Mapping of a surface-exposed B-cell epitope to the variable sequent 3 of the major outer-membrane protein of *Chlamydia trachomatis*

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A B-cell epitope, AEFPLDIT, was located to the variable sequent 3 of the major outer-membrane protein (MOMP) using the monoclonal antibody L3-1, raised to the *Chlamydia trachomatis* serovar L3 MOMP. By Western blot and inclusion immunofluorescence assay the monoclonal antibody recognized all the C complex and C-related complex serovars of *C. trachomatis*, except serovar C. Dot-blot and ELISA data using native elementary bodies indicated that the epitope was surface exposed. The monoclonal antibody, at concentrations of 10 and 100 µg per 10⁷ chlamydial inclusion-forming units, was able to neutralize the infectivity of chlamydia in an *in vivo* assay but did not neutralize chlamydia *in vitro* or in a mouse toxicity assay. A peptide corresponding to the variable sequent 3 has previously been shown to also elicit a T-cell response; thus, careful consideration should be given to inclusion of this region of the major outer-membrane protein in a subunit vaccine.

Introduction

Chlamydia spp. are obligate intracellular Gram-negative bacteria responsible for a variety of diseases in humans and several species of animals. Three species of chlamydiae are now recognized, *Chlamydia trachomatis*, *Chlamydia psittaci* and *Chlamydia pneumoniae*. Based on serological analysis, the human *C. trachomatis* isolates have been subdivided into a C complex (A, C, H, I and J), C-related complex (K and L3), B complex (B, Ba, D, E, L1 and L2) and B-related complex (F and G) (Wang & Grayston, 1984). This classification is based mainly on the immunogenic characteristics of the major outer-membrane protein (MOMP).

Several studies have indicated that the MOMP is strongly antigenic and can elicit neutralizing antibodies (Peterson *et al.*, 1988, 1991; Zhang *et al.*, 1987). Thus, great interest exists in characterizing this protein with the ultimate goal of producing a subunit vaccine (Taylor-Robinson & Ward, 1989). DNA sequence analysis of the MOMP gene of the B, C and L2 serovars of *C. trachomatis* demonstrated the presence of four variable sequents (VSs) interspersed with five constant sequents

(CSs) (Baehr *et al.*, 1988; Stephens *et al.*, 1987). Using monoclonal antibodies (mAbs) several serovar-, subcomplex- or subspecies-specific B-cell epitopes have been mapped to the VS1 and VS2 and subspecies- and species-specific epitopes have been located to the VS4 (Baehr *et al.*, 1988; Carlson *et al.*, 1989; Cheng *et al.*, 1992; Conlan *et al.*, 1988, 1989; Peterson *et al.*, 1988, 1991). Newhall *et al.* (1990) have, in a preliminary report, described two mAbs that mapped to the surface-exposed VS3.

mAbs with serovar and subspecies specificity have been found in general to neutralize the infectivity of the homologous serovars while mAbs with species specificity do not seem to be protective. One of the few exceptions to this is the mAb E4 which recognizes the sequence TLNPTIA, as well as the N-terminus of the serovar E VS4, binds to all serovars by Western blot and neutralizes isolates in the B, B-related and C-related complex but not those in the C complex (Peterson *et al.*, 1988, 1991). Thus, the need to locate neutralizable epitopes for the members of the C complex with potential use in a subunit vaccine remains an important task. Here, we report on the identification of a surface-exposed B-cell epitope located in the VS3 of MOMP present in most of the C- and C-related complex serovars.

Methods

Chlamydiae. *C. trachomatis* serovars L1 (440), L2 (434), L3 (404), A (G-17), B (TW-5), Ba (Apache-2), C (TW-3), D (IC-Cal-8), E (Bour), F (UW-6), G (UW-57), H (UW-4), I (UW-12), J (UW-36), K (UW-31)

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Abbreviations: CS, conserved sequent; EB, elementary body; HRP, horseradish peroxidase; IFA, indirect fluorescent assay; IFU, inclusion-forming unit; i.p., intraperitoneal(ly); mAb, monoclonal antibody; MOMP, major outer-membrane protein; VS, variable sequent.

and mouse pneumonitis (Nigg II), and *C. psittaci* (Texas turkey) and *C. pneumoniae* (TWAR-183) were grown in HeLa-229 cells (American Type Culture Collection) as previously described (Peterson *et al.*, 1988).

