Generation and evaluation of vaccine candidates against *Chlamydophila pneumoniae*

Ph. D. Thesis

Ildiko Faludi

Department of Medical Microbiology and Immunobiology

Faculty of Medicine

University of Szeged

Szeged, Hungary

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Introduction

Chlamydophila pneumoniae is an obligate intracellular human pathogen, which causes respiratory tract infections, i.e. pneumonia, bronchitis, pharingitis and sinusitis. Seroepidemiological studies show that *C. pneumoniae* is widespread and nearly everybody becomes infected with it during his life. *C. pneumoniae* can also cause chronic infections, which might lead to atherosclerosis and heart disease. It is suspected to have a role in the pathogenesis of Alzheimer disease and multiple sclerosis. These are the reasons why it is important to develop vaccine against *C. pneumoniae* infection.

C. pneumoniae possesses a type III secretion system (TTSS), which allows them to secrete effector molecules into the inclusion membrane and the host cell cytosol. Low calcium response proteins E (LcrE) and H (LcrH) are parts of TTSS. The LcrE is surface exposed in *C. pneumoniae* elementary body (EB) and capable of eliciting protective antibodies in infected hosts, and therefore has potential as a candidate vaccine component to prevent infection with this significant human pathogen. LcrH is a TTSS chaperone protein, expressed in the middle to late stages of the developmental cycle. 6His-tagged LcrE and LcrH were cloned from *C. pneumoniae* CWL029, expressed in *Escherichia coli* (*E. coli*) and purified from the supernatant of *E. coli* lysate using the HIS-select TALON CellThru Resin.

The immunogenicity and protective effect of recombinant LcrE protein combined with either Freund's or Alum adjuvants were investigated in a mouse model of *C. pneumoniae* infection.

The immunogenicity of recombinant LcrH protein mixed with Freund's adjuvant and the protective effect of the immune response was also investigated in mice.

Mycobacterium smegmatis is a species of rapidly growing saprophyte with a number of properties that make it an effective vaccine vector. Recombinant *M. smegmatis* expressing protective antigens of different pathogens and molecules modulating the immune responses offers some potential for reduction of the burden of tuberculosis, HIV and hepatitis B infections. Recombinant *M. smegmatis* expressing chlamydial proteins can be used as a vaccine vector. For this reason we studied over-expression of homologues and heterologous proteins and RNA metabolism in *M. smegmatis*.

Aim

The purpose of this study was:

- the over-expression and purification of chlamydial LcrE and LcrH proteins in large quantities;

- investigation of the immunogenicity and the protective ability of subcutaneous vaccination with recombinant LcrE protein in combination with Freund's or Alum adjuvants against *C. pneumoniae* infection in mice;
- generation of recombinant *M. smegmatis* expressing chlamydial proteins which can be used as vaccine:
    - for this purpose over-expression of proteins in *M. smegmatis* was studied, i.e. *M. smegmatis* Rne/Rng family protein (RNase E) was cloned and expressed and some of its associated proteins were identified;
    - furthermore chlamydial proteins were cloned into *M. smegmatis*.

Materials and methods

I. Bacterial strains and growth conditions

M. smegmatis MC2 155, *E. coli* DH5α, *E. coli* HB101 and *C. pneumoniae* CWL029 (ATCC) strains were used. *E. coli* strains were routinely grown in LB (Luria–Bertani) medium supplemented with the appropriate antibiotic(s). *M. smegmatis* MC2 155 was grown in LB medium or in Middlebrook 7H9 broth or 7H11 agar (Difco) supplemented with 10% Middlebrook OADC (oleic acid/albumin/dextrose/catalase) enrichment (Difco) and 0.05% Tween 80 (Sigma).

II. Plasmid constructions

A 3.1 kb DNA fragment containing the MSMEG_4626 gene (Gene ID: 4531291; Locus tag: MSMEG_4626) encoding the ribonuclease (RNase) Rne/Rng family protein was amplified by PCR, using the following oligonucleotide primers: S1 5'-GTG CAT ATG GCC GAA GAT GCC CAT-3' and S2 5'-ACC GGA TCC GTG ATG CTC GTC TAG-3' and *M. smegmatis* MC2 155 DNA as template.

