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Partial Protection Against Chlamydial Reproductive Tract Infection by a recombinant-Major Outer Membrane Protein/CpG/Cholera Toxin Intranasal Vaccine in the Guinea Pig *Chlamydia caviae* Model

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ABSTRACT

Chlamydia trachomatis is a major cause of sexually transmitted diseases worldwide. There currently is no vaccine to protect against chlamydial infection of the female reproductive tract. Vaccine development has predominantly involved using the murine model, however infection of female guinea pigs with *Chlamydia caviae* more closely resembles chlamydial infection of the female reproductive tract, and presents a better model to assess potential human chlamydial vaccines. We immunised female guinea pigs intranasally with recombinant major outer membrane protein (r-MOMP) combined with CpG-10109 and cholera toxin adjuvants. Both systemic and mucosal immune responses were elicited in immunised animals, with MOMP-specific IgG and IgA present in the vaginal mucosae, and high levels of MOMP-specific IgG detected in the serum. Antibodies from the vaginal mucosae were also capable of neutralising *C. caviae in vitro*. Following immunisation, animals were challenged intravaginally with 10^2 inclusion forming units of live *C. caviae*. We observed a decrease in duration of infection and a significant ($p<0.025$) reduction in infection load in r-MOMP immunised animals, compared to animals immunised with adjuvant only. Importantly, we also observed a marked reduction in upper reproductive tract (URT) pathology in r-MOMP immunised animals. Intranasal immunisation of female guinea pigs with r-MOMP was able to provide partial protection against *C. caviae* infection, not only by reducing chlamydial burden but also URT pathology. This data demonstrates the value of using the guinea pig model to evaluate potential chlamydial vaccines for protection against infection and disease pathology caused by *C. trachomatis* in the female reproductive tract.

KEYWORDS

1. INTRODUCTION

Chlamydia trachomatis is the most common sexually transmitted bacterial pathogen worldwide and presents a serious health threat due to the high burden of disease that can result from infection of the female reproductive tract (WHO, 2001). In women, *C. trachomatis* normally infects the columnar epithelium of the endocervix (Soong et al., 1990). Following replication, the bacteria can ascend to the upper reproductive tract (URT)[1], with a recent mathematical model suggesting that the degree of ascension is dependent on the interaction between the innate immune response and the passive movement of *Chlamydia* (Mallet et al., 2009). Once in the URT, the organism can establish a persistent infection that may lead to pelvic inflammatory disease, ectopic pregnancy or infertility (Weström et al., 1992). Infection can be resolved with antimicrobial treatment, however 70-90% of infected women are asymptomatic, resulting in a large number of cases going undiagnosed and untreated (Stamm and Holmes, 1990). It has been proposed that the development of a prophylactic chlamydial vaccine, that protects against URT pathology, would significantly reduce the disease impact and associated economic cost of *C. trachomatis* infections (de la Maza and de la Maza, 1995; Gray et al., 2009).

Early studies of chlamydial vaccines utilised whole inactivated elementary bodies to induce protection against ocular *C. trachomatis* infection. However in these trials, a stronger immune response actually correlated with a more pronounced disease state (Grayston et al., 1961). Subsequent vaccine research has focused on selecting specific proteins of the pathogen that are immunogenic and avoid the immune related pathology associated with use of the whole chlamydial organism. The major outer membrane protein (MOMP) has frequently been identified as a promising vaccine candidate (Baehr et al., 1998; de la Maza and Peterson, 2002). It is present throughout all stages of the *C. trachomatis* developmental cycle and is recognised in most species as an immunodominant antigen (Zhang et al., 1993). Our group has previously demonstrated that combining MOMP with CpG and cholera toxin adjuvants, and delivering via either the intranasal (Cunningham et al., 2009) or transcutaneous route (Berry et al., 2004), stimulates a strong immune response and provides partial protection against *Chlamydia muridarum* reproductive tract infection in mice.

