Published in final edited form as:

Vaccine. 2011 March 21; 29(14): 2519–2522. doi:10.1016/j.vaccine.2011.01.074.

Vaccination with the Defined Chlamydial Secreted Protein CPAF Induces Robust Protection Against Female Infertility Following Repeated Genital Chlamydial Challenge

Ashlesh K. Murthy^{*,‡}, Weidang Li^{*,‡}, M. Neal Guentzel^{*}, Guangming Zhong[†], and Bernard P. Arulanandam^{*}

^{*}South Texas Center for Emerging Infectious Diseases, Department of Biology, University of Texas at San Antonio, San Antonio, TX 78249

[†]Department of Microbiology and Immunology, University of Texas Health Science Center, San Antonio, TX 78229

Abstract

We previously have shown the efficacy of recombinant (r) chlamydial protease-like activity factor (CPAF) vaccination against hydrosalpinx development following primary genital chlamydial challenge. In this study, we evaluated further the protection induced by rCPAF vaccination against infertility. Following primary challenge, fertility levels were not significantly different between the mock- and CPAF-vaccinated and *Chlamydia* alone challenged mice. However, following secondary genital chlamydial challenge, mock (PBS) immunized mice displayed a significant reduction of fertility compared to age-matched naïve mice, while mice vaccinated intranasally with rCPAF+CpG displayed significant prevention of infertility. These results suggest that hydrosalpinx may be a reliable indicator of impending infertility, and that rCPAF is a promising candidate to prevent infertility resulting from repeated genital chlamydial infections.

Keywords

Chlamydia; genital infection; secondary challenge; repeated infections; vaccine; intranasal; chlamydial protease-like activity factor; infertility; fertility

Introduction

Chlamydia trachomatis has been recognized as an important worldwide cause of reproductive tract disease, and repeated episodes of infection have been reported to be associated with female infertility [1, 2]. A preventive vaccine is considered the ideal solution to reduce the morbidity induced by this pathogen, but a licensed vaccine is not yet available. We previously have demonstrated the efficacy of a recombinant form of chlamydial protease-like activity factor (rCPAF), a defined secreted chlamydial protein, administered

© 2011 Elsevier Ltd. All rights reserved.

¹Corresponding Author: Bernard Arulanandam, Department of Biology, University of Texas at San Antonio, One UTSA circle, San Antonio, TX 78249, Bernard.arulanandam@utsa.edu; Ph: (210)458-5492; Fax: (210)458-5523.

[‡]AKM and WL contributed equally

Potential conflicts of interest: None.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

with a T helper (Th) 1 type adjuvant in inducing significantly enhanced bacterial clearance following vaginal challenge with homologous or heterologous serovars/species of *Chlamydia* [3-5]. Moreover, the vaccination induced robust protection against upper genital tract (UGT) pathology, including hydrosalpinx and inflammatory cellular infiltration which have been suggested to be indirect markers of infertility.

In this study, we compared the protective immunity induced by intranasal immunization with rCPAF+CpG against infertility following primary and secondary genital *C. muridarum* challenge in female BALB/c mice. We found that vaccination with rCPAF+CpG helps maintain normal levels of fertility following repeated genital chlamydial infections and significantly reduces the incidence of total infertility.

Materials and methods

Chlamydia.

Chlamydia muridarum was grown on confluent HeLa cell monolayers as described previously [6]. Cells were lysed using a sonicator (Fisher Scientific, PA) and elementary bodies (EBs) were purified on discontinuous density gradients of Renograffin-76 as described previously. Aliquots of bacteria were stored at -70°C in sucrose-phosphate-glutamine (SPG) buffer.

rCPAF and CpG

Recombinant CPAF was purified as described previously [7]. Briefly, rCPAF constructs from *C. trachomatis* L2 genome with a 6X-Histidine tag (His) were cloned into pBAD vectors and Ni-NTA agarose beads (Amersham, NJ) were used for purification of rCPAF. The fusion protein was concentrated using Centrplus YM-10 tubes (Millipore, MA), suspended in PBS with proteinase inhibitor cocktail (Roche, CA), aliquoted, and then stored at -20°C . The purity of rCPAF was evaluated by SDS-polyacrylamide gel electrophoresis and by Western blot using anti-CPAF mouse antibodies [3]. CpG (TCCATGACGTTCTCTGACGTT) was obtained from Sigma Genosys (St. Louis, MO) [4].

Mice

Four-to-six week-old, female BALB/c mice were obtained from Charles River Laboratory (Bar Harbor, ME). Mice were housed and bred at the University of Texas at San Antonio and provided food and water *ad libitum*. Animal care and experimental procedures were performed in compliance with the Institutional Animal Care and Use Committee (IACUC) guidelines.

