Original Article

Identification of immunodominant linear B-cell epitopes within the major outer membrane protein of *Chlamydia trachomatis*

Shanli Zhu, Jun Chen, Meixia Zheng, Wenci Gong, Xiangyang Xue, Wenshu Li, and Lifang Zhang*

Department of Microbiology and Immunology, Wenzhou Medical College, Wenzhou 325035, China
*Correspondence address. Tel: +86-577-86689910; Fax: +86-577-86689910; E-mail: wenzhouzlf@126.com

*Chlamydia trachomatis* is one of the most prevalent sexually transmitted pathogens. Chlamydial major outer membrane protein (MOMP) can induce strong cellular and humoral immune responses in murine models and has been regarded as a potential vaccine candidate. In this report, the amino acid sequence of MOMP was analyzed using computer-assisted techniques to scan B-cell epitopes, and three possible linear B-cell epitope peptides (VLKTDVNKE, TKDASIDYHE, TRLIDERAAH) with high predicted antigenicity and high conservation were investigated. The DNA coding region for each potential epitope was cloned into pET32a(+) and expressed as Trx-His-tag fusion proteins in *Escherichia coli*. The fusion proteins were purified by Ni-NTA agarose beads and followed by SDS-PAGE and western blot analysis. We immunized mice with these three fusion proteins. The sera containing anti-epitope antibodies from the immunized mice could recognize *C. trachomatis* serovars D and E in ELISA. Antisera of these fusion proteins displayed an inhibitory effect on invasion of serovar E by *Chlamydia trachomatis* in vitro. Neuturalized antibodies [10] may represent potential candidates for the development of novel vaccine formulations. Among them, the major outer membrane protein (MOMP) is regarded as a promising candidate, because the cysteine-rich MOMP makes up 60% of the total outer membrane protein and harbors genus-, species-, and serotype-specific epitopes that elicit T-cell responses and neutralizing antibodies [10]. However, data from previous publications indicated that it is difficult to produce recombinant MOMP in a native form with intact, conformationally relevant epitopes on a scale large enough to be commercially viable. These particular problems have greatly delayed the development of *C. trachomatis* recombinant protein vaccines.

From the published data, we proposed that antigenic complexity could be designed to include protective B-cell and T-cell epitopes and exclude potentially immunosuppressive and immunopathogenic determinants. The epitope-based vaccine containing selected immunogenic targets represents an alternative and novel strategy against chlamydia. Considering the importance of MOMP, we believed that a detailed analysis of MOMP epitopes is necessary for understanding immunological events, the development of

Introduction

*Chlamydia trachomatis* is an obligate intracellular pathogen that infects the epithelium of the genital and ocular mucosa. According to the WHO report, genital chlamydia infection is the most common sexually transmitted infections in several industrialized nations, accounting for more than 92 of the 340 million annual new sexually transmitted diseases (STDs) worldwide [1]. In addition, infection with *C. trachomatis* facilitates the transmission of human immunodeficiency virus [2] and might be a cofactor in human papillomavirus-induced cervical neoplasia [3,4].

Host immune response contributes significantly to protective anti-chlamydia responses and has been blamed for pathologic changes associated with chlamydial infection. In particular, Th1 type cytokines IFN-γ is a major player in promoting chlamydia persistence and immunopathology [5,6]. Neutralization antibody may play a role in host defense against chlamydial infection [7]. New tools need to be developed to control the chlamydia persistent infection and its rapid global spread. Vaccination is one such tool that may control and even eradicate the disease.

Several surface-associated proteins highly conserved among the different serotypes of *C. trachomatis* [8,9] may represent potential candidates for the development of novel vaccine formulations. Among them, the major outer membrane protein (MOMP) is regarded as a promising candidate, because the cysteine-rich MOMP makes up 60% of the total outer membrane protein and harbors genus-, species-, and serotype-specific epitopes that elicit T-cell responses and neutralizing antibodies [10]. However, data from previous publications indicated that it is difficult to produce recombinant MOMP in a native form with intact, conformationally relevant epitopes on a scale large enough to be commercially viable. These particular problems have greatly delayed the development of *C. trachomatis* recombinant protein vaccines.
epitope-based experimental vaccines and clinical diagnosis of chlamydia infections.

