Published in final edited form as:
Vaccine. 2010 November 10; 28(48): 7606–7612. doi:10.1016/j.vaccine.2010.09.060.

A new murine model for testing vaccines against genital *Chlamydia trachomatis* infections in males

Sukumar Pal, **Annahita K. Sarcon**, and **Luis M. de la Maza***
Department of Pathology and Laboratory Medicine, Medical Sciences, Room D440, University of California, Irvine, Irvine, CA 92697-4800, USA

Abstract

Two groups of 50 BALB/c male mice were immunized with live *Chlamydia trachomatis* mouse pneumonitis (MoPn) using the intranasal (i.n.) or the meatus urethra (intraurethral: i.u.) routes. As a control group, 100 male mice were sham-immunized in parallel. Both groups of animals vaccinated with live organisms developed strong *Chlamydia*-specific humoral and cell mediated immune responses. Based on the IgG2a/IgG1 ratio and the levels of IFN-γ both groups mounted a Th1 immune response. At six weeks following the immunization, all mice were challenged in the meatus urethra. The urethra, urinary bladder, testes and epididymides were harvested at weekly intervals and tested for the presence of *C. trachomatis*. Based on the culture results from these four organs both groups of *Chlamydia*-immunized mice showed significant protection. In the group immunized i.u., 10% (5/50) had positive cultures, while in the group immunized i.n. 28% (14/50) had positive cultures during the 5 weeks of observation. In contrast, in the sham-immunized animals 47% (47/100) had positive cultures ($P<0.005$) during the study period. In addition, the number of positive organs, the length of time that the animal had positive cultures, and the total number of inclusion forming units (IFU) recovered were overall significantly lower in the i.u. or i.n. groups in comparison with the sham-immunized animals. However, in relation to the i.u. immunized group, the protection elicited in the i.n. group was delayed and not as robust. In conclusion, immunization of mice in the meatus urethra may provide the gold standard for testing *Chlamydia* vaccines in a male model.

Keywords

Chlamydia; vaccines; males; mice

INTRODUCTION

Historically male urethritis has been called gonococcal urethritis when *Neisseria gonorrhoeae* was detected, and non-gonococcal urethritis (NGU) when *N. gonorrhoeae* was not isolated [1,2]. Chronic urethritis associated with the "inclusion forming agents" was first described by Halberstaedter and von Prowazek in 1907 [3] and confirmed by Linder in 1910 [4]. In 1964 Jones isolated *Chlamydia* from a man with urethritis [5]. Since then, *C. trachomatis* has been found to be the most common sexually bacterial pathogen in males. It is estimated that 2 million cases of symptomatic male acute urethritis occurs in the USA

*Corresponding author: Department of Pathology and Laboratory Medicine, Medical Sciences, Room D440, University of California, Irvine, Irvine, CA 92697-4800, USA, Telephone: (949) 824-7450, Fax: (949) 824-2160, lmdelama@uci.edu.

every year [6,7]. Approximately 50% of the urethritis cases are due to *C. trachomatis*. Additionally, 50–70% of the acute urethritis cases due to *C. trachomatis* in males are asymptomatic [2,6,7]. An increase in the incidence of male urethritis due to *C. trachomatis* has recently been observed in several countries [6,8–11]. For example, starting in 1996, Massari et al. [10] observed an increase in the annual incidence of male urethritis in France. The authors ascribe this upsurge to a return to unprotected sexual practices following the decline resulting from the AIDS epidemic.

Complications may develop in some males with *C. trachomatis* urethritis. Approximately 500,000 cases of epididymitis occur yearly in the USA and of these, probably 50% are due to *C. trachomatis* [12]. Sexually active young males account for approximately 70% of the cases. In general, the epididymitis is unilateral and the patient has scrotal and inguinal pain. As a result of epididymitis abscess formation and infarction of the testicle may developed [12–14]. The role of *C. trachomatis* in male infertility is not well understood [15]. Transit of the sperm through the epididymides is necessary for development of normal sperm function. Thus, acute inflammation of the epididymides could lead to decreased fertility even in the absence of occlusion. Hosseinzadeh et al. [16] have proposed that *C. trachomatis*, by increasing tyrosine phosphorylation, may lead to premature capacitation of the spermatozoa and failure of conception. In addition to infertility, other possible complications associated with *Chlamydia* infections in males include proctitis, Reiter's syndrome, and sexually acquired reactive arthritis (SARA) [1,2,17].

