Vaccine 28 (2010) 1598–1605

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Novel *Chlamydia pneumoniae* vaccine candidates confirmed by Th1-enhanced genetic immunization

Yihang Li[a], Sudhir K. Ahluwalia[a], Alexandre Borovkov[b], Andrey Loskutov[b], Chengming Wang[a], Dongya Gao[a], Anil Poudel[a], Kathryn F. Sykes[b], Bernhard Kaltenboeck[a,*]

[a] *Department of Pathobiology, College of Veterinary Medicine, Auburn University, Auburn, AL 36849-5519, USA*
[b] *The Biodesign Institute, Arizona State University, Tempe, AZ 85287-5901, USA*

ARTICLE INFO

Article history:
Received 26 June 2009
Received in revised form 30 October 2009
Accepted 18 November 2009
Available online 2 December 2009

Keywords:
Chlamydia pneumoniae
Vaccine candidate
Th1 immunity

ABSTRACT

Identification of highly immunogenic antigens is critical for the construction of an efficacious subunit vaccine against *Chlamydia pneumoniae* infections. A previous project used a genome-wide screen to identify 12 protective *C. pneumoniae* candidate genes in an A/J mouse lung disease model (Li et al. [14]). Due to insufficient induction of Th1 immunity, these genes elicited only modest protection. Here, we used the *Escherichia coli* heat-labile enterotoxin as a Th1-enhancing genetic adjuvant, and re-tested these 12 genes, in parallel with six genes identified by other investigators. Vaccine candidate genes *cutE* and Cpn0420 conferred significant protection by all criteria evaluated (prevention of *C. pneumoniae*-induced death, reduction of lung disease, elimination of *C. pneumoniae*). Gene *oppA_2* was protective by disease reduction and *C. pneumoniae* elimination. Four other genes were protective by a single criterion. None of the six genes reported elsewhere protected by reduction of lung disease or elimination of *C. pneumoniae*, but three protected by increasing survival.

1. Introduction

Chlamydia (*C.*) *pneumoniae* is an obligate intracellular bacterium that causes community-acquired respiratory infection and pneumonia in humans [1]. It has also been strongly associated with chronic inflammatory diseases such as atherosclerosis [2]. These public health concerns indicate a need for control of such infections.

Antibiotic therapies have only limited success against *C. pneumoniae* infections [3], especially after infection and pathology are established, in which case antibiotics may even enhance chlamydial dissemination [4,5]. For instance, in large scale field trials, antibiotic treatment did not reduce atherosclerosis, despite its association with increased *C. pneumoniae* antibody levels and detection of agent in lesions [6].

Genetic vaccines have been explored against chlamydial infections, due to inocula consistency and ease of manipulation, production, storage, and delivery [7]. A number of rationally selected *C. pneumoniae* genes, based on their known or presumed surface location, have been tested for protection in rodent models. In one study, heat-aggregated CopN (chlamydial outer protein N) protein, when intranasally administered in high dose together with *Escherichia coli* heat-labile toxin (LT), protected BALB/c mice against intranasal *C. pneumoniae* challenge [8]. In a different BALB/c mouse study, immunization with plasmids encoding the major outer membrane protein (MOMP) or an ADP/ATP translocase (Npt1) of *C. pneumoniae* resulted in a reduced bacterial load in the lung after challenge [9]. Finco et al. [10] showed that subcutaneous immunization with recombinant *C. pneumoniae* enolase (*Eno*) and several other proteins significantly decreased the amount of *C. pneumoniae* after an intraperitoneal challenge in hamsters. Svanholm et al. [11] showed that intranasal immunization with plasmid DNA encoding chlamydial heat shock protein 60 (HSP-60) reduced the *C. pneumoniae* lung loads by 5–20-fold in C57BL/6 mice, while also decreasing disease severity. Rodriguez et al. [12] showed that intranasal, but not intraperitoneal, genetic immunization with *C. pneumoniae* MOMP or HSP-60 conferred protection against *C. pneumoniae* infection, probably due to induction of cell mediated immune responses. Finally, Thorpe et al. [13] used recombinant LcrE, a potential component of the chlamydial type III secretion system to intraperitoneally immunize BALB/c mice. While a number of presumed surrogate parameters appeared to suggest protection, no statistically valid data indicated reduction of *C. pneumoniae* or any other form of actual protection of the mice. Overall, none of these antigens mediated protection that is close to the protection conferred by natural immunity after asymptomatic low-level *C. pneumoniae* infection, in which *C. pneumoniae* lung burdens are