Several HLA-restrict CTL epitopes in MOMP have been identified [11–13]. However, few reports were focused on B-cell epitopes of MOMP [14,15]. And published data have indicated that C. trachomatis serovars D, E, and F were the most predominant serovars prevalent worldwide [16–18]. In this report, we screened and identified the linear B-cell epitopes on MOMP protein of C. trachomatis serovar E, using a bioinformatics method in combination with molecular biology methods, which could gain further understanding of the antigenic structure of the proteins for developing novel vaccines and designing diagnostic tools.

Materials and Methods

Materials

Restriction enzymes and T4 DNA ligase were purchased from MBI fermentas (Burlington, Canada). Mouse monoclonal anti-His-tag antibody (mAb), Horseradish peroxidase (HRP)-labeled goat anti-mouse IgG and labeled goat anti-human IgG were purchased from KPL (Gaithersburg, USA). pET32a (+) expression vector was purchased from Novagen (Darmstadt, Germany). Ni-NTA agarose was purchased from Qiagen (Hilden, Germany). Chlamydia trachomatis serum E (ATCC VR-348B), serum D (ATCC VR-885) and HeLa 229 cells (ATCC CCL-2.1) were purchased from the American Type Culture Collection (ATCC) (Manassas, USA). Chlamydial antigens were prepared by purifying elementary body over ultracentrifugation and inactivation by 60°C for 1 h.

Human sera samples from women patients diagnosed with C. trachomatis urogenital infection were provided by the second affiliated hospital of Wenzhou Medical College (Wenzhou, China). Patients were informed that the tests requested in the study were designed for research only.

Prediction of B-cell epitopes of C. trachomatis

Prediction of B-cell epitope of C. trachomatis MOMP was performed according to Zhu et al. [19]. Briefly, the amino acid sequences of MOMP from C. trachomatis serovars D–K were obtained from the National Center of Biotechnology Information. Multiple alignments were carried out using the software of the European Bioinformatics Institute website (http://www.ebi.ac.uk/Tools/clustalw2). Secondary structure of MOMP was predicted by GOR (Garnier-Osguthorpe-Robson) method [20], hydrophilicity and flexibility were analyzed by the methods of Hoop and Woods [21] and Zimmerman et al. [22], respectively. All the programs are provided in the server EXPASY (http://www.expasy.org/tools). The transmembrane domains of MOMP were analyzed by the methods of artificial neural network (http://strucbio.biologie.uni-konstanz.de) [23]. Antigenic propensity value was further carried out using the approach of Kolaskar and Tongaonkar (http://bio.dfci.harvard.edu/Tools/antigenic.pl) [24]. The procedure for epitope mapping was as follows: only peptides defined by all the algorithms were considered; continuous amino acid residues were chosen with high antigenicity, flexibility, and hydrophilicity; extracellular peptides in β-turn and random coils were chosen [25].

Plasmid construction

To express the predicted peptides, the amino acid sequences of each peptide were translated into DNA sequences. Then the complementary oligonucleotide pairs encoding each peptide were synthesized. The coding strand and antisense strand targeting each peptide were designed to include BamHI and XhoI restriction sites, respectively. And termination codon of TAA was added in the antisense strand before XhoI sites. After the complementary oligonucleotides were annealed and confirmed, the genes were cloned into pET32a(+) digested with the appropriate restriction enzymes. All constructs were sequenced prior to further use.

Protein expression and affinity purification of Trx-His-tagged proteins

For expression of epitope fusion proteins, E. coli BL21 (DE3) transformed with positive plasmids was induced by isopropyl-β-D-thiogalactopyranoside (IPTG). The expression and purification of Trx-His-epitope fusion proteins were performed under denaturing conditions following the procedure recommended by Qiagen. Proteins were renatured by dialysis against phosphate buffered saline (PBS), fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and detected by western blot analysis with an anti-His antibody.