Monoclonal antibody production and characterization. To produce mAbs, 4–6-week-old female BALB/c (H2^d) mice (Simonsen Laboratories, Gilroy, CA, USA) were immunized with 30 µg *C. trachomatis* L3 MOMP extracted by the octyl-glucopyranoside (OGP) method (Bavoil *et al.*, 1984). The first inoculation was given intraperitoneally (i.p.) with complete Freund's adjuvant. Three weeks later the mice were given a second dose with the same amount of protein with incomplete Freund's adjuvant. The animals were boosted with purified MOMP via the tail vein once more 3 d before they were killed. Hybridomas were produced as previously described and cloned by limiting dilution (Peterson *et al.*, 1988). Ascites fluid was produced in 6-week-old female BALB/c mice by injecting i.p. 10⁶ hybridoma cells into pristane-treated mice. The peritoneal fluid was harvested and purified through a protein A column (Bio-Rad) following the manufacturer's procedure. The subclass of the mAb was determined using the Bio-Rad ELISA kit.

Immunoassays. Western blots were performed as previously described using nitrocellulose membranes (Peterson *et al.*, 1988). Following transfer, the non-specific sites were blocked with BLOTTO (bovine lacto transfer technique optimizer; 5% (w/v) nonfat dried milk and 0.02% sodium azide in water) and the ascites fluid added. The mAb binding was visualized using a horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Cappel) developed with 0.01% hydrogen peroxide and 4-chloro-1-naphthol.

For the dot-blots, purified chlamydial elementary bodies (EBs) were adsorbed to a nitrocellulose membrane directly after harvesting or following heat treatment (56 °C for 30 min) (Zhang *et al.*, 1987).

ELISA was performed in 96-well plates (Corning) as described elsewhere (Das *et al.*, 1984) except that the wells were coated with native EBs in phosphate-buffered saline (0.01 M-PBS, pH 7.4). EBs of *C. trachomatis*, *C. psittaci* and *C. pneumoniae* were diluted in PBS at a concentration of 40 µg ml⁻¹ and 100 µl added per well. After washing, the mAb or normal mouse ascites was incubated and the EB-antibody complexes were detected by adding HRP-conjugated goat anti-mouse IgG (see above) and the binding was measured using an ELISA reader (Bio-Rad) at 405 nm. For colour development, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) was used as a substrate.

Mapping of the epitope. A commercially available kit (Cambridge Research Biochemical) based on the technique developed by Geysen *et al.* (1987) was used to map the binding site of mAb L3-1. Overlapping hexameric peptides were synthesized to represent the *C. trachomatis* serovar L3 MOMP amino acid sequence (Fielder *et al.*, 1991; Peterson *et al.*, 1991). Goat anti-mouse IgG conjugated to HRP served as the second antibody, ABTS as the substrate, and the reactions were read as indicated above. The assay was repeated twice using sonication in hot SDS and 2-mercapthoethanol to remove the antibody between each assay (Peterson *et al.*, 1991).

Neutralization assays. The *in vitro* neutralization assay was performed as previously described (Peterson *et al.*, 1988), with some modifications. Briefly, tenfold serial dilutions (1, 10 and 100 µg) of mAb L3-1 were made in duplicate using PBS (10 mM, pH 7.4). Purified normal mouse IgG (Sigma) was used as a negative control. *C. trachomatis* EBs were incubated with the mAb dilutions at 37 °C for 45 min. The mixture was then used to inoculate by centrifugation HeLa 229 cells or stationary HaK monolayers grown in glass vials (15 × 45 mm) on a 12 mm glass coverslip. The monolayers were incubated for 48 h at 37 °C in Eagle's minimal essential medium supplemented with 5% foetal bovine serum (FBS), gentamicin (50 µg ml⁻¹) and cycloheximide (1 µg ml⁻¹). The monolayers were fixed in methanol and the chlamydial inclusions stained using mAb E4 and a goat anti-mouse peroxidase stain. The number of inclusion-forming

units (IFUs) was counted in ten 200 × fields and the experiment was repeated three times on separate days.

mAb L3-1 was also assayed for its ability to neutralize chlamydial toxicity in mice (Peterson *et al.*, 1988). Briefly, 100 µl of mAb L3-1 containing 1, 10 or 100 µg of purified IgG was mixed with 10⁸ IFUs of *C. trachomatis* serovar L3 and incubated at 37 °C for 45 min (Peterson *et al.*, 1988). A total of three mice were tested for each concentration of mAb. After incubation the whole mixture was injected into 6–7-week-old BALB/c mice via the tail vein. Controls were run in parallel, with purified normal mouse IgG. The mice were monitored for survival during the next 48 h. The experiment was done three times.