To date, the majority of chlamydial vaccine studies have been performed in mice (Cochrane et al., 2010), however reproductive tract infection of this species does not accurately reflect sexually transmitted chlamydial infection in humans. Potential vaccines need to be evaluated in a model that more closely parallels humans in terms of infection course and pathological response. Very few vaccine candidates have been explored in the guinea pig model of *C. caviae* reproductive tract infection (Batteiger et al., 1993; Rank et al., 1990), despite the model being used strategically to investigate immune responses and infection

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[1] **ABBREVIATIONS**

ATCC (American Type Culture Collection), CI (Confidence Interval), CpG (cytosine phosphate guanine), CT (cholera toxin), DTT (Dithiothreitol), EPT (end point titre), H+E (haematoxylin and eosin), H+L (heavy and light chains), HI (heat inactivated), HMRC (Herston Medical Research Centre), HRP (horseradish peroxidase), IFU (inclusion forming unit), IMAC (immobilised metal affinity chromatography), IMVS (Institute of Medical and Veterinary Science), LB (Lysogeny broth), LRT (lower reproductive tract), MOMP (major outer membrane protein), PBS-T (phosphate buffered saline containing 0.05% Tween-20), PCB (phosphate citrate buffer), PMSF (phenylmethanesulfonylfluoride), ROES (random outbred English short haired), SPG (sucrose-phosphate-glutamate), TMB (3,3',5,5'-Tetramethylbenzidine), URT (upper reproductive tract), WHO (World Health Organization)

kinetics (Rank et al., 1979, 1982, 1988, 1989, 2000; Wang et al., 2010). Female guinea pigs have a 15-17 day oestrous cycle (Stockard and Papanicolaou, 1919), comparable to the 28 day menstrual cycle in humans. This is important as the vaginal mucosae is affected by the release of sex hormones, which can influence immune response to infection (Rank et al., 1982). Chlamydial infection of the reproductive tract in guinea pigs can also progress to a disease state that closely resembles clinical disease seen at this site in humans (Rank and Sanders, 1992). In addition, guinea pigs are the only known species to have had the sexually transmitted infective dose of *Chlamydia* characterised (Rank et al., 2003). Animals are typically given a high infective dose of *Chlamydia*, as well as progesterone pre-treatment in the murine model, to guarantee infection (Rank et al., 2003; Vanrompay et al., 2006). However, this approach does not accurately assess the protective function of a vaccine (Rank et al., 2003) and can lead to inconsistent findings across a particular model (Ahmad et al., 1977). By challenging guinea pigs intravaginally with a natural infective dose of *C. caviae* post immunisation, it is possible to correlate the infection course and pathological response to that observed in humans, presenting a better model with which to evaluate chlamydial vaccines.

Results of a previous study (Batteiger et al., 1993) showed that female guinea pigs produced an immune response and a reduced infection course following subcutaneous immunisation with native MOMP, however protection against URT pathology was not investigated. We report here the first intranasal immunisation of guinea pigs with recombinant *C. caviae* MOMP (r-MOMP) combined with CpG and CT, which stimulated both systemic and mucosal immune responses and provided partial protection against *C. caviae* intravaginal infection, measured as a decrease in duration and intensity of infection, as well as reduced pathology in the URT of these animals.

2. MATERIALS AND METHODS

2.1. Guinea Pigs

Sexually mature (600-800 g), female, random outbred English short haired (ROES) guinea pigs were obtained from the Institute of Medical and Veterinary Science (IMVS; Gilles Plain, SA, AU) and housed at the Herston Medical Research Centre (HMRC; Brisbane, QLD, AU). Animals were placed in individual mini isolators (Able Scientific, Canning Vale, WA, AU) and kept in an environmentally controlled room at 21°C on a 12 h light/12 h dark cycle. They were fed standard guinea pig pellets and tap water supplemented with 4 mg/20 L ascorbic acid *ad libitum*. All procedures involving animals were approved by the Queensland University of Technology University Animal Ethics Research Committee (QUT UAEC No. 0700000346).