When implemented in a timely fashion, antibiotics are effective against *Chlamydia*. However, since many infections are asymptomatic the patients go untreated. Even in symptomatic cases unless the patient is treated early in the infection, with the correct type of antibiotic, long-term complications may occur. Therefore, development of a vaccine to prevent chlamydial infections in males should be a priority. Pal et al. [18] have recently established a new murine model of *C. trachomatis* urogenital tract (UGT) infection by inoculating male mice in the meatus urethra with C. trachomatis MoPn. This pathogen, originally isolated by Nigg [19] from mice inoculated with human respiratory specimens, was considered by Nigg and Eaton [20] to be, most likely, of human origin. In female and male mice MoPn produces UGT infections that closely resemble those produced in humans by *C. trachomatis* [18,21,22]. Here, using this isolate, we explored new vaccination strategies with the goal of establishing a gold standard for testing chlamydial vaccines for males.

MATERIALS AND METHODS

Organisms

The *C. trachomatis* mouse MoPn strain Nigg II (MoPn; also called *C. muridarum*) was purchased from the American Type Culture Collection (Mannassas, VA) and was grown in HeLa-229 cells [23]. Stocks of elementary bodies (EB) were prepared as described by Caldwell et al. [24] and stored at −70°C in SPG (0.2 M sucrose, 20 mM sodium phosphate pH 7.2 and 5 mM glutamic acid).

Immunization and challenge of mice

Three week-old male mice BALB/c (H-2^d) were purchased from Charles River Laboratories (Wilmington, MA). The animals were housed in isolation cubicles at a constant temperature of 24°C with a cycle of 12 h of light and 12 h of darkness and were fed chow ad libitum. For i.u. immunization, the animals were anesthetized with Ketamine plus Xylazine and, while lying on their backs, the prepuce was pulled and using a 4x magnifying glass 10^6 inclusion forming units (IFU; $20\times ID_{50}$) of *Chlamydia* MoPn was placed on the meatus urethra [18].

Mice were kept on their backs until the inoculum was observed to reflux into the urethra. For i.n. immunization the mice were anesthetized and 10^4 IFU of MoPn ($20\times ID_{50}$) in 20 μl of EMEM were placed on the nostrils [23,25]. A control group of male BALB/c mice of the same age was sham-immunized with SPG. Six weeks after the immunization the mice were challenged with 10^6 IFU i.u. as described above. The experiments were repeated twice. The animal protocols were approved by, the University of California Irvine, Animal Care and Use Committee.

Organ culture

The male mice were euthanized at weekly intervals following the challenge and the penile and membranous urethra, urinary bladder, epididymides and testes were harvested and placed in 2 ml of SPG [18]. The tissues were homogenized using a Stomacher Lab-Blender 80 (Tekmar Co., Cincinnati, OH) and duplicates of 10-fold dilutions were inoculated by centrifugation (1,000 × g, 1 h at 24°C) onto HeLa-229 cells grown in 48-well tissue culture plates. Each well was inoculated with 100 μl of the homogenate. The cells were incubated for 30 h at 37°C and the *C. trachomatis* inclusions stained with a pool of monoclonal antibodies (mAb) prepared in our laboratory [18]. This pool included mAb to the major outer membrane protein (MOMP), the 60-kDa cysteine-rich protein (crp), a 150-kDa putative outer membrane protein and the lipopolysaccharide (LPS) of *C. trachomatis* MoPn. The limit of detection was 20 IFU per organ.

Immunoassays

Following euthanasia blood was collected at weekly intervals from the heart and the serum from each mouse for each group of mice was pooled. The *C. trachomatis* MoPn specific antibody titer in serum was determined by an enzyme linked immunosorbant assay (ELISA) [23]. In brief, a 96-well plate was coated with 100 μl of *C. trachomatis* MoPn EB in PBS at a concentration of 10 μg of protein/ml. Serum (100 μl) was added to each well in 2-fold serial dilutions. After incubation at 37°C for 1 h the serum was discarded and the wells washed three times with PBS. Subsequently, the plates were incubated with horseradish peroxidase-conjugated goat antimouse IgM, IgA, IgG, IgG1, IgG2a, IgG2b and IgG3 (Southern Biotechnology Associates, Inc. Birmingham, AL). The binding was measured in an EIA reader (Labsystem Multiscan, Helsinki, Finland) using 2′-2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) as the substrate. Preinfection sera were used as negative controls. The titers are expressed as the reciprocal of the dilution.