A 1200 bp and a 700 bp fragments containing the *lcrE* (GeneID: 895078; Locus tag: CPn0324) and *lcrH* (GeneID: 894648; Locus tag: CPn0811) genes respectively were amplified by PCR, using the following primers: E1 5'-GGA GGC ATA TGG CAG CAT CA-3' and E2 5'-CAC AGG ATC CGT ATT GGT TTT GCA TGG C-3' for LcrE; and: H1 5'-TCT CAT ATG AGC AAG CCC TCT C -3' and H2 5'- TCT GGA TCC CTC CTT AGA ATC TTA CTA ACG -3' for LcrH, with *C. pneumoniae* CWL029 DNA as template.
PCR was performed in a GeneAmp II thermocycler (Applied Biosystems, Foster City, CA, USA) with Advantage GC cDNA polymerase (Clontech, Mountain View, CA, USA) and the amplification conditions were set as recommended by the manufacturer.
The amplified DNAs were digested with NdeI and BamHI and inserted into the plasmid p6HisF-11d(icl) by digesting it with the same enzymes and replacing the *icl* gene, the resulting plasmids were pSRNE1, pLCRE1 and pLCRH. p6HisF11d(icl) is a pET-11d

(Novagen) based plasmid carrying the 6His and FLAG tags in the N-terminal of the cloned insert.

Since pET plasmids can not be used in mycobacteria we constructed pSRNE2 as well as pLCRE2 which carry MSMEG_4626 or *lcrE,* respectively downstream of the mycobacterial *icl* promoter (Rv0467) and 6His and FLAG tags in the *E. coli-mycobacterium* shuttle plasmid pMV262. I1 5'-ACT ATC TAG ATC CGC AGG ACG TCG A-30 and I2 50-GAC AGC CAT GGA CAA CTC CTT A-3' primers were used to amplify the *icl* promoter with *M. tuberculosis* H37Rv chromosomal DNA as template. The amplified DNA was cut with XbaI and NcoI and inserted into pSRNE1 and pLCRE1 opened with the same enzymes. The XbaI–BamHI fragment of pSRNE1 and pLCRE1 containing the icl promoter sequence and the genes of the candidate proteins were inserted into pMV262. The resulting plasmids were pSRNE2 and pLCRE2.

III. Expression of Rne, LcrE and LcrH in E. coli

For over-expression, *E. coli* HB101(pGP1-4) cells carrying either the pSRNE1, pLCRE1 or the pLCRH plasmids were grown and treated according to the method of Tabor and Richardson. Briefly, cells containing the plasmids were grown at 32°C in LB medium in the presence of the required antibiotics (ampicillin and kanamycin). Over-expression of proteins was induced by shifting the temperature to 42°C for 20 min. After induction, the temperature was shifted down to 37°C for an additional 90 min or longer, cells were harvested by centrifugation and cell pellets were frozen.

IV. Purification of Rne, LcrE and LcrH proteins

Cell lysates were prepared by resuspending the frozen cell pellets in lysis buffer (50 mM sodium phosphate, 300 mM sodium chloride, pH 7,0) containing protease inhibitor cocktail (Sigma) and lysozime (Sigma) (0,75 mg/ml). Bacteria were opened by sonication. After centrifugation Rne, LcrE and LcrH proteins were purified from the supernatant using the TALON CellThru Resin (Clontech), following the vendor's instructions.

V. Protein detection

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli. Gels were stained with Coomassie brillant blue.

VI. Generation of recombinant M. smegmatis expressing either RNase E or LcrE

Competent *M. smegmatis* MC2 155 prepared in 10% glycerol was transformed with either the pSRNE2 or the pLCRE2 plasmids by electroporation with a Gene Pulsar (Bio-Rad) set at 2.5 kV and 25 μF and with the pulse controller resistance set at 1,000 Ω. Transformed *M. smegmatis* was selected on Middlebrook 7H10 (Difco, Sparks, MD) agar plates supplemented with 30 μg/ml kanamycin. To monitor the expression of RNase E or LcrE,