2.2. Recombinant MOMP Expression and Purification

The CCA-00047 gene *(C. caviae* MOMP; GenBank ID: AAP04799.1) was synthesised and cloned into a pCold TF expression vector (performed by GenScript Corporation, Piscataway, NJ, US). pCold TF-CCA-00047 was transformed into *Escherichia coli* Origami B (DE3) cells (Novagen, Darmstadt, DE) for expression of recombinant *C. caviae* MOMP. Transformed *E. coli* were incubated in Lysogeny broth (LB) at 37°C to reach mid-log phase, then induced with 5 mM IPTG (Quantum Scientific, Murarrie, QLD, AU) for 4 h at 15°C. Bacterial cultures were harvested by centrifugation at 4,650 *g* for 10 mins at 4°C. *E. coli* were lysed by incubation with lysozyme (Sigma-Aldrich), then sonicated following addition of Protease Inhibitor Cocktail (Sigma-Aldrich) and PMSF (Sigma-Aldrich). Inclusion bodies were solubilised with 4 M urea for 4 h at 4°C, then centrifuged at 4,650 *g* for 10 mins at 4°C. Supernatant was purified and concentrated as previously described (Barker et al., 2008). r-MOMP was quantified via BCA protein assay (Thermo Scientific, Scoresby, VIC, AU).

2.3. Immunisation Protocol

Guinea pigs were lightly sedated with 2-4% Isoflurane inhalation anaesthesia (Abbott Australasia, Botany, NSW, AU) in 4 L/min oxygen. Synthetic CpG-10109 (Gu et al., 2007) oligonucelotides (Sigma-Aldrich) and cholera toxin (CT; Sapphire Bioscience, Waterloo, NSW, AU) were used as adjuvants in each immunisation. r-MOMP/CpG/CT guinea pigs (n=5) were administered 100 µg r-MOMP, 25 µg CpG, 10 µg CT. CpG/CT guinea pigs (n=5) were given 25 µg CpG, 10 µg CT only. Each group was successfully immunised intranasally with 150-200uL three times at weekly intervals, and given a final immunisation booster two weeks following third immunisation. Immunisations were equally distributed to nares of each animal using a micropipette.

2.4. Live *C. caviae* Infection Challenge and Vaginal Swab Collection

C. caviae (ATCC VR-813; Manassas, VA, US) was grown on McCoy cells, then semi-purified (Rank et al., 1988). Animals were lightly sedated as described in section 2.3. Each

guinea pig then was given 25 µL sterile PBS containing 10^2 inclusion forming units (IFUs) of live *C. caviae*, via micropipette into the vagina. Infection was monitored by collecting cervico-vaginal swabs every three days until day 27. Swabs were placed in 500 µL sucrose-phosphate-glutamate (SPG) with 3x sterile glass beads, and vortexed before storage at -80°C. The *C. caviae* infection course was determined by infecting McCoy cells with swab eluate, then stopping with 100% methanol fixation 30 h post infection and staining with a FITC-conjugated monoclonal antibody (Chlamydia Cel LPS Reagent; Cellabs Pty Ltd, Brookvale, NSW, AU). IFUs were counted using a fluorescence microscope.

2.5. Collection of Serum and Vaginal Washes

Blood and vaginal washes were obtained from anaesthetised guinea pigs prior to immunisation and one week following the final immunisation booster. Blood was collected from the saphenous leg vein (Lopez and Navia, 1997) and allowed to clot at room temperature for 1 h. Serum was acquired by centrifuging clotted blood at 12,000 *g* for 30 mins. Vaginal washes were collected by flushing the vagina 3x with 200 µL sterile PBS. Serum and vaginal washes were stored at -20°C until required.