Immunoblots were performed by using 250 μg of protein from purified *C. trachomatis* MoPn EB in tricine-SDS polyacrylamide gels (10% acrylamide, 0.3% bis-acrylamide (w/v) [23,26]. Following transfer to nitrocellulose membranes the nonspecific sites were blocked with BLOTTO (Bovine Lacto Transfer Technique Optimizer: 5% (w/v) non fat dried milk, 2 mM $CaCl_2$ and 50 mM Tris-HCl, pH 8.0). Serum samples were diluted 1:100 and incubated overnight at 4°C. Antibody binding was detected with horseradish peroxidase-conjugated goat anti-mouse antibody developed with 0.01% hydrogen peroxide and 4-chloro-1-naphthol [23].

A T-cell lymphoproliferative assay (LPA) was performed using splenocytes as described [23]. Briefly, the spleens of two to three mice from each group were collected, teased and enriched for T-cells by passage over a nylon wool column. T-enriched cells were counted and 10^5 cells were aliquoted into a 96-well plate. Antigen presenting cells (APC) were prepared by irradiating splenocytes with 3,300 rads. UV inactivated *C. trachomatis* MoPn EB were added at a concentration of 10 EB to 1 APC. Control wells received medium alone as negative control, or concanavalin A, as a positive control, at a concentration of 5 μg/ml.

Cell proliferation was measured by addition of 1 μCi of (methyl ^{3}H) thymidine per well. The mean count was determined for triplicate cultures.

Cytokine measurements

Levels of IFN-γ, IL-4 and TNF-α were determined by EIA using commercial kits following the manufacturer instructions (BD-Pharmingen, San Diego, CA). T-cells from the spleen were stimulated as described above and the cytokines were measured in tissue culture supernatants collected after 48 h of incubation.

Statistics

The two-tailed unpaired Student's *t*-test, Fisher's exact test, Chi square test and the Mann-Whitney's *U* test were used to determine the significance of differences between the groups using the SigmaStat 3.5 software program on a PC computer. A p value of <0.05 was considered to be significant.

RESULTS

Assessment of the humoral immune response following immunization

Male BALB/c mice were immunized with live *Chlamydia* by the i.u. or i.n. routes and serum samples were collected the day before the animals were challenged in the meatus urethra. Results of the ELISA performed to detect serum antibodies specific for *C. trachomatis* MoPn EB are shown in Table 1. The titer of IgG antibodies in the mice immunized i.u. was 3,200 and in the mice immunized i.n. the titer was 12,800. To determine if the animals had developed a Th1 or a Th2 biased immune response the levels of *Chlamydia*-specific IgG2a and IgG1 were determined. Titers of IgG2a were 8-fold higher than those of IgG1, 6,400/800 and 12,800/1,600, in the groups immunized i.u. and i.n., respectively. Serum IgA titers were equivalent in the mice immunized i.n. (800) and in the animals inoculated i.u. (400). No *Chlamydia*-specific IgM antibodies were detected in either group of male mice. No *Chlamydia*-specific antibodies were detected in the serum samples form the sham-immunized animals.

The *Chlamydia*-specific neutralizing titers were also determined the day before the i.u. challenge. As shown in Table 1, the neutralizing titer was the same (1,250) in the i.u. and the i.n. immunized groups. The serum from the sham-immunized group was used as the background control.

Figure 1 shows the results of the Western blot performed with the serum samples collected the day before the challenge. The serum from the animals immunized i.n detected several bands with a MW in the range of 90–150 kDa. In this MW range a few faint bands were also observed with the serum from the animals immunized i.u. In addition, the doublet corresponding to the 60-kDa crp, the 60-kDa-heat shock protein (hsp), MOMP, the 28 kDa and LPS were detected in both groups of animals. The Western blot was negative for the sham-immunized mice.