Western blot assay

To confirm the presence and the apparent molecular mass of the recombinant proteins, western blotting was carried out using anti-His tag mAb. Purified fusion proteins were separated by 12% SDS-PAGE, electrotransferred onto a nitrocellulose membrane, and blocked with 5% nonfat dry milk in TBS at 37°C for 60 min. After washing with PBST, the membrane was incubated with anti-His mAb for 2 h. After washing, the filters were further incubated with HRP-conjugated goat anti-mouse IgG, and immunoreactive protein bands were visualized with 3,3’-diaminobenzidine tetrahydrochloride (DAB) kit from Santa Cruz Biotechnologies (Santa Cruz, USA).

To evaluate the antigenicity of expressed epitope fusion proteins, the purified samples were detected with 1:100 dilution of sera from women patients diagnosed with C. trachomatis urogenital infection and HRP-conjugated goat anti-human IgG. Trx-His-tag protein purified from
E. coli BL21 transformed with pET32a(+) vector was used as control.

**Production of anti-epitope sera**

Antisera to the three fusion proteins were raised in female BALB/c mice (SLACCAS, Shanghai, China) \((n = 7\) for each peptide). Mice received three subcutaneous injections of 100 \(\mu\)l of immunogen containing 50 \(\mu\)g fusion proteins emulsified in complete Freund’s adjuvant on Day 0 or 50 \(\mu\)g in incomplete Freund’s adjuvant on Days 14 and 28. Tail blood was collected on Day 42 and the antisera were used in ELISA and neutralization assays. Sera of mice inoculated with Trx-His-tag protein were used as negative controls.

**Indirect ELISA**

An indirect ELISA technique was used to evaluate the ability of immune sera of epitope fusion proteins to recognize the native chlamydial antigen. Microplates were coated with heat inactivated *C. trachomatis* serovars E and D at \(1 \times 10^4\) inclusion-forming units (IFU)/ml resuspended in carbonate buffer (pH 9.6) at 4°C overnight. After washing to remove excess antigen and blocking with 2% BSA in PBS, sera (diluted 1:100) were added to the antigen-immobilized microplates for 1 h at 37°C, followed by four PBST washes. The serum antibody binding was detected with a goat antimouse IgG conjugated with HRP in combination with soluble substrate Ortho-phenylenediamine and was quantitated by reading the absorbance at 490 nm using an ELx800™ microplate reader (BIO-TEK, Winooski, USA). The mean value of triplicate determinations was calculated.

**In vitro neutralization assays**

The *in vitro* neutralization assay was performed as described previously [26]. Briefly, mouse polyclonal antisera were prepared in our laboratory to potential B epitopes and chlamydia were heated to inactivate at 56°C for 30 min before use. Antisera were diluted in PBS containing 5% guinea pig serum (complement). Chlamydiae (\(10^4\) IFU) were added to the appropriate antiserum dilutions and the control mixtures, which contained anti-His-tag antibody. The antigen-antibody mixtures were incubated at 37°C for 45 min and inoculated into duplicate confluent HeLa 229 cell monolayers contained in six well plates, which had been washed twice with PBS immediately before inoculation. Cells were infected by centrifugation at 1000 g for 1 h followed by stationary incubation at 37°C for 1 h, then cultures were mixed with 1 ml of DMEM containing cycloheximide (1 mg/ml). Infected monolayers were incubated for 48 h, fixed, and stained with iodine. IFU were counted in 10 fields at a magnification of 200x, an average was taken, and the results were expressed as a percentage of the IFU of control monolayers. Each test was repeated at least three times on separate days.