For the *in vivo* neutralization assay, 200 µl mAb L3-1 containing 1, 10 or 100 µg purified IgG, was incubated at 37 °C for 1 h with 10⁷ IFUs of *C. trachomatis* serovar L3. Controls, consisting of normal mouse ascites fluid, undiluted and diluted 1/10 and 1/100 with PBS, or 1, 10 and 100 µg affinity-purified normal mouse IgG, were processed in parallel. Following incubation the EBs were inoculated i.p. into 4–6-week-old female BALB/c mice. A total of eight mice were inoculated with each of the three concentrations of mAb and also seven mice were included in each control group. The animals were killed at 3 d post-inoculation; the lungs, kidneys, liver and spleen were harvested and the yield of chlamydial IFUs was assayed in HeLa 229 cells (Zhong *et al.*, 1988). The Mann-Whitney U test was used for statistical analyses.

Results

Immunoassays

Of the several positive hybridomas obtained by screening the tissue culture fluids by IFA and ELISA mAb L3-1 was chosen for further testing. This mAb was found to be of the IgG1 isotype with a κ light chain. In Western blots mAb L3-1 recognized the MOMP of all the serovars of the C and C-related complex, except the C serovar, while

Fig. 1. Western blot analysis of mAb L3-1 with *C. trachomatis* serovars [A–L3 and mouse pneumonitis (MP)], *C. pneumoniae* (TWAR-183; Pn) and *C. psittaci* (Texas turkey; Ps). Molecular size markers are shown on the left (kDa).

Fig. 2. Dot-blot assay of mAb L3-1 against native (rows A and B) and heat-treated (rows C and D) chlamydial EBs. The order of the *C. trachomatis* serovars and the other chlamydial species in rows A and C is: 1, A; 2, B; 3, Ba; 4, C; 5, D; 6, E; 7, F; 8, G; 9, H; 10, I; 11, J; 12, K. Rows B and D contain 1, L1; 2, L2; 3, L3; 4, HeLa cell protein control; 5, mouse pneumonitis; 6, *C. pneumoniae* (TWAR-183); 7, *C. psittaci* (Texas turkey).

Fig. 3. Histogram of the ELISA of mAb L3-1 against native chlamydial EBs. A to L3, serovars of *C. trachomatis*; MOPN, mouse pneumonitis; TT, *C. psittaci* (Texas turkey); TWAR, *C. pneumoniae* (TWAR-183). The error bars represent 1 SD.

no reactivity was obtained with the B and B-related group serovars (Fig. 1).

Dot-blot analysis was performed using both native and heat-treated EBs. As shown in Fig. 2, mAb L3-1 reacted with both native and heat-treated EBs of the serovars A, H, I, J, K and L3. No reactivity was noted with the serovars in the B and B-related groups.

When the reactivity of the mAb L3-1 against native EBs of different serovars of *C. trachomatis* was measured by ELISA, only serovars in the C and C-related complex, i.e. A, H, I, J, K and L3, showed an A_{405} value significantly higher ($A_{405} \geq 0.25$) than the negative control (Fig. 3).

Fig. 4. ELISA results for mAb L3-1 with the overlapping hexameric peptides corresponding to amino acids 211 to 309 of the *C. trachomatis* L3 serovar.

Table 1. Amino acid sequence of the chlamydia serovars corresponding to the region of the B-cell epitope

Major group	Serovar	Peptide sequence*
C complex	A	AEFPLDIT
	C	AEFPLNIT
	H	AEFPLDIT
	I	AEFPLDI
	J	AEFPLDIT
	K	VEFPLDIT
	L3	AEFPLDIT
B complex	B	<u>KE</u> LPLDLT
	Ba	<u>KE</u> LPLDLT
	E	<u>QE</u> FPLALI
	D	<u>KE</u> FPLDLT
	G	<u>QE</u> FPLALT
	F	<u>KE</u> FPLDLT
	L1	<u>KE</u> FPLDLT
Other	L2	<u>QE</u> FPLDLK
	MoPn† <i>C. psittaci</i>	<u>QE</u> FPLNIK <u>SN</u> FPLPIT

* Underlined amino acids are different from the *C. trachomatis* L3 amino acid sequence.