Determination of the cellular immune response following immunization

To characterize the cellular immune response following the i.u. and i.n. immunizations, T cell-enriched splenocytes were collected and tested for their proliferative response to EB. As controls, splenocytes from the sham-immunized males were used. As shown in Table 2 a very strong proliferative response to EB was observed in both groups immunized with *C. trachomatis* when compared to the sham-immunized control animals. The stimulation index (SI) in the group immunized i.u. was 54 (12,058/225) and the SI for the animals inoculated i.n. was 58 (9,511/163).

Levels of IFN-γ, IL-4 and TNF-α were measured in the supernatants from stimulated T-cells from the spleen. As shown in Table 2, high levels of IFN-γ and TNF-α were detected in the supernatants of the lymphocytes from the immunized mice in comparison to those in the sham-infected mice ($p<0.05$). In contrast, levels of IL-4 were below the limit of detection for the *C. trachomatis* vaccinated and the sham-immunized animals.

Characterization of the course of the i.u. challenge

Six weeks after the i.u. and the i.n. immunizations the BALB/c male mice were challenged in the meatus urethra with 1×10^6 IFU of *C. trachomatis* MoPn. At weekly intervals groups of mice were euthanized, their UGT harvested and cultured for *C. trachomatis*. As shown in Tables 3 and 4, overall, significant protection was observed in the mice immunized by either the i.u. or the i.n. routes. Interestingly, the animals immunized i.u. showed significant protection at 7 days after the challenge while the mice immunized i.n. showed significant protection starting at 14 days post-challenge. For example, at 7 days post-challenge 85% (17/20) of the sham-immunized mice had positive cultures from at least one UGT organ while only 30% (3/10) of the animals immunized i.u. had positive cultures ($P<0.05$). In contrast, in the i.n. immunized group 90% (9/10) of the mice had positive cultures ($P>0.05$). Also, only 15% (6/40) of the UGT organs cultured were positive in the i.u. immunized group while 59% (47/80) were positive in the control sham-immunized animals ($P<0.05$). Of the organs cultured at 7 days after the challenge from the i.n. immunized mice, 48% (19/40) were positive ($P>0.05$). Furthermore, a significant decrease in the number of IFU recovered from the urethra, bladder, epididymides and testes was also observed in the i.u. immunized group at 7 days post-challenge in comparison with the sham-immunized animals ($P<0.05$). Importantly, in the i.u. immunized group only 10% (1/10) of the epididymides were positive and all the testes were negative (0/10) while in the sham immunized group 55% (11/20) and 50% (10/20) of the animals had positive cultures from those two organs, respectively ($P<0.05$). A similar trend was also noted at 14, 21 and 28 days post-challenge. At days 21, 28 and 35 after the challenge all the mice immunized i.u. had negative cultures. Therefore, in the group immunized i.u., the total number of mice with positive cultures, the number of positive organs, the length of time that the animal had positive cultures, and the total number of IFU recovered were overall significantly lower in comparison with the sham-immunized animals (Table 3).

Animals immunized i.n. with *C. trachomatis* were also significantly protected against an i.u. challenge. However, the protection was not observed immediately after the challenge. At day 7 post-challenge no statistically significant differences were observed between the animals immunized i.n. and the sham immunized animals. The percentage of animals with positive cultures, the number of organs that were positive for *Chlamydia*, and the number of IFU recovered per organ, were not significantly different between the two groups. Nevertheless, at day 14 post-challenge the percentage of animals with positive cultures 20% (2/10) versus 60% (12/20), and the number of positive organs, 8% (3/40) versus 33% (26/80), were significantly lower in the groups immunized i.n. with *Chlamydia* in comparison with the sham-immunized group ($P<0.05$). A significant decrease in the number of IFU recovered from the urethra and epididymides was also noted in the i.n. immunized group at 14 days post-challenge ($P<0.05$). A similar trend was observed at day 21 post-challenge. By day 35 post-challenge all mice immunized i.u. or i.n. with *Chlamydia* had negative cultures while 20% (4/20) of the sham-immunized animals had positive cultures from the urethra.

Table 4 shows the percentage of mice with positive cultures in the 5 weeks of observation and the total number of *C. trachomatis* IFU recovered from the four UGT organs each week. A significant decrease in the number of mice with positive cultures was observed in the groups immunized i.u. 10% (5/50) and i.n. 28% (14/50) versus the sham immunized animals

47% (47/100); P<0.05). In addition, at days 7, 14 and 21 post-challenge statistically significant differences were observed in the number of IFU recovered from all UGT organs cultured between the i.u. *Chlamydia* immunized and the sham immunized groups (P<0.05). For the group immunized i.n. significant differences with the sham-immunized group were observed at day 14 and 21 post-challenge.