**Results**

**Prediction and selection of B-cell epitopes**

Flexibility, hydrophilicity, and extracellular regions are important features of antigenic epitopes. The existence of flexible regions, such as random coil and turn regions, provides powerful evidence for epitope identification. In this study, the secondary structure of MOMP was predicted by the methods of GOR, on the basis of the sequence of MOMP protein obtained from NCBI (GenBank/GenPept accession no. DQ064286). Results showed that random coil was the major characteristics of the flexibility region in the secondary structure of MOMP. Analysis of transmembrane domain indicated that MOMP was a transmembrane protein, which coincided with previous work by Rodríguez-Marañón et al. [27]. The amino acid sequence of MOMP was analyzed by the methods of Hopp and Woods, Zimmerman, and Kolaskar and Tongaonk algorithms in combination with structural information, five peptides were identified and defined as potential epitopes, they are located in the regions of 73–81, 161–175, 217–225, 261–270, and 377–386 in the N-terminal region (Table 1). When the amino acid sequences of MOMP were compared with those from the most prevalent serovars (D–F) worldwide, high conservation of amino acids was found within the four predicted epitope peptides, except one in the region of 161–175 (Fig. 1). As the region of 161–175 has been reported to contain a B-cell epitope (NQSTVKT) by Batteiger [15], we investigated only three (73–81, VLKTDVNKE; 261–270, TKDASIDYHE; and 377–386, TRLIDERAAH) epitope peptides experimentally according to the following criteria: high conservation among serovars, high predicted antigenic and physicochemical scores.

**Production of Trx-His-fused proteins carrying the predicted epitopes**

The DNA fragments encoding each polypeptide were synthesized and cloned into the BamHI and XhoI sites of pET32a(+) (Fig. 2), resulting in three recombinant

<table>
<thead>
<tr>
<th>Dominant region of B-cell epitope</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>73–81</td>
<td>VLKTDVNKE</td>
</tr>
<tr>
<td>161–175</td>
<td>DNENQSTVKTNSVPN</td>
</tr>
<tr>
<td>217–225</td>
<td>QSKPKVEEL</td>
</tr>
<tr>
<td>261–270</td>
<td>TKDASIDYHE</td>
</tr>
<tr>
<td>377–386</td>
<td>TRLIDERAAH</td>
</tr>
</tbody>
</table>
Figure 1 Alignment of predicted B-cell epitope peptides of MOMP among various *C. trachomatis* isolates  The peptides epi-1, epi-2, and epi-3 corresponding to amino acids 73–81, 261–270, and 377–386 of *C. trachomatis* serovar E MOMP were compared with amino acid sequences in the corresponding MOMP regions of strain E/Bour (DQ064286), strain D/IC-Cal-8 (X62920), strain F/IC-Cal3 (X52080), strain G/UW57 (DQ064299), strain I/UW-12 (DQ064290), strain J/UW-36 (DQ064292), strain H/580 (DQ064289), and strain K/UW-31 (DQ064293) from Genbank. Amino acid sequences were aligned using Clustalw. Dots in the alignments correspond to conserved amino acids and short lines to gaps. B-cell epitopes, predicted for the sequences obtained from E/Bour, are shown using the corresponding residue letters and shaded in gray.
plasmids. Sequencing the recombinant plasmids confirmed the successful construction of these expression plasmids.

These epitope proteins were expressed as fusion proteins with the Trx-His-tag, which can be detected easily with mAb. Figure 3 shows that all three recombinant proteins containing epitope-1, 2, and 3, respectively, and were efficiently induced and purified under the denatured conditions from transformed *E. coli* cells. These Trx-His-tag fusion proteins were purified by Ni-NTA agarose affinity chromatography and analyzed by SDS-PAGE [Fig. 3(A)]. The expected molecular mass of the recombinant proteins was about 23 kDa, which was verified by western blotting with anti-His tag mAb [Fig. 3(B)].

Immunogenicity of predicted epitopes in animals
Epitopes can be identified by their ability to bind to certain antibodies. Almost all of the *C. trachomatis* urogenital infection individuals’ sera were MOMP positive [28]. In order to define the predicted epitopes on chlamydia MOMP, sera from three women patients diagnosed with *C. trachomatis* urogenital infection were tested for the reactivity of the epitope-fused proteins by western blot assay. As shown in Fig. 4, all these three epitope-fused proteins could be specifically recognized by patient’s sera. The fusion proteins reacted with human sera and showed an intensive band at the position of 23 kDa, accompanied by weak bands in larger sizes that are possibly protein dimers of the fusions, as determined by the molecular weight [Fig. 4(B), lanes 1–3].