individual colonies of recombinant *M. smegmatis* were grown in Middlebrook 7H9-OADC-Tween broth in the presence of 30 μg/ml of kanamycin and were harvested by centrifugation. After a rinse with sterile phosphate-buffered saline (PBS), mycobacterial cells were lysed by using a MiniBeadbeater-8 cell disrupter (BioSpec Products, Inc, Barlesville, Oklahoma), with glass beads (106 μm, Sigma) in the presence of a protease inhibitor cocktail (Sigma). Cell lysates were cleared by centrifugation. Proteins in the lysate of recombinant *M. smegmatis* were separated by 10% SDS-PAGE and the expression of the LcrE protein was tested by Western blot.

VII. Western blot

Purified LcrE and LcrH proteins and concentrated *C. pneumoniae* elementary bodies (prepared as described earlier) were heated to 95°C for 5 min in a sample buffer, and the proteins were separated by 10% SDS-PAGE. The separated proteins were blotted onto a polyvinylidene difluoride membrane (SERVA Heidelberg, Germany). The membranes were blocked overnight at 4°C with PBS containing 5% skim milk and 0.05% Tween 20. Membranes were probed with sera of LcrE or LcrH-immunized and non-immunized mice at 1:50 dilution in PBS containing 5% skim milk and 0.05% Tween 20. After washings, the filter was incubated with HRP-conjugated anti-mouse IgG, and the colour was developed using diaminobenzidine tetrahydrochloride (DAB, Sigma–Aldrich Chemie GmbH, Steinheim, Germany) with hydrogen peroxide in 10 mM Tris pH 7.5.

VIII. Identification of proteins by mass spectrometry

The gel slices containing the corresponding polypeptides copurified with LcrE, LcrH from *E. coli* and with RNase E from *M. smegmatis* were cut from the gel and analysed by mass spectrometry. Briefly, the proteins were digested with trypsin directly in the gel slices, and the resulting products of digestion were eluted and analysed by matrix-assisted laser desorption ionization/time-of-flight (MALDI-TOF) mass spectrometry. The resulting peptide mass lists were subjected to a database search. Post source decay analysis was used to confirm the MALDI-TOF results. Homology search was carried out with the computer programs of Altschul *et al.*. Protein parameters were calculated by the ExPASY Server.

IX. Immunization either with LcrE or with LcrH proteins or with recombinant and wild type M. smegmatis

Specific-pathogen-free 6-8-week-old, female BALB/c mice were obtained from Charles River Laboratories UK (Kent England). Mice were maintained under standard husbandry conditions at the animal facility of the Department of Medical Microbiology and Immunobiology, University of Szeged and were given food and water *ad libitum*. The mice in groups of 25 were immunized subcutaneously into the tail base with the purified LcrE protein

diluted in PBS at a dose of 20 μg mixed with 25 μl Alum (Aluminum hydroxide Gel, Sigma) or 75 μl Freund's adjuvants (FCA) (Chemicon International, Temecula, CA, USA; 1st inoculation with complete and 2nd and 3rd inoculations with incomplete Freund's adjuvant) in 150 μl volume 3 times at 3-week intervals.

Mice in groups of 5 were immunized subcutaneously at the tail base either with the purified LcrH protein diluted in PBS at a dose of 20 μg in 75 μl mixed with 75 μl Freund's adjuvant (1st inoculation with complete; 2nd and 3rd inoculations with incomplete Freund's adjuvant) 3 times at 3-week intervals.

Mice in groups of 5 were immunized intraperitoneally either with the recombinant *M. smegmatis* or with wild type *M. smegmatis* (10^8 colony forming unit (CFU) in a 100-μl total injection volume). Four weeks after the first immunization, mice were boosted with the same quantity of the same mycobacteria. Mice were sacrificed 14 days after last immunization. Blood was collected in heparinized capillaries from the retro-orbital plexus.