DISCUSSION

The main goal of this study was to establish a new murine model to test vaccines against *C. trachomatis* genital infections in males. The results show that i.n. or i.u immunization with live *Chlamydia*, can induce immune responses in male mice that are protective against a challenge in the meatus urethra. Overall, male mice that were immunized by the i.u. route, showed a stronger protection than animals immunized by the i.n. route. This is, to our knowledge, the first time that vaccine-induced protection in male mice has been shown against a UGT infection with *C. trachomatis*.

Two species of *Chlamydia*, *C. trachomatis* and *C. psittaci*, have been used to characterize immune-induced protection against a UGT infection in males. Non-human male primates have been immunized with live *C. trachomatis* and subsequently challenged [27–29]. For example, Digiacomo et al. [27] inoculated the urethra of two sexually mature male baboons (*Papio cynocephalus*) with the *C. trachomatis* serovar D. Baboon 1 developed an infection as shown by the isolation of *Chlamydia* from the urethra, and a positive antibody titer, while the second animal remained negative. After reinfecting the second animal, both baboons had positive urethral cultures for almost 3 months and positive antibody titers. No changes were noted in the epididymides of the two animals. Over a period of several months the animals were subsequently challenged with the D and I serovars. Based on serological and culture findings the authors concluded that, following a primary infection, the baboons developed an immune response that was protective, not only against the serovar used for the primary inoculation, but also against a distantly related serovar.

In the case of the guinea pig model, males have been infected with the *C. psittaci* guinea pig inclusion conjunctivitis (GPIC) agent (also called *Chlamydia caviae*) [30–32]. For example, Howard et al. [32] inoculated male guinea pigs in the urethra with GPIC. The animals developed urethritis as shown by shedding of *Chlamydia* from the urethra for a period of 8–14 days. When these guinea pigs were challenged 42 days after the primary inoculation none of the animals shed *Chlamydia*. The authors concluded that the finding clearly demonstrated immunity to a urethral reinfection, after immunization at the same site. Further, based on this finding the authors stated: "Resistance to infection in males alone may be sufficient to disrupt the sexual transmission of chlamydial agents". Similarly, to analyze the protective immune response to a reinfection in the guinea pig model, Patterson and Rank [31] inoculated the urethra of male animals with the GPIC agent. Positive cultures were obtained in all animals with the infection peaking at 9 days p.i.. By 21 days all animals had negative cultures. Three different groups of guinea pigs were then challenged at 30, 75 or 150 days post immunization. On these dates, only 20%, 10% and 30%, respectively, of the animals became reinfected. The authors concluded that male guinea pigs are more resistant to a genital reinfection and for a longer period of time than female animals [31].

The results we obtained with the male murine model have significant similarities with the female murine model but there are also interesting differences. In both, male and female models, inoculation with live *C. trachomatis* by all routes elicits a strong immune Th1 response [18,33]. In males and females high IFN-γ levels can be detected in supernatants from EB-stimulated lymphocytes and levels of chlamydial specific IgG2a antibodies in serum are significantly higher than those of IgG1. These findings suggest that formulation of

a subunit vaccine to be used in males, similar to that to be used in females, should include adjuvants that favor a Th1 response. This premise is postulated on the assumption that natural infection provides the best model for a vaccine. This assumption however, may or may not be correct. Like with other infections, induction of "unnatural immunity" by a vaccine may elicit better protection than that resulting from a natural infection [34] .