2.6. ELISA Analysis

MOMP-specific IgG and IgA titres were determined for serum and vaginal washes using previously described methods (Carey et al., 2009). ELISA 96 well plates (Grenier Bio-One med. binding; Interpath Services, Heidelberg West, VIC, AU) were coated with 0.5 µg r-MOMP protein/well. Serum and vaginal washes initially were diluted 1:2000 and 1:25 respectively. Samples then were serially diluted twofold in PBS-Tween (PBS-T) and added to wells in duplicate. For IgG detection, 1:1000 rabbit anti-guinea pig IgG (H+L) HRP (MP Biomedicals, Solon, OH, US) in PBS-T was added to each well. This was repeated for detection of IgA, but with rabbit anti-guinea pig IgA (α-chain) (MP Biomedicals) in PBS-T instead. Following incubation, 1:1000 goat anti-rabbit IgG (H+L) HRP (MP Biomedicals) in PBS-T was added to IgA wells. TMB substrate (Sigma-Aldrich), diluted 1:100 with Phosphate Citrate Buffer (PCB; Sigma-Aldrich), was added to all wells. Colour development was stopped by adding 1 M sulphuric acid. Absorbance values were measured at 450 nm with a Bio-Rad xMark™ Microplate Spectrophotometer. Samples collected from animals prior to immunisation were used as negative controls to determine end point titre (EPT) values. EPT was calculated as the inverse of the dilution, where absorbance values were equivalent to the mean of the negative control + two standard deviations.

2.7. *in vitro* Neutralisation Assay

Serum and vaginal washes collected from each animal prior to immunisation (pre), and one week following the final immunisation booster (post), were diluted 1/5 and 1/25 respectively in media in 96 well microplates. Each well was incubated with 7.5×10^3 IFUs of *C. caviae* for 1 h at 37°C. Following incubation, the contents of each well were transferred to corresponding wells of 96 well microplates containing 80% confluent McCoy cell

monolayers. Infection proceeded to 30 h as described in section 2.4. Following staining, infectivity (the number of *C. caviae* IFUs per the number of McCoy cells) was determined using a fluorescence microscope. Neutralisation was calculated by comparing the change in infectivity between pre and post samples for each animal.

2.8. Histopathology Assessment of Reproductive Tract

Guinea pigs were euthanised by intracardiac injection of 1 mL/2 kg sodium pentobarbitone (Lethabarb; Abbott Australasia) four weeks following *C. caviae* infection. Reproductive tracts were surgically removed and fixed in 10% Neutral Buffered Formalin for one week. For each animal; cervix, uterus (uterine corpus, 2x uterine horns), 2x oviducts and 2x ovaries, were embedded in paraffin wax, sectioned at 5 µm and haematoxylin and eosin (H+E) stained (performed by Dr. John Mackie). Each anatomic site was scored for acute inflammation, chronic inflammation and plasma cell infiltration using a histopathological assessment method adapted from a previously published system (Rank et al., 1992): trace of parameter, +0.5 (low); presence of parameter, +1 (mild); presence of parameter at 1 to 4 foci (moderate), +2; presence of parameter at >4 foci, +3 (marked); confluent presence of parameter, +4 (severe).

2.9. Statistics

All statistical analyses were performed using GraphPad Prism v.5. Statistical significance was determined using unpaired, two-tailed Student's t-test with 95% confidence interval (C.I.).

3. RESULTS

3.1. r-MOMP immunisation stimulates MOMP-specific antibody response

Serum collected from r-MOMP immunised animals one week following immunisation booster had significantly higher (p<0.001) levels of MOMP-specific IgG (>100 000 EPT) when compared to serum from animals immunised with CpG/CT only (Fig. 1A). In vaginal washes, MOMP-specific IgG and IgA antibodies were present at significantly higher (p<0.001) levels in animals that received r-MOMP immunisation, compared to those immunised with CpG/CT (Fig. 1B,1C). No MOMP-specific IgA was recorded in the serum of r-MOMP or CpG/CT immunised animals. All animals were negative for MOMP-specific IgG and IgA antibodies in the serum and vaginal washes prior to immunisation.

3.2. r-MOMP induced vaginal antibodies partially neutralise *C. caviae* infection *in vitro*

The infectivity was determined in both pre- and post- immunisation samples of individual animals at 1/25 dilution, and neutralisation was calculated by comparing the change in infectivity between these two samples.   r-MOMP immunised animals showed a 22.5% increase in *in vitro* neutralisation of *C. caviae* compared to CpG/CT immunised animals (Fig. 2). All immunisation parameters and components for guinea pigs receiving CpG/CT immunisation were kept consistent with r-MOMP immunised animals; therefore any difference in neutralisation between these groups is attributed to immunisation with r-MOMP protein. Whilst a difference in *in vitro* neutralisation in vaginal washes was observed between r-MOMP and CpG/CT immunised animals, no difference in neutralisation occurred when testing the serum collected from these animals (data not shown).