X. Challenging the LcrE and LcrH immunized and non-immunized mice

Two weeks after the last immunization with LcrE the immunized and non-immunized mice were challenged with $4x10^5$ inclusion forming units (IFU) of *C. pneumoniae* in 25 μl PBS intranasally under pentobarbital sodium anaesthesia. At this time point serum samples were collected in heparinised capillaries by retro-orbital bleeding for testing the antibody production and groups of 5 mice were euthanized and spleens were dissected and homogenized by pressing through nylon mesh into RPMI 1640 (Sigma) growth medium supplemented with 10% foetal calf serum, 10 mM HEPES (Sigma), L-glutamine (0.3 mg/ml; Sigma), gentamicin (60 μg/ml, Sanofi Aventis, Hungary) and 50 μM 2-mercaptoethanol (Sigma) for testing cell mediated immunity. The remaining mice were sacrificed 7 days after infection. By heart puncture blood was collected in heparin. The lungs were removed and homogenized mechanically in 2 ml of sucrose-phosphate-glutamic acid buffer (SPG) for cultivation of bacteria and LcrE-specific IgA antibody and cytokine detection.

On day 14 after the last immunization LcrH-immunized mice were challenged with $4x10^5$ IFU *C. pneumoniae* in 25 μl PBS intranasally under pentobarbital sodium anaesthesia. At this time point serum samples were collected in heparinised capillaries by retro-orbital bleeding for testing the antibody production. At 7 days after infection the mice were sacrificed. The lungs were removed and homogenized mechanically in 2 ml of SPG for cultivation of bacteria.

All animal experiments complied with the University of Szeged Guidelines for the Use of Laboratory Animals.

XI. Inoculum preparation and culturing of C. pneumoniae from the lungs

C. pneumoniae was propagated on HEp-2 cells (ATCC) as described earlier. The partially purified and concentrated EBs were aliquoted and stored at –80 °C until use. A mock preparation was prepared from uninfected HEp-2 cell monolayer processed in the same way as the infected cells. The titre of the infectious EBs was determined by inoculation of serial dilutions of the EB preparation onto HEp-2 monolayers, and after 48 h culture cells were fixed with acetone and stained with monoclonal anti-*C. pneumoniae* antibody (DAKO Ltd. Ely, UK) and FITC-labelled anti-mouse IgG (Sigma). The number of *C. pneumoniae* inclusions was counted under a UV microscope, and the titre was expressed as IFU/ml. Lung homogenates from individual mice were centrifuged (10 min, 400 g), serial dilutions of the supernatants were inoculated onto HEp-2 cell monolayers and the titre (IFU/ml) of *C. pneumoniae* was determined as described for the titration of the *C. pneumoniae* inoculum.

XII. ELISA tests

Recombinant protein-specific antibodies were detected by ELISA, using plates coated with the purified protein (100 ng/well), and horseradish-peroxidase (HRP)-conjugated secondary antibodies (α-mouse IgG-HRP; α-mouse IgA-HRP, Sigma and α-mouse IgG1 and IgG2a-HRP, Biosource) were used for detection. Purified mouse IgG1 and IgG2a (Cappel, Laboratories, Downington, PA, USA)-coated plates were used for determination of the corresponding secondary antibody dilutions that gave quantitatively similar reactivity in the ELISA assay. The titres were determined at dilutions demonstrating an optical density (OD) higher than 0.1. Cytokines (IL-4, IL-6, IL-10, IFN-γ – BD OptEIA set, BD Biosciences Pharmingen, San Diego, CA, USA; KC, MIP-2 – R&D Systems Minneapolis, MN, USA) were determined in the lung homogenates by ELISA according to the manufacturers instructions.

XIII. Lymphocyte proliferation assay

Proliferation of spleen cells *in vitro* stimulated with LcrE and *C. pneumoniae* was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT, Sigma-Aldrich) method. Single-cell suspensions from two spleens were pooled and resuspended in the complete growth medium used during homogenization of spleen cells. The proliferative response of $5x10^5$ splenocytes to 2 μg/ml concentration of LcrE recombinant protein, purified formalin-inactivated *C. pneumoniae* EBs (1 μg/ml) and HEp-2 mock preparation in three parallel wells were detected after incubation for 3 days. The proliferation was determined by MTT assay according to the manufacturer's instructions (Boehringer Mannheim Biochemica, Mannheim, Germany). Proliferation index (PI) was calculated

according to the following formula: ODs obtained in the presence of the test antigen divided by the ODs obtained in the presence of culture medium alone.