Intranasal immunization procedure

Animals were immunized as described previously [3-5, 7-11]. Specifically, groups of mice were anesthetized (3% isofluorane) and immunized i.n. on day 0 with $15\text{ }\mu\text{g}$ of rCPAF in $25\text{ }\mu\text{l}$ of sterile PBS or PBS alone (mock). This was accompanied on days -1, 0, and +1 with $10\text{ }\mu\text{g}$ of CpG in PBS alone (mock) containing 1% normal mouse serum. Mice were boosted i.n. with the same doses on days 14 and 28. The dose of rCPAF that was selected ($15\text{ }\mu\text{g}/\text{mouse}$) provided optimal protection against genital *C. muridarum* challenge in our studies using BALB/c mice [3].

Fertility analyses

One month following the final vaccination with rCPAF+CpG or PBS (mock), female BALB/c mice were challenged intravaginally (i.vag.) with 5×10^4 IFU of *C. muridarum* in $5\text{ }\mu\text{l}$ of SPG buffer as described previously [3-5, 7-11]. All animals were injected with depo-

progesterone (Pharmacia Upjohn, MI) on days -10 and -3 before challenge. Then, on day 80 following primary genital challenge, the immunized animals were evaluated for levels of fertility. Female mice were mated with proven breeder males for a period of 18 days. The females that became pregnant, based on body weight gain, were removed from the breeding cages. The mice that did not get pregnant in this first mating session were rested for a period of one week, and then mated again with the proven male breeders for another session of 18 days. The pregnant female mice were euthanized, genital tracts dissected, and the number of implantations per uterine horn was assessed. One set of mice were allowed to resolve the primary infection and rested for a period of one month. These mice were then rechallenged and fertility was assessed at day 80 following the secondary genital challenge. Each set of fertility assessments included age-matched mice that were left unimmunized and unchallenged, but treated with the depo-progesterone regimen, and which served as positive controls for levels of fertility. The numbers of pregnant mice after the first and second session of mating, along with the percentage pregnant per group, and the expected number of pregnant mice based on age-matched naïve controls was determined. The results are expressed as mean $\pm$ SD of the number of implantations in the right (R) and (L) uterine horn, and the mean $\pm$ SD total implantations per mouse group.

Statistical analyses

The numbers of pregnant mice in different groups were compared using the Chi-square test. The number of pregnant age-matched naïve mice in each set of experiments was considered as the expected number of pregnant mice for comparison to immunized/challenged groups. The reduction in observed (actual) number of pregnant mice in the challenged mock (PBS) immunized group was considered the effect of chlamydial infection *per se* on infertility. Based on this reduction, the protective effect of rCPAF+CpG vaccination at day 80 after primary or secondary genital chlamydial challenge was assessed using Chi-square analyses, and the two-tailed *p* value reported. Additionally, the total numbers of implantations in pregnant mice were compared between all groups using one-way analysis of variance (ANOVA). Fertility assessment following primary challenge was conducted once, whereas two independent experiments were carried out following secondary challenge. The data from experimental repeats were pooled and analyzed, since incidence of pregnancy is a categorical data set. However, the significant differences shown here are reflective of those found in each individual experiment.

Results

Groups of mice were vaccinated i.n. with rCPAF+CpG or PBS (mock) on day 0 with booster immunizations given on days 14 and 28. The mice were rested for one month and challenged i.vag. with *C. muridarum* [(*C. mur*), primary challenge (1^o Ch)]. Consistent with our previous reports [3-5, 7-11], rCPAF+CpG vaccinated mice displayed complete resolution of vaginal bacterial shedding on day 18, compared to day 30 after challenge in mock (PBS) immunized mice (data not shown). A group of depo-progesterone alone treated age matched (naïve) mice also were evaluated for levels of fertility. All mice were mated with proven male breeders on day 80 after primary challenge. As shown in Table 1, there was a reduction, albeit not statistically significant, in the number of pregnancies and the number of implantations in pregnant mice following primary genital chlamydial challenge in rCPAF+CpG vaccinated and mock (PBS) immunized mice, compared to depo-progesterone alone treated unchallenged control mice. Moreover, there was a reduction, albeit not statistically significant, in the number of pregnancies and the number of implantations in pregnant mice following primary genital chlamydial challenge in mock (PBS) immunized mice compared to rCPAF+CpG vaccinated mice. These results suggest that fertility is not affected significantly following primary genital chlamydial infection in this model.