3.3. r-MOMP immunisation reduces the intensity and duration of live *C. caviae* infection

Following intranasal immunisation with r-MOMP, animals were challenged intravaginally with a low dose (10^2 IFU) of live *C. caviae*. Some animals did not become infected, determined by the absence of *C. caviae* IFUs from the vaginal swab samples collected. Only 2/5 (40%) of r-MOMP immunised animals, and 3/5 (60%) of CpG/CT immunised animals demonstrated an active infection. CpG/CT immunised animals had a peak of infection at day 18 (Fig. 3A), with shedding levels >4.0×10^5 IFU. r-MOMP/CpG/CT immunised animals showed signs of reduced infection intensity, with shedding levels peaking significantly lower (p<0.025) at <1.5×10^5 IFU, 6 days earlier. This group was able to clear infection by day 21, whereas CpG/CT immunised animals did not clear the infection until day 27. Area under the curve analysis of *C. caviae* infected animals (Fig. 3B) demonstrates that the r-MOMP/CpG/CT immunised group had a reduced course of infection compared to the CpG/CT immunised group.

3.4. r-MOMP immunisation reduces URT pathology

In the lower reproductive tract (LRT) of both r-MOMP and CpG/CT immunised animals, moderate acute inflammation was seen in the cervix four weeks follow infection (Fig. 4A). However, only low chronic inflammation was observed in r-MOMP immunised animals, whilst moderate chronic inflammation was seen in CpG/CT immunised animals.

In the URT of animals that received r-MOMP immunisation, mild acute inflammation was observed in the uterus (Fig. 4B), and mild acute and chronic inflammation in the ovaries (Fig. 4C + 4D). Conversely, in those animals that received CpG/CT immunisation only, moderate acute inflammation was observed in the uterus, with marked acute and chronic inflammation in the ovaries.

Whilst tissues were collected, processed and analysed from all animals, only those that demonstrated an active infection following *C. caviae* challenge were included in the results of this study. Hydrosalpinx development was not seen in the fallopian tubes and no signs of pathology were observed in the fallopian tubes microscopically. No fibrosis or mucosal erosion was present in any samples.

4. DISCUSSION

This is the first report of partial protection against live intravaginal *C. caviae* challenge by intranasal r-MOMP immunisation in the female guinea pig model of reproductive tract infection. Intranasal immunisation of female guinea pigs with r-MOMP/CpG/CT induced MOMP-specific IgG and IgA responses in the vaginal mucosae, and a strong MOMP-specific IgG response in the serum. This indicates that the subunit vaccine stimulated both the systemic and mucosal immune systems when delivered intranasally. Antibodies generated in the vaginal mucosae were also able to partially neutralise *C. caviae* infection *in vitro*, suggesting that these antibodies could recognise live *C. caviae* locally and prevent an active infection. Although *in vitro* neutralisation of *C. caviae* was observed in vaginal washes, no neutralisation occurred when immune serum was assayed, which may be due to the absence of MOMP-specific IgA in serum.