XIV. ELISPOT assay for the determination of IFN-γ producing cells

ELISPOT assay was performed to define the number and the phenotype of the spleen cells producing IFN-γ after *in vitro* re-stimulation with LcrE protein or with viable *C. pneumoniae* at a multiplicity of infection of 0.2 IFU, or with an equivalent amount of HEp-2 mock preparation. To determine the phenotype of IFN-γ producing cells, the spleen cell suspensions were depleted of CD4+ and CD8+ cells, respectively by using micro-beads coated with the respective antibody [CD4 (L3T4), CD8a (Ly-2), Miltenyi Biotec, Bergisch, Germany] and applying the magnetic cell sorting (MACS) system of Miltenyi Biotec. The outcome of the procedure was controlled by FACS analysis after direct staining of the depleted cells by α-CD4-TC and α-CD8-rPE antibodies (Caltag Laboratories, Burlingame, CA, USA). Ninety-six-well filter plates with a cellulose ester membrane (MAHAS4510, Millipore Corporation, Bedford, MA, USA) were coated with the capture antibody of the mouse IFN-γ ELISPOT set (BD Biosciences) overnight at 4 °C. The stimulated spleen cells ($4x10^5$) were distributed into each well in triplicate and incubated. After 20 h, the plates were washed, and biotinylated detection antibody, HRP-streptavidin and substrate solution were then added, as suggested by the protocol. The mean number of spots counted in triplicate wells under a dissecting microscope was used to calculate the number of spot-forming cells (SFC) per 1 million (1M) spleen cells.

XV. Statistical analysis

Statistical analysis of the data was carried out by GraphPad Prism 5 software using Wilcoxon-Mann-Whitney two-sample test. Differences were considered significant at $P<0.05$.

Results

I. Over-expression of LcrE and LcrH proteins in E. coli

LcrE is a protein consisting of 399 amino acids. Its calculated molecular mass is 43 kDa; because of the 6His and FLAG tags, our fusion protein is 4 kDa larger. LcrH protein is smaller, it contains 230 amino acids. Its calculated molecular mass is 26 kDa; it also contains the 6His and FLAG tags. Since these proteins carry the 6His and FLAG tags, both tags can be exploited during purification. After heat-induction a strong over-expression occurred after 90 minutes in case of LcrE and after 150 minutes in case of LcrH.

II. Chlamydial protein purification and identification by MALDI-TOF MS

The purifications were carried out with the TALON CellThru Resin. In case of the crude extract of LcrE-expressing *E. coli* the SDS-PAGE analysis demonstrated a strong band at the right position (around MW 45 kDa). In case of the purified LcrE strong bands were

visible at the same positions in the eluted fractions. Despite the use of protease inhibitors, the LcrE molecule seemed to be unstable. LcrH-*E. coli* crude extract and the eluted fractions showed strong bands below MW 35 kDa marker. With this purification method we were able to purify up to 20 mg proteins from a 3-L bacterial culture.

The eluted fractions containing LcrE were concentrated 10 times using concentrators with 5 000 MW cut-off and then subjected to 10% SDS–PAGE. After Coomassie blue staining, 4 protein bands were identified. In the purified LcrH two protein bands were detected by SDS-PAGE. Gel slices containing the proteins of LcrE and LcrH samples were cut from the gel. The proteins extracted from the gel fragments were analysed by MALDI-TOF MS. The results suggested that a large proportion of the identified proteins were *C. pneumoniae* proteins. The majority of the associated proteins identified in our study were *E. coli* ribosomal proteins, other co-purified proteins were those involved in stress response, which were presumably evoked by the heat-induction.

III. Western blot analysis of LcrE and LcrH proteins

The immunogenicity of the purified proteins in mice and the *Chlamydia*–specificity of the produced antibodies were confirmed by Western blot. The proteins were used for immunization of mice, and sera reacted only with the proteins in the appropriate positions in the concentrated *C. pneumoniae* preparation.