Since repeated genital chlamydial infections are common and are associated with induction of infertility, we also examined the effect of rCPAF+CpG vaccination on preservation of fertility levels following a secondary chlamydial challenge. Groups of mice were vaccinated i.n. with rCPAF+CpG or PBS (mock) as before, rested for one month, and then challenged i.vag. with *C. muridarum* [(*C. mur*), primary challenge (1° Ch)]. All mice had resolved the primary infection by day 30 after primary challenge. The mice were rested for an additional period of one month and rechallenged (2° Ch) i.vag. with *C. muridarum*. All mice receiving secondary challenge displayed complete cessation of vaginal chlamydial shedding by day 9 following the secondary chlamydial challenge. The mice were then mated at day 80 after challenge with proven male breeder mice. Depo-progesterone alone treated age matched (naïve) mice also were evaluated for levels of fertility. As shown in Table 2, rCPAF+CpG vaccination resulted in significantly ($p = 0.0049$) greater number of pregnancies compared to challenged mock (PBS) immunized mice. While both CPAF+CpG (61%) and mock (30.7%) immunized mice displayed significant reductions in the numbers of pregnancies, compared to age-matched unchallenged naïve control animals (81.5%), there was a significantly greater reduction in pregnancy in the challenged mock-immunized versus rCPAF+CpG vaccinated group ($p < 0.0001$ versus $p = 0.029$, respectively). Importantly, the number of implantations in rCPAF+CpG immunized pregnant mice following secondary vaginal chlamydial challenge (8.0 ± 1.9) was comparable to that in age-matched unchallenged control naïve mice (9.1 ± 1.8). However, challenged pregnant mock (PBS) immunized mice displayed significantly ($p < 0.05$, each) reduced number of implantations (4.3 ± 1.1) compared to rCPAF+CpG vaccinated or age-matched naïve mice. These results demonstrate that rCPAF+CpG vaccination prevents a significant reduction in fertility following repeated chlamydial infection in the female genital tract.

Discussion

A licensed vaccine has been considered the ideal solution to reduce transmission and prevent reproductive morbidity caused by genital chlamydial infections [1, 2, 12]. Historical trials using killed whole chlamydial EBs induced reductions in the incidence of infection, but led to exaggerated pathology [1]. Therefore, there is significant interest in the development of a subunit vaccine. The defined secreted protein, chlamydial protease-like activity factor (CPAF) displays a high level of amino acid identity across chlamydial serovars and species [13] and may be used to induce broad-spectrum protective immunity [9]. We previously have demonstrated the efficacy of immunization with recombinant CPAF plus a Th1 type adjuvant in inducing CD4⁺ T cell/IFN- γ dependent [10], and antibody independent [11], protective immunity against the infection. Specifically, protection was demonstrated as a significant enhancement in vaginal chlamydial clearance, as well as reduction of reproductive tract sequelae including hydrosalpinx and inflammatory cellular infiltration [3], following primary genital chlamydial challenge. In this study, we confirm and extend those observations by directly demonstrating that rCPAF+CpG vaccination significantly reduces the incidence of infertility, and importantly leads to preservation of normal levels of fertility in protected animals following a repeated genital chlamydial challenge. These results suggest that (a) the reduction in hydrosalpinx in rCPAF+CpG vaccinated mice following primary genital chlamydial challenge [3] may be a reliable indirect indicator of the risk of impending infertility following subsequent challenge, (b) rCPAF+CpG vaccination induces robust protection against development of infertility following a repeated genital chlamydial infection, and that (c) rCPAF should be evaluated further as a potential component of an anti-chlamydial vaccine for human use.

Acknowledgments

Financial Support: This work was supported by National Institutes of Health grant 1R01AI074860.