* Corresponding author at: Department of Pathobiology, College of Veterinary Medicine, 270 Greene Hall, Auburn University, Auburn, AL 36849, USA.
Tel.: +1 334 844 2665; fax: +1 334 844 2652.
E-mail address: kaltebe@auburn.edu (B. Kaltenboeck).

0264-410X/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2009.11.046

reduced at least 100-fold as compared to mock-vaccinated mice 10 days after inoculation. Thus, truly highly protective *C. pneumoniae* vaccine antigens still need to be identified as components of a vaccine with reasonable probability for successful human application.

In previous experiments, we used expression library immunization to identify from the *C. pneumoniae* genome a total of 12 vaccine candidate genes that are capable of conferring high level protection to mice, as indicated by lower lung weights and better chlamydial elimination as compared to the mock-vaccinated controls [14]. In a subsequent re-test, however, these antigens did not confer complete protection, either by gene gun or a combined intramuscular–intradermal genetic immunization. We speculated that the poor vaccine efficacy was due to Th2-biased immunity elicited by gene gun vaccination [14]. However, early and robust induction of a Th1 response is critical for protective immunity against chlamydial infections. This has prompted us to use a vaccine adjuvant that particularly promotes Th1 immune responses.

Arrington et al. [15] have used both the A and B subunits of cholera toxin (CT) or the *E. coli* heat-labile enterotoxin (LT) as genetic adjuvants for particle-mediated genetic vaccines. Co-immunization with either of these vectors significantly elevated Th1 cytokine (IFN-γ) and Th2 cytokine (IL-4) levels. While both Th1 and Th2 cytokine production were enhanced in this experiment, the LT vectors have elicited more Th1-like biased responses in other systems. For example, HBcAg-specific IgG2a/IgG1 ratios were elevated and the IFN-γ (but not IL-4) responses were augmented [15]. Therefore, we used the LT subunit A and B plasmid vectors as a genetic adjuvant for re-evaluation of the *C. pneumoniae* vaccine candidates. In this investigation, we have re-tested the genes ranked highest for protection against *C. pneumoniae* in our previous genome-wide screen [14] delivered in the Th1-modulated vaccination regimen. We have identified gene vaccine candidates that confer protection levels comparable to a live *C. pneumoniae* vaccine.

2. Materials and methods

2.1. C. pneumoniae

C. pneumoniae strain CDC/CWL-029 (ATCC VR-1310) was grown in Buffalo Green Monkey Kidney monolayer cell cultures, purified by differential centrifugation, and quantified as previously published [16]. Purified infectious EBs were suspended in sucrose-phosphate-glutamate (SPG) buffer, stored in aliquots at −80 °C, and their infectivity was confirmed in female A/J mice.

2.2. Animals

Inbred A/J female mice were obtained from the Jackson Laboratory (Bar Harbor, ME) at 5 weeks of age and maintained in ventilated cages of 5 mice each with ad libitum access to water and a 19% protein/1.33% L-arginine standard rodent maintenance diet. Two weeks prior to the challenge infection the mice were started on a custom diet containing 24% protein/1.8% L-arginine (Harlan Teklad, Madison, WI). All animal protocols were approved by the Auburn University Institutional Animal Care and Use Committee (IACUC).