IV. Level of LcrE and LcrH-specific IgG in the sera and isotype of LcrE-specific IgG antibodies in immunized mice

High-titre LcrE-specific IgG was detected by ELISA in the mouse sera at the time of *C. pneumoniae* challenge, with no significant difference between the antibody levels induced by the different adjuvants (titres as geometric mean: LcrE-Freund's: 172216; LcrE-Alum: 164540). Both Alum and Freund's immunized sera reacted with the band corresponding to LcrE protein in the purified LcrE preparation used for immunization and also with LcrE in purified chlamydia elementary body. Sera of mice infected 3 times with *C. pneumoniae* reacted with proteins of EB but did not recognize the LcrE protein band in Western blot, although the production of LcrE-specific antibodies during the infection was clearly detectable by ELISA using LcrE protein as antigen (mean OD of 5 mouse sera = 0.479, with a range of OD 0.222–2.469 at a dilution of 1:50).

Irrespective of the applied adjuvant high IgG1 level was detected in the sera; however the IgG2a titres were higher in Freund's adjuvant-immunized mice. Both adjuvants induced a mixed Th1/Th2 isotype pattern; a higher relative IgG2a level indicates a Th1-biased response in the case of Freund's adjuvant-immunized mice.

LcrE-specific IgA was found in the sera and in the lungs. Freund's adjuvant was more effective in inducing LcrE-specific IgA at both sites; however IgA levels in the lungs showed high variation in Freund's adjuvant-immunized mice.

Mice immunized with LcrH protein mixed with Freund's adjuvant responded with antibody production. LcrH-specific IgG was detected by ELISA with a mean titre of 5300.

V. The local cytokine response

To investigate whether the immunization affects the local cellular reaction in response to the infection, the cytokine production was analyzed in the supernatant of the lung homogenates of mice after *C. pneumoniae* challenge. IL-4 was present at a marginal concentration while IL-10 levels were similarly increased in the lungs of all groups of mice. The IL-6 and IFN-γ content of the lungs was slightly diminished in the LcrE-Freund's immunized mice which might suggest a lower level of inflammation in these animals. Suppression of pro-inflammatory cytokines, keratinocyte-derived chemokine (KC) and macrophage-inflammatory protein-2 (MIP-2) in immunized compared to the non-immunized mice were also observed.

VI. Cellular immune response

For comparison and quantitation of the antigen-specific T-cell reactivity following immunization a lymphoproliferation assay was performed. The spleen cells of LcrE immunized mice collected 2 weeks after the last immunization were re-stimulated *in vitro* with LcrE protein, *C. pneumoniae* or mock antigens. Proliferation indices were determined. Proliferative responses were detected after both immunization methods upon stimulation with LcrE protein and also with *C. pneumoniae* antigen. Stimulation with LcrE protein induced proliferation of spleen cells from non-immunized but *C. pneumoniae* infected mice.

To estimate the number of Th1 subset of T cells activated by LcrE-immunization, IFN-γ producing cells were enumerated in the spleens by ELISPOT assay. The number of LcrE-specific IFN-γ producing cells was higher in LcrE-immunized mice than in non-immunized mice and more IFN-γ producing cells were counted after LcrE-Alum immunization.

In order to define the phenotype of T cells which release IFN-γ ELISPOT assay was carried out with the spleen cells of LcrE immunized mice depleted of CD4+ or CD8+ cells before *C. pneumoniae* challenge. Depletion of CD4+ cells resulted in a major reduction in the number of SFCs in LcrE and *C. pneumoniae*-stimulated spleen cell cultures. In the case of LcrE-Freund's immunized mice the depletion of CD8+ cells also caused a measurable decrease in the number of IFN-γ secreting cells.

VII. Protection against C. pneumoniae infection as measured by culturing of C. pneumoniae from the lungs

To find out whether LcrE protein applied in combination either with Freund's or Alum adjuvants can induce protective immune response against *C. pneumoniae* infection, *C. pneumoniae* titres in the lungs of immunized or non-immunized and *C. pneumoniae*-infected mice were compared. The reduction in *C. pneumoniae* burden in the lungs calculated as geometric mean (GM) was 60% when Freund's adjuvant was used and 63% when Alum adjuvant was used compared to non-immunized mice. The protection was significant (LcrE-Freund's: $P<0.0007$; LcrE-Alum: $P<0.0003$) and showed no difference irrespective of the used adjuvant.