References

1. Brunham RC, Rey-Ladino J. Immunology of *Chlamydia* infection: implications for a *Chlamydia trachomatis* vaccine. *Nat Rev Immunol*. 2005 Feb; 5(2):149–61. [PubMed: 15688042]
2. Morrison RP, Caldwell HD. Immunity to murine chlamydial genital infection. *Infect Immun*. 2002 Jun; 70(6):2741–51. [PubMed: 12010958]
3. Murthy AK, Chambers JP, Meier PA, Zhong G, Arulanandam BP. Intranasal vaccination with a secreted chlamydial protein enhances resolution of genital *Chlamydia muridarum* infection, protects against oviduct pathology, and is highly dependent upon endogenous gamma interferon production. *Infect Immun*. 2007 Feb; 75(2):666–76. [PubMed: 17118987]
4. Cong Y, Jupelli M, Guentzel MN, Zhong G, Murthy AK, Arulanandam BP. Intranasal immunization with chlamydial protease-like activity factor and CpG deoxynucleotides enhances protective immunity against genital *Chlamydia muridarum* infection. *Vaccine*. 2007 May 10; 25(19):3773–80. [PubMed: 17349723]
5. Chaganty BK, Murthy AK, Evani SJ, et al. Heat denatured enzymatically inactive recombinant chlamydial protease-like activity factor induces robust protective immunity against genital chlamydial challenge. *Vaccine*. 2010 Mar 8; 28(11):2323–9. [PubMed: 20056182]
6. Murthy AK, Sharma J, Coalson JJ, Zhong G, Arulanandam BP. *Chlamydia trachomatis* pulmonary infection induces greater inflammatory pathology in immunoglobulin A deficient mice. *Cell Immunol*. 2004 Jul; 230(1):56–64. [PubMed: 15541719]
7. Murthy AK, Cong Y, Murphey C, et al. Chlamydial protease-like activity factor induces protective immunity against genital chlamydial infection in transgenic mice that express the human HLA-DR4 allele. *Infect Immun*. 2006 Dec; 74(12):6722–9. [PubMed: 17015458]
8. Murphey C, Murthy AK, Meier PA, Guentzel MN, Zhong G, Arulanandam BP. The protective efficacy of chlamydial protease-like activity factor vaccination is dependent upon CD4⁺ T cells. *Cell Immunol*. 2006 Aug; 242(2):110–7. [PubMed: 17116296]
9. Li W, Guentzel MN, Seshu J, Zhong G, Murthy AK, Arulanandam BP. Induction of cross-serovar protection against genital chlamydial infection by a targeted multisubunit vaccination approach. *Clin Vaccine Immunol*. 2007 Dec; 14(12):1537–44. [PubMed: 17942608]
10. Li W, Murthy AK, Guentzel MN, et al. Antigen-specific CD4⁺ T cells produce sufficient IFN-gamma to mediate robust protective immunity against genital *Chlamydia muridarum* infection. *J Immunol*. 2008 Mar 1; 180(5):3375–82. [PubMed: 18292563]
11. Murthy AK, Chaganty BK, Li W, et al. A limited role for antibody in protective immunity induced by rCPAF and CpG vaccination against primary genital *Chlamydia muridarum* challenge. *FEMS Immunol Med Microbiol*. 2009 Mar; 55(2):271–9. [PubMed: 19281569]
12. Debattista J, Timms P, Allan J, Allan J. Immunopathogenesis of *Chlamydia trachomatis* infections in women. *Fertil Steril*. 2003 Jun; 79(6):1273–87. [PubMed: 12798871]
13. Dong F, Zhong Y, Arulanandam B, Zhong G. Production of a proteolytically active protein, chlamydial protease/proteasome-like activity factor, by five different *Chlamydia* species. *Infect Immun*. 2005 Mar; 73(3):1868–72. [PubMed: 15731091]

Table 1
Effect of rCPAF+CpG vaccination on fertility following primary genital chlamydial challenge

Vaccination	I ° C. mur challenge	Pregnant mice		Mean±SD implantations (pregnant mice only)				
		Pregnant/Total (%)		Observed	Expected	R	L	
Mock (PBS)	Yes	4/6 (67%)		4/6 (67%)	6 (100%)	1.3±1.5	4±0.8	7.3±4.6
rCPAF+CpG	Yes	5/6 (83%)		5/6 (83%)	6 (100%)	3.8±0.8	4.6±1.7	8.4±1.5
None	None			6/6 (100%)		4.7±1	4.5±1	9.2±1
								Total
								7.3±4.6
								8.4±1.5
								9.2±1

Table 2
Effect of rCPAF+CpG vaccination on fertility following secondary genital chlamydial challenge

Vaccination	1°/2° <i>C. mur</i> challenge	Pregnant mice		Mean±SD implantations (pregnant mice only)		
		Pregnant/Total (%)	Expected	R	L	Total
Mock (PBS)	Yes	3/12 (30.7%)	9.8 (81.5%)	2.3±0.6	2.0±1	4.3±1.1
rCPAF+CpG	Yes	11/18 (61%)	14.7 (81.5%)	3.7±1.4	4.3±1	8.0±1.9
None	None	22/27 (81.5%)		4.3±1.2	4.8±1.1	9.1±1.8

Chi-square test for number of pregnant mice:

two-tailed $p=0.0049$ rCPAF+CpG versus mock (PBS)

two-tailed $p=0.029$ rCPAF+CpG versus age-matched naïve mice

two-tailed $p<0.0001$ mock (PBS) versus age-matched naïve mice

One-way ANOVA: $p<0.001$ between all groups

$p<0.05$ rCPAF+CpG versus mock (PBS)

$p<0.05$ mock (PBS) versus age-matched naïve mice