These data indicated that peptides of VLKTDVNKE, TKDASIDYHE, and TRLIDERAAH were immunodominant B-cell epitopes within MOMP. These epitope peptides were further verified by indirect ELISA technique and showed a good reactivity to mouse immune sera after third immunization of epitope fusion proteins. The specific antibodies to recombinant protein could be induced in all these mice (data not shown). As shown in Fig. 5, all induced antibodies of fusion proteins could bind to native chlamydial antigen serovars D and E, and the best with the immune sera of epitope-3 fused protein contained a sequence of TRLIDERAAH. These results further indicated that these three predicted epitopes were immunodominant B-cell epitopes of MOMP.

In vitro neutralization assays
In vitro neutralization assays were undertaken to determine whether or not anti-epitope fusion protein antibodies produced in the mice serum could prevent *C. trachomatis* infection of HeLa 229 cells. The assays were done using the same antisera for indirect ELISA (Fig. 5). Sera from mice immunized with *C. trachomatis* serovar E and Trx-His-tag were used as positive and negative controls, respectively. Neutralization defined as a 50% reduction in the percentage of infected cells in the test assay when compared with the negative control at the same dilution. At 1:10 and 1:40 dilutions, these anti-epitope protein sera showed a 50% reduction of infected cells when compared with the serum immunized with Trx-His-tag (Fig. 6).
Identification of immunodominant linear B-cell epitopes within the MOMP

**Discussion**

MOMP was demonstrated to play a key role in chlamydia infection and pathogenesis by host cell interaction and inhibition of phagosome–lysosome fusion [29]. Owing to its immunogenicity and surface distribution [30–32], MOMP has been the most widely targeted antigen as a vaccine candidate against chlamydial infection. However, it is difficult to obtain recombinant MOMP in a native form with intact, conformationally relevant epitopes due to its cysteine-rich structure. The purpose of this study was to locate linear B-cell epitopes on MOMP, which might be potentially useful in diagnosis, vaccine design, and immunological studies, even though a few B-cell epitopes of MOMP has been reported.

Epitopes are continuous (or linear) and discontinuous (or conformational) epitopes. Methods used for mapping B epitopes include homolog-scanning mutagenesis, scans of overlapping peptides, phage display peptide libraries, and so on. Many of those methods used for characterizing epitopes are time consuming and demand large resources. With the aid of software and bioinformatics techniques, we are able to map linear B-cell epitopes of the MOMP from *C. trachomatis* serovar E. We have identified five peptide

---

**Figure 4** SDS-PAGE and western blot analysis of the fusion proteins

Affinity purified epitope fusion proteins and Trx-His-tag alone expressed from *E. coli* were run in 12% SDS-PAGE with molecular weight markers in lane M. One of the gels was stained with Coomassie blue (A). Protein bands in the other gel were transferred onto nitrite membranes for western blot with sera from patient infected with *C. trachomatis* (B). Epi-1 fusion protein (lane 1), Epi-2 fusion protein (lane 2), Epi-3 fusion protein (lane 3), and Trx-His-tag alone (lane 4).

**Figure 5** ELISA analysis of immune sera of epitope fusion proteins

Mice were immunized with each epitope fusion protein by subcutaneous injection. Trx-His-tag alone served as a negative control. Sera from each mouse group were evaluated by ELISA with *C. trachomatis* serovars D and E at $1 \times 10^5$ IFU/ml as described in ‘Materials and Methods’. *P* < 0.05, compared with sera from Trx-His-tag alone (n = 7).

