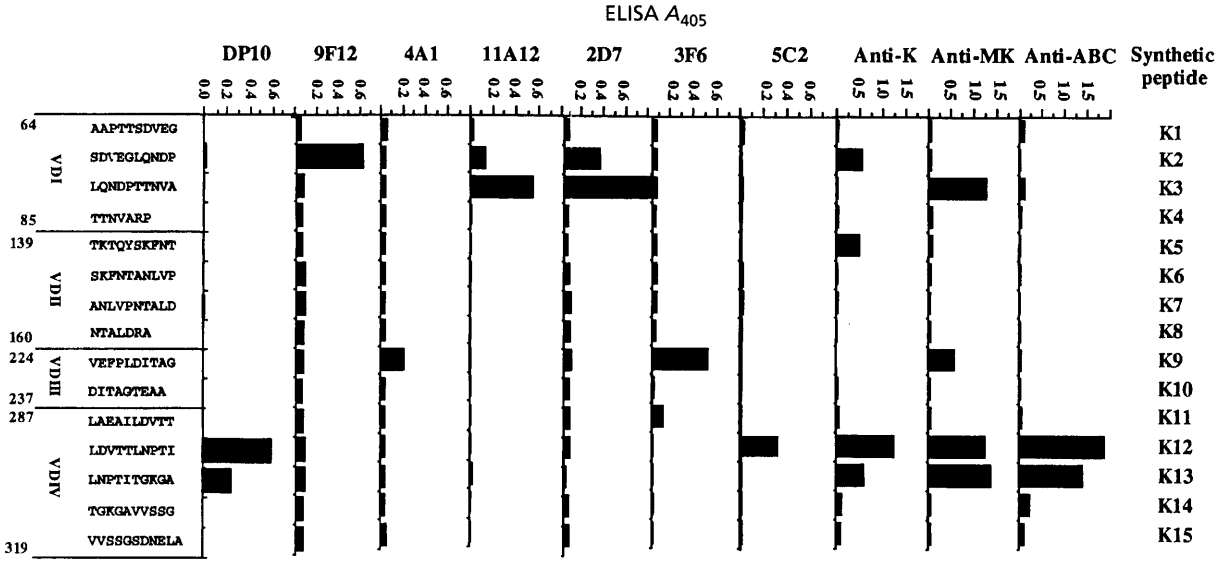

Fig. 3. Mapping of seven anti-MOMP mAbs and three rabbit polyclonal antibodies using overlapping synthetic peptides. Fifteen peptides of ten amino acid residues (named K1 to K15), overlapping by five residues, corresponding to the four VDs of *C. trachomatis* serovar K, were used in this ELISA. mAbs (1 μg ml^{-1}) and polyclonal antibodies (1:1000) were incubated over solid phase-fixed synthetic peptides (50 μg ml^{-1}). Anti-MOMP mAb 5C2 reacted only with the BSA-conjugated form of peptide K12.

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

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

Mouse or rat antiserum	ELISA Ig antibody titre against serovar K*				Neutralizing titre†
	K2‡	K12	MOMP	EBs K	
Mouse anti-K2	3200	ND	400	1600	640
Rat anti-K2	1600	ND	800	1600	ND
Mouse anti-K12	ND	800	400	800	160
Rat anti-K12	ND	200	200	400	ND

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 had neutralizing 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, ^{296}TTLNPTIAG^{304}, 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 ^{69}SDVEGLQNDP^{78}, 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 ^{71}VAGLEK^{76}, a serovar A-specific neutralizing epitope, which has been reported to induce a protective immune response in mice when injected colinearly with a species-conserved T cell epitope (Baehr *et al.*, 1988; Su & Caldwell, 1992; Su *et al.*, 1990a). However, it differs from ^{71}VAGLEK^{76} as these three mAbs do not react with serovar A. Also, Qu *et al.* (1993) recently reported the ^{71}VAGLQNDPT^{79} 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, ^{69}SDVAGLQ^{75} and ^{74}LQNDPTTN^{81}. 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 VDI 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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