In order to find out the protective effect of LcrH-specific immunity LcrH-immunized mice were infected with *C. pneumoniae.* The amount of recoverable *Chlamydia* from the lungs did not show significant reduction compared to that in non-immunized controls.

VIII. Protein over-expression and purification from M. smegmatis

1. Rne protein

Before cloning chlamydial proteins into pMV262 vector, preliminary experiments were carried out to check the conditions of the protein over-expression in *M. smegmatis.* Based on our previous experience, for this purpose an Rne family protein was chosen. The gene of Rne protein of *M. smegmatis* (MSMEG_4626) was cloned into pET (pSRNE1) or pMV262 (pSRNE2) and expressed in *E. coli* and *M. smegmatis*, respectively. The MSMEG_4626 gene product is a large protein consisting of 1,037 amino acids. Its calculated molecular weight is 112.7 kDa; because of the 6His and FLAG tags attached to it, our fusion protein is 4 kDa larger. In SDS-PAGE gels, it migrates as a 180 kDa protein. *M. smegmatis* Rne is highly similar to Rne proteins from *Rhodococcus sp.*, *Streptomyces coelicolor*, *Frankia sp.* and *Corynebacterium diphtheriae*. The central portion of *M. smegmatis* Rne (amino acids 402–805) exhibits 37% identity and 59% similarity to the amino terminal catalytic region of *E. coli* RNase G. In the case of *C. pneumoniae*, the corresponding values are 33% and 53%.

6His-tag labelled Rne from lysates of *M. smegmatis* were purified with Talon Metal Affinity Resin. The purified preparation separated by SDS-PAGE furnishes different protein bands besides RNase E. These bands were cut out from the gel and the proteins were identified by mass spectrometry. Proteolytic fragments of RNase E were found in all bands checked. GroEL, the major associated protein, is a chaperonin involved in the productive folding of proteins. In *M. smegmatis*, no heat shock treatment was applied during the expression of RNase E.

2. LcrE protein

M. smegmatis was transformed with pLCRE2 which carries LcrE after the mycobacterial *icl* promoter. LcrE protein expression by the recombinant *M. smegmatis* was confirmed by Western blot. The LcrE-immunized mouse sera reacted only with the band at the appropriate position in the concentrated recombinant *M. smegmatis*-LcrE preparation. Immunization with this recombinant *M. smegmatis* did not resulted in production of LcrE-specific antibodies by the inoculated mice.

Discussion

C. pneumoniae has generated huge attention during the last decade, not only as a respiratory pathogen but because of its association with a number of acute and chronic diseases, including atherosclerosis, Alzheimer's disease and multiple sclerosis. Since antibiotics cannot fully inhibit chlamydial growth and because of the incomplete protection induced by natural infection, the development of an effective vaccine would be desirable to control the infections caused by this highly prevalent pathogen. High priority vaccine candidates, include proteins such as the type III secretion system proteins, Hsp60 and proteins with analogous function in other organisms that are antigenically distinct.

As shown, we have cloned, expressed and purified efficiently with high purity in large quantity the LcrE protein encoded by *lcrE*, which is a putative secreted effector and negative regulator of TTSS of *C. pneumoniae* and the LcrH protein encoded by *lcrH* gene, which is one of the specialized chaperones with function to stabilize and assure efficient secretion of translocator proteins and also to regulate expression of some of the TTSS genes.

The immunogenicity of these proteins and *Chlamydia*-specificity of the immune response was proved by Western blot.