**Figure 6** *In vitro* neutralization assays

*In vitro* neutralization of *C. trachomatis* infectivity by anti-epitope sera in the presence of complements. Chlamydia were mixed with the following mice sera with 5% GPS: sera from mice immunized with Trx-His-tag alone as a negative control; individual anti-epitope fusion protein serum; and sera from mice immunized with *C. trachomatis* as a positive control. The chlamydia-serum mixtures were incubated with HeLa 229 monolayers. After 48 h, IFU was determined by microscopy of iodine-stained smears and inhibition efficiency (%) of each serum was calculated from three separate experiments. Neutralization assays demonstrated that these anti-epitopes antisera effectively inhibited *C. trachomatis* infectivity at 1:10 and 1:40 dilutions, similar to that observed with antibodies from mice immunized with *C. trachomatis* serovar E.
epitopes. One of them has been identified as a B-cell epitope [15]. Three peptides VLKTDYNKE (MOMP_{73–81}), TKDASIDYHE (MOMP_{261–270}), and TRLIDERAAH (MOMP_{377–386}), with high conservation among serovars, high predicted antigenic and physicochemical scores, were confirmed further by western blotting, ELISA, and in vitro neutralization assays.

The epitope peptides were expressed as Trx-His-tag fusion proteins in prokaryotic expression system induced by IPTG. SDS-PAGE showed that these epitope fusion proteins could be efficiently purified by Ni-Sepharose affinity chromatography. Western blot confirmed the fusion proteins reactive with the anti-His mAb.

The antigenicity and immunogenicity of these potential B-cell epitopes of MOMP from C. trachomatis were determined by western blotting and indirect ELISA, respectively. As mentioned above, the sera from patients infected with C. trachomatis contained the antibodies to the whole MOMP of C. trachomatis. If the protein of native MOMP contained the predicted B-cell epitopes, antibodies in the sera would recognize the epitope fusion proteins in western blotting. As a consequence, all these epitope fusion proteins were recognized by the sera of patients infected with C. trachomatis. Furthermore, mouse immune sera of epitope fusion proteins could respond to native MOMP antigen obtained from C. trachomatis, which further confirmed the three predicted epitopes. Based on the physicochemical characteristics of amino acid residues, such as molecular secondary structure, hydrophilicity, flexibility, surface probability, and transmembrane region, we inferred that the peptide of MOMP_{377–386} (TRLIDERAAH) is the strongest immunogen among them in combination with the data of indirect ELISA.

In addition, homology searches have indicated that three immunodominant peptides of C. trachomatis MOMP, located within the sequence of 73–81, 261–270, and 377–386, are well conserved among several serotypes D–K. The data of indirect ELISA showed that serum antibodies from mice inoculated with these epitope fusion proteins were able to cross-react with C. trachomatis both serovars D and E. Western blot assays showed all of the epitope fusion proteins were recognized by sera from patients infected with C. trachomatis, despite serovar of C. trachomatis in the patient infection was unknown.

Numerous studies have shown that antibodies produced in serum against C. trachomatis MOMP are able to neutralize the infectiousness of C. trachomatis in vitro and in vivo [33,34]. In present study, antibodies to epitope fusion proteins from the immunized mice were able to recognize the epitope recombinant proteins as well as the native chlamydial MOMP protein, and in vitro neutralization assays demonstrated that these anti-epitopes antisera effectively inhibited C. trachomatis infectivity at 1:40 dilution, almost similar to that observed with antibodies from mice immunized with C. trachomatis serovar E. These data not only further indicated these peptides of 73–81 (VLKTDYNKE), 261–270 (TKDASIDYHE), and 377–386 (TRLIDERAAH) were immunodominant B-cell epitopes within C. trachomatis MOMP, but also implied these peptides might be the potential target for the diagnosis and epitope vaccine of C. trachomatis.

In summary, the specific recognition of the peptide fusion proteins by sera from patients and the ability to stimulate antibodies production in mice demonstrates that the C. trachomatis MOMP contains predicted B-cell epitopes. Thus, our study indicated that the production of recombinant proteins could be served as diagnostic tools and candidate vaccines to stimulate protective immunity against C. trachomatis. It will be of great interest to investigate the ability of anti-epitope antibodies in conferring protective immunity against C. trachomatis infection in animal models.

Funding

This work was supported by grants from the National Natural Science Foundation of China (no. 30972669) and Natural Science Foundation of Zhejiang province (no. Y205659).

References

9 Swanson KA, Taylor LD, Frank SD, Sturdevant GL, Fischer ER, Carlson JH and Whitmire WM, et al. Chlamydia trachomatis polymorphic membrane