Whilst it is known that CT and CpG adjuvants induce innate immunity (Klinman et al., 2000; Matsuo et al., 2000), we specifically wanted to measure the effect of the chlamydial antigen above the adjuvant only effect in providing protection in guinea pigs against chlamydial reproductive tract infection. As such, we chose to compare r-MOMP/CpG/CT immunised animals with an adjuvant only (CpG/CT) control group. All animals were challenged intravaginally with 10^2 IFU of live *C. caviae*. Of the r-MOMP immunised animals, 40% became infected, compared to 60% in the adjuvant only (CpG/CT) group. The peak of infection in the r-MOMP group was significantly lower (p<0.025) than the adjuvant only group. This may have led to enhanced development of URT pathology in CpG/CT immunised animals, as a higher infectious load could ascend from the LRT (Mallet et al., 2009). Increased acute and chronic inflammation was seen in the ovaries and uterus of the adjuvant only group, although the fallopian tubes showed no signs of inflammation, and no gross abnormalities were observed. The majority of chlamydial studies investigating female URT pathology have frequently identified the fallopian tubes as being one of the most affected areas, in guinea pigs (Rank et al., 1993) as well as other species (Cunningham et al., 2010; Schautteet et al., 2010). The lack of fallopian tube pathology may have been due to the low chlamydial infective dose administered. This is the first study to investigate URT pathology following challenge with a realistic chlamydial dose that approximates the natural infective dose from sexual transmission (Rank et al., 2003), and was not expected to cause the same pathology seen in animals that are given a high infective dose of live *Chlamydia*. This characterises what may be seen in human females, as not all *C. trachomatis* infections may ascend to the URT (Brunham and Rey-Ladino, 2005). Recent reports also highlight the need for additional modifications to our current guinea pig model of chlamydial reproductive tract infection, to further reflect important biological processes in women that impact chlamydial pathogenesis. This includes defining the stage of the menstrual cycle at the time of infection and the potential effect of innate immune mediators present in the male seminal fluid that would be transferred at coitus (Lyons et al., 2009), as well as establishing prolonged infections following immunisation (Miyairi et al., 2010), which would likely improve the efficacy of the guinea pig model in testing vaccine efficiencies

MOMP has been established in most species as a dominant antigen recognised by the humoral immune system during chlamydial reproductive tract infection (Caldwell and Schachter, 1982). Previous work by our group has shown that combining MOMP with the potent CpG and cholera toxin adjuvants that induce T_H1 and T_H2 responses respectively, and delivering via intranasal immunisation, is able to generate a MOMP-specific immune

response in mice systemically and in the vaginal mucosae (Barker et al., 2008; Cunningham et al., 2009). We have confirmed those findings in this study, showing that intranasal immunisation of guinea pigs with a subunit vaccine elicits both systemic and mucosal antibody responses that provide partial protection. We are currently developing methods to analyse vaccine-induced mucosal cell-mediated immunity in outbred guinea pigs, to evaluate the role of T cell responses in protection.

A vaccine that provides protection against upper reproductive tract pathology caused by *C. trachomatis*, would considerably reduce the global disease burden and associated impact of this sexually transmitted infection (de la Maza and de la Maza, 1995; Gray et al., 2009). The guinea pig model of chlamydial reproductive tract infection closely parallels humans in terms of infection course and pathological response (Rank and Sanders, 1992) and should continue to be used to explore the efficacy of potential vaccine candidates against *C. trachomatis* infection of the human female reproductive tract.

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FIGURE LEGENDS

Fig. 1 - MOMP-specific antibody levels in serum and vaginal washes collected one week following immunisation booster: (A) MOMP-specific IgG EPT in serum, (B) MOMP-specific IgG EPT in vaginal washes, (C) MOMP-specific IgA EPT in vaginal washes. Serum and vaginal washes collected from animals prior to immunisation were used as controls. n = 5 animals per group. Bars represent SEM. Statistical significance was obtained using unpaired, two-tailed Student's T-test with 95% C.I.

Fig. 2 - *in vitro* neutralisation of *C. caviae* by vaginal washes. Samples diluted 1/25. Percentage neutralisation for individual animals determined using equation; (pre immune infectivity – post immune infectivity)/pre immune infectivity. n = 5 animals per group. Bars represent SEM.

Fig. 3 - (A) Partial protection observed in r-MOMP immunised animals against intravaginal *C. caviae* challenge; reduced duration and intensity of infection compared to CpG/CT immunised animals. Swabs collected every three days following challenge until day 27. (B) Area under the curve analysis of course of infection. n (CpG/CT) = 3 animals, n (r-MOMP) = 2 animals. Bars represent SEM. Statistical significance was obtained using unpaired, two-tailed Student's T-test with 95% C.I.

Fig. 4 - Histopathology assessment of LRT and URT, scored for acute inflammation (neutrophil infiltration), chronic inflammation (lymphocyte infiltration) and plasma cells: (A) cervix - LRT, (B) uterus (uterine corpus, 2x uterine horns) - URT, (C) ovaries (left and right) - URT. n (CpG/CT) = 3 animals, n (r-MOMP) = 2 animals. Bars represent SEM.