2.3. Confirmation of the Th1 immunostimulatory effect of the LT adjuvant

The *Chlamydia abortus* protective vaccine candidate gene *dnaX2* was cloned into genetic immunization vector, pCMVi-UB as described earlier [17]. Plasmid-coated gold particles for gene gun immunization were prepared in a standard protocol (Bio-Rad Laboratories, Hercules, CA) using endotoxin free plasmid DNA preparations. Each vaccine dose contained a total of 1 μg of a plasmid DNA mix. The mix contained 0.9 μg of the DnaX2-encoding plasmid and 0.1 μg of the LT genetic adjuvant. This adjuvant was a 1:4 mixture of two plasmids encoding the B and A subunits of *E. coli* heat-labile toxin (LT A + B), which has been shown to induce a strong and Th1-biased immune response [15]. The coding sequence for subunit A was modified to change the R at position 192 to G to detoxify the gene [18]. DNA was delivered by gene gun (Bio-Rad Laboratories, Hercules, CA) into each ear lobe of each mouse (5 mice/group). An accelerated vaccination schedule was used to immunize mice on days 0, 21, and 42. Sera from mice were obtained by saphenous vein bleeding 4 weeks after the last vaccination.

For large-scale protein production of recombinant *C. abortus* DnaX2 antigen, sequence-confirmed DnaX2 was subcloned into pEXP5-NT (Invitrogen, Carlsbad, CA). The expression construct was used to transform the host strain BL21(λ)DE3. Cells were grown to mid-logarithmic phase and induced with 0.5 mM IPTG according to recommended protocols. Cells were harvested 3–4 h after induction by centrifugation and the resulting cell pellet lysed by resuspension in PBS containing 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride (PMSF), and protease inhibitors (Roche Applied Sciences, Indianapolis, IN). Cell walls were permeabilized with 10 mg of lysozyme and subjected to 3 freeze/thaw cycles between −80 °C and room temperature. The viscous lysate was cleared in a 1 h incubation at 4 °C with 10 g/mL of DNase I and 20 mM $MgCl_2$. The lysate was centrifuged at 27,000 × *g* for 10 min at 4 °C, and the supernatant containing the soluble material was transferred to a fresh tube. The insoluble material, remaining in the pellet of the cleared lysate, was washed 4 times in PBS containing 1% Triton X-100 and 0.5 M guanidine followed by 3 washes with PBS. Cells were collected between washes by centrifugation at 3000 × *g* for 5 min at room temperature. After the final PBS wash, the inclusion bodies were resuspended in PBS, flash-frozen in liquid nitrogen, and stored at −80 °C until ready for use. To solubilize the inclusion bodies, the pellets were resuspended in PBS containing 8 M urea and 10% glycerol. Insoluble material was removed by centrifugation at 14,000 × *g* for 5 min at room temperature, and the soluble protein was collected in the supernatant and dialyzed against PBS.

Total IgG, IgG1, and IgG2a antibody concentrations against *C. abortus* DnaX2 were determined by ELISA of 1:2000 diluted sera. Briefly, 0.1 μg of recombinant *C. abortus* DnaX2 protein was coated per well by dilution in 0.05 M $NaHCO_3$, pH 9.6. After incubation of diluted sera, bound antibodies were detected by use of horseradish-peroxidase conjugated goat antibodies against mouse IgG, IgG1 and IgG2a (Southern Biotechnology Associates, Birmingham, AL) followed by TMB substrate (Thermo Scientific Pierce, Rockford, IL). The substrate reaction was stopped with sulfuric acid, and antibody concentrations were determined as absorbance at 450 nm. The background signal of antisera in a well without DnaX2 antigen was subtracted from the data.

2.4. Confirmatory C. pneumoniae vaccine candidate screen

Previously, the genome sequence of *C. pneumoniae* isolate CDC/CWL-029 (ATCC strain VR-1310) was extracted from Genbank (AE001363, 1,230,230 bp) and all ORFs were tested in two rounds of screening through expression library immunization (ELI) [14]. In Round 1, the 1263 ORFs of 1.5 kb or less were PCR amplified and constructed as linear expression elements (LEEs) by linking to a CMV promoter and a human growth hormone terminator sequence. The LEE library was arranged in three different sets of 30 random pools, each with ~42 ORFs, and used as inocula for 3 gene gun immunizations in groups of 5 mice. Each test inoculum contained 200 ng of a mixture of ~42 ORFs and 800 ng of pUC118 carrier DNA. All mice were challenged by intranasal inoculation of 1×10^8 *C. pneumoniae*