Protective effect of LcrE-specific immunity against *C. pneumoniae* infection in a mouse model has been studied. The results of our study are in agreement with previous reports that LcrE protein is an antigen with considerable potential as a vaccine subunit. The relatively poor immunogenicity of subunit vaccines, however, requires effective delivery systems e.g. adjuvants. In our study, the type and the protective capacity of immune response induced by recombinant LcrE protein formulated with Alum was determined and compared to that induced by LcrE mixed with Freund's adjuvant. Aluminum hydroxide was chosen because it is the only adjuvant used widely with human and veterinary vaccines and it has excellent safety and adjuvanticity records. Comparison was made with the effect of FCA, since it is still the gold standard for adjuvants producing excellent antibody response and the T-cell response induced is mainly Th1 type in experimental animals. Cell mediated immunity is crucial in host defence against intracellular pathogens such as *C. pneumoniae*. The correct

mechanism of cell-mediated immune (CMI) response in this disease is not clear. One of the potential effector mechanisms is the inhibition of chlamydial growth by cytokines such as IFN-γ. Expression of this cytokine is a bench-mark of a Th1 response, while for Th2 it is IL-4, IL-6 and IL-10, these cytokines are more effective in stimulating B cells to produce antibody. Both CD8+ and CD4+ T cells were shown to be activated during *C. pneumoniae* primary infection in humans.

The immunogenicity and protective effect of recombinant LcrE protein combined either with Freund's or Alum adjuvants were investigated in mice. The immunization with both protocols resulted in a significant reduction of the number of viable *C. pneumoniae* in the lungs after challenge. The LcrE immunization with either adjuvants was successful in respect of antibody response (generated a high IgG level in mice). These IgG antibodies recognized LcrE protein in purified *C. pneumoniae* EB-s as demonstrated by Western blot assay. In agreement with earlier data, these results showed the localization of LcrE protein in *C. pneumoniae* EBs. The IgG isotype profile, namely the high IgG2a/IgG1 ratio closely correlates with Th1 type CMI response. We observed the high level of Th2 related IgG1 production after FCA and even higher after Alum immunization, although increased IgG2a levels were also detected after immunization using FCA, compared to Alum immunization. Lower IgG2a/IgG1 ratio in Alum-immunized mice suggested a shift towards Th2 type immune response, but the presence of LcrE-specific IFN-γ producing cells in LcrE-Alum-immunized mice indicated Th1 type response also. The phenotype of LcrE-specific IFN-γ producing cells was CD4+ in Alum- and Freund's-immunized mice, but CD8+ cells were also detected in Freund's-immunized mice.

IgA is well known to protect mucosal surfaces during bacterial and viral infections. Here we report that LcrE protein immunization induces IgA production at mucosal surfaces.

In this study we have evaluated whether *M. smegmatis* was an effective vector for the delivery of homologous and heterologous antigens. Based on the results we got with *M. smegmatis* Rne cloning and expression, we developed a recombinant *M. smegmatis* strain carrying an LcrE-expressing plasmid. Production of the LcrE protein by this recombinant *M. smegmatis* was proved by Western blot. The capability of this construct to induce *C. pneumoniae*-specific immune responses and to provide protection against *C. pneumoniae* infection was evaluated in mice.

Immunization with this recombinant *M. smegmatis* was not successful, immunized mice did not produce LcrE-specific antibodies. The presumable explanation is that the *M. smegmatis* vector expressed inadequate levels of LcrE protein to induce a detectable immune response.

New statements

- We have cloned, expressed and purified in large quantity the LcrE protein encoded by *lcrE* and the LcrH protein encoded by *lcrH* gene of *C. pneumoniae.* The mass spectrometry showed that cloned LcrE and LcrH were expressed.
- The immunogenicity of these proteins and *Chlamydia*-specificity of the immune response was proved by Western blot.
- The protective effect of LcrE-immunization was shown as significantly decreased number of *C. pneumoniae* (IFU) in the lungs of immunized and *C. pneumoniae*-infected mice compared to non-immunized mice,
- LcrE-immunization in combination with Alum adjuvant provided an equal level of protection against *C. pneumoniae* infection to immunization with LcrE+Freund's adjuvant, in spite of the differences between the induced IgG isotypes, IgA levels and induction of LcrE-specific IFN-γ secreting CD8+ cells in the spleen of LcrE+Freund's-immunized mice additionally to CD4+ IFN-γ secreting T cells.
- We have cloned, expressed and purified Rne protein encoded by MSMEG_4626 gene of *M. smegmatis.* The mass spectrometry showed that full-length Rne was expressed.
- LcrE protein of *C. pneumoniae* has been cloned into pMV262, expressed in *M. smegmatis* as it was proved by Western blot.

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