† MoPn, mouse pneumonitis.

Mapping of the epitope with hexameric peptides

The complete amino acid sequence of the *C. trachomatis* L3 serovar MOMP was synthesized using overlapping hexameric peptides. By this method mAb L3-1 had the highest reactivity with the peptide AEFPLD located in the VS3 of MOMP and decreasing reactivity with the next two consecutive peptides EFPLDI and EFPLDIT (Fig. 4). No reactivity was observed with the rest of the MOMP.

Table 2. *In vivo* neutralization assayResults are mean log₁₀ [IFU (g tissue)⁻¹] ± 1 SD.

Antibody concn (µg)	Lung		Liver		Spleen		Kidney	
	mAb	Control	mAb	Control	mAb	Control	mAb	Control
1	3.1 ± 0.6	3.1 ± 0.7	3.6 ± 0.8	4.7 ± 1.4	5.8 ± 1.0	6.9 ± 0.7	2.8 ± 0.7*	3.8 ± 0.8
10	2.9 ± 0.7	3.5 ± 0.8	3.0 ± 1.0*	4.5 ± 0.9	5.4 ± 0.7*	6.8 ± 0.5	3.0 ± 1.2	3.5 ± 0.8
100	2.5 ± 0.1*	3.5 ± 0.6	2.6 ± 0.9*	4.6 ± 0.9	4.6 ± 1.5*	6.8 ± 0.7	2.3 ± 0.2*	3.7 ± 0.4

* Values significant by the Mann-Whitney U test ($P < 0.05$).

In addition, hexameric peptides with all the possible amino acid substitutions corresponding to the same region on the other *C. trachomatis* isolates were synthesized (Table 1; Yuan *et al.*, 1989). While a substitution of the A with a V did not affect mAb L3-1 binding, as was seen with serovar K, replacement of this amino acid in the B-complex serovars with a K or a Q completely abolished recognition by mAb L3-1. Furthermore, substitution of the D with an N, as in serovar C, and replacement of the F by an L, as in serovars B and Ba, also abrogated antibody binding. This suggests that A/VFD are critical residues for antibody recognition.

Neutralization assays

To assess the ability to block the *in vitro* infectivity of chlamydia, EBs were incubated with mAb L3-1 and their infectivity subsequently assayed in HeLa 229 and HaK cells. No *in vitro* neutralization was obtained with the serovars tested, L3, C, H, I, J, A and K. This mAb also failed to protect mice in a toxicity assay, even at an IgG concentration of 100 µg per 10⁸ *C. trachomatis* L3 IFUs.

To determine if mAb L3-1 could block *in vivo* the infectivity of chlamydia, three different concentrations of the mAb (1, 10 and 100 µg in 200 µl PBS) were incubated with 10⁷ *C. trachomatis* serovar L3 IFUs and inoculated i.p. into three groups of BALB/c mice. The liver, spleen, lungs and kidneys were harvested 3 d post-inoculation and the yield of chlamydial IFUs determined for each organ. With 100 µg of the mAb, there was a significant reduction in the yield of IFUs from the four organs harvested and, with 10 µg of the mAb, the yield of chlamydial IFUs from the liver and spleen was also significantly reduced ($P < 0.05$) as shown in Table 2.

Discussion

Significant efforts have been focused on the characterization of B- and T-cell epitopes of the *C. trachomatis* MOMP with the eventual goal of identifying those regions necessary for inclusion in a recombinant

subunit vaccine. B-cell epitopes have been mapped with mAbs to the VSs 1, 2 and 4 of the *C. trachomatis* MOMP and T-cell epitopes have been described in several of the VSs and CSs of MOMP (Allen *et al.*, 1991; Baehr *et al.*, 1988; Carlson *et al.*, 1989; Conlan *et al.*, 1988, 1989; Peterson *et al.*, 1988, 1991; Su *et al.*, 1990). Here we report a surface-exposed B-cell epitope identified in VS3. Of the eight amino acids recognized by mAb L3-1, AEFPLDIT, four, EFPL, are conserved in all the serovars of *C. trachomatis* including the mouse pneumonitis biovar, except for the B and Ba serovars, where the F has been replaced by a L (Table 1). It is interesting that Newhall *et al.* (1990), using fusions proteins, located the epitopes of two mAbs to the region, GKEFPLDLTAGTDA, that overlaps the epitope of mAb L3-1. The B, Ba, D, F, L1 and subspecies (-B, Ba) epitopes were found to be surface-exposed as determined by immunoelectron microscopy (Newhall *et al.*, 1990). All the C and C-related complex serovars have the same eight amino acid sequence except for the C serovar, which has a N instead of a D, and the K serovar, which has a V replacing the A. The finding by Western blot, ELISA, peptide pins and by dot-blot using native and heat-treated EBs that the C serovar is negative suggests that replacement of D, an acidic amino acid, by N, a neutral amino acid, significantly alters the configuration of the epitope. Similarly in the B, B-related complex and in the mouse pneumonitis serovar the A has been replaced by a K or a Q and the I by a L and these substitutions also resulted in lack of binding. P and D are two amino acids frequently found in β -turns. P is present in all the *C. trachomatis* serovars while D is replaced by an N, an amino acid also frequently found in β -turns, in the C and the mouse pneumonitis serovars. In contrast, in serovars E and G, both members of the B group, the D is substituted by an A, an amino acid not often found in β -turns. Thus, most likely the overall conformation and the surface exposure of this region of the MOMP are significantly different in the serovars of the C and C-related group, except C, when compared with the other *C. trachomatis* serovars.

Analyses of the amino acid sequence of the B and L2

serovars indicated that trypsin cleavage sites, either K or R residues, were present in all the VSs (Su *et al.*, 1988). Digestion of the B serovar with trypsin however, showed that only the VS2 and VS4 could be cleaved, suggesting that the VS1 and VS3 trypsin-sensitive cleavage sites were buried in the membrane and as a result not available for trypsin digestion. Furthermore, the serovar L2 MOMP was cleaved only in the VS4. These findings suggested that the VS3 was not surface exposed. We obtained mAb L3-1 following the inoculation of OGP-extracted MOMP and thus, the possibility existed that a hidden site in the native chlamydia was exposed during extraction. Our findings with dot-blot analysis and ELISA using native EBs show that mAb L3-1 was able to bind to all the members of the C and C-related complex serovars, except C, suggesting that this epitope located in the VS3 is surface-exposed in these isolates. Although our data can only suggest that this epitope is surface-exposed, Newhall *et al.* (1990), using immunoelectron microscopy, determined that the VS3 was surface exposed by probing with two mAbs to this region. Furthermore, the fact that this epitope was also recognized by the mAb on Western blots, on methanol-fixed chlamydial inclusions and on the synthesized peptide pins suggests that the primary configuration of the epitope is critical for antibody binding, an important fact for including epitopes in subunit vaccines where the tertiary and quaternary conformation of the epitope would not be present.

The mAbs with serovar and subspecies specificity have been found in general to neutralize the infectivity of homologous serovars while mAbs with species specificity do not seem to be protective (Baehr *et al.*, 1988; Peterson *et al.*, 1988, 1991; Zhang *et al.*, 1987). One of the few exceptions to this is mAb E-4, which recognizes the species-conserved amino acid sequence TLNPTIA in the VS4, binds to all serovars in Western blots and neutralizes all the *C. trachomatis* serovars except those in the C complex (Cheng *et al.*, 1992; Peterson *et al.*, 1988, 1991). As a result, the search for broad-reacting, neutralizable epitopes, particularly for the C-complex serovars, has continued. The *in vitro* and toxicity assay data do not indicate that mAb L3-1 described here can neutralize the infectivity of *C. trachomatis*. On the other hand, the neutralization obtained in the *in vivo* assay was significant. Thus, the ability of a peptide containing this epitope to elicit protective antibodies should be further explored, particularly since this region of the MOMP is known also to be able to elicit a strong T-cell response (Allen *et al.*, 1991; Ishizaki *et al.*, 1992; Su *et al.*, 1990). The mechanisms involved in the *in vitro* and *in vivo* infectivity neutralization assays and in the toxicity assay are poorly understood and it is not surprising that there is not always a complete correlation between the three

methods. It is very likely that the mechanisms of attachment to and entry into cells are different *in vivo* and *in vitro*. Furthermore, in the *in vivo* model other clearing mechanisms such as opsonization and antibody-dependent cytotoxicity may be important. Discrepancies between the *in vivo* and *in vitro* functional activities of mAbs have been also found in other systems (Isaacs *et al.*, 1992).

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