An unexpected finding that may be significant for implementing a vaccine in humans, is the difference in the ability of the i.n. versus i.u immunization to protect males. In 1994 Pal et al. [23] first reported that an i.n. immunization of female mice with live *C. trachomatis* MoPn elicited a very strong protection against a genital challenge. Similarly, Igietseme et al. [35] compared various routes of immunization, including the vaginal, oral and sucutaneous routes, with live *C. trachomatis* MoPn in the female murine model and concluded that the i.n. route was the most efficacious in inducing protection against a vaginal challenge. Based on the results obtained with the female and male models, it appears that in males, a genital immunization better protects against a genital challenge while in females an i.n. immunization is the most efficacious. The results obtained with the non-human primates and the guinea pig male models support these findings with the male mouse model [27,30,36]. This apparent discrepancy between the male and the female models may be due to several factors. For example, the UGT of the males may be a better inductive site than the UGT of the female. This is not surprising since the UGT of the females needs to tolerate sperm and the fetus. While higher number of *Chlamydia* IFU were used to immunize animals i.u. compared to the i.n. route (10^6 versus 10^4 IFU, corresponding to $20\times ID_{50}$ for both sites) we do not attribute the observed dissimilarity in protection to the dose difference. I.N. inoculation with 10^4 IFU results in the production of up to 10^{10} *Chlamydia* IFU in the lung at 10 days p.i. versus a maximum recovery of 10^7 IFU from the urethra following i.u. inoculation with 10^6 IFU, as shown in this study and the publication by Pal et al. [25]. Therefore, the local immune response, rather than the *Chlamydia* immunization dose, most likely accounts for the observed differences in protection.

Several laboratories have evaluated the capacity of the male UGT to act as an inductive site. For example, Pate et al. [37] collected penile urethral swabs from PCR-confirmed cases of *C. trachomatis* infection and looked for the presence of cytokines, including IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18. In addition, they measured TGF-β, TNF-α, INF-γ, and antigen-specific antibodies. In comparison with the non-infected control group they only detected elevated levels of IL-8 and IgA and IgG *Chlamydia*-specific antibodies. Based on these results the authors concluded that the male genitourinary tract should be considered a weak inductive site. This observation contrasts with the results reported by other groups [38,39]. For example, Pudney and Anderson [38] characterized the immunobiology of the human penile urethra and concluded that this region probably plays a key role in protecting males against infections since it contains all the necessary components for antigen presentation and humoral and cellular immune responses. Specifically, they determined that the penile urethra is an active site for the production of secretory IgA and contains dendritic cells, macrophages and T lymphocytes, in particular $CD8^+$ cells. Most of the T lymphocytes are positive for CD45RO, a memory marker, and for αEβ7 integrin, a mucosal associated antigen.

Our findings indicate that the genitourinary tract may be a better inductive site than the respiratory tract for eliciting a protective immune response in male mice against a urethral challenge. In contrast, in female mice the respiratory route appears to be more effective that the vaginal route for inducing protection [23,36,40]. The fact that the findings in males do not correlate with the observations in the female murine model should not be surprising. In humans a vaccine for genital herpes simplex virus has shown differences in its ability to protect females and males. For example, Stanberry et al. [41] showed that a vaccine

formulated with glycoprotein D protected women but not men against genital herpes. Until now, most of the human vaccines were not directed against sexually transmitted pathogens and therefore, for the most part, did not take into account the anatomical and physiological differences between males and females. With the implementation of vaccines for sexually transmitted pathogen such as, herpes simples virus, human immunodeficiency virus, human papillomavirus and *Chlamydia* these differences are factors that will need to be taken into consideration.

Both the non-human primate model and the guinea pig model offer several advantages over the murine model for testing vaccines in males. For example, in non-human primates it is possible to infect the animals with the human serovars of *C. trachomatis* and characterize the ability of a vaccine to induce cross-protection against the various human serovars [27,28]. In addition, the immune system of these animals probably more closely parallels that of humans. In the case of guinea pigs these animals are larger than mice and therefore allow for easier urethral inoculations and collection of urethral specimens with a swab [31,42]. Also, for studies that require large amounts of tissues fewer animals are required when studying guinea pigs rather than mice. However, both the non-human primate and the guinea pig model have some limitations. For example, the lack of inbred animals and immunological reagents limits the types of studies that can be conducted with these animals. Furthermore, they are more expensive to acquire and maintain. Therefore, the mouse model can be used to perform the initial testing of vaccination protocols. Formulations found to be protective in mice can then be tested in the guinea pig model and later on in non-human primates.

In conclusion, we have established a new mouse model for testing vaccines in males against *C. trachomatis* genital infections. Our data show that, at least with a live vaccine, it is possible to induce protection in male mice against a UGT infection with *Chlamydia*. The challenge now is to formulate a subunit vaccine that can induce protection in this model and eventually in humans.

Acknowledgments

This work was supported by Public Health Service grants AI-32248 and AI-67888 from the National Institute of Allergy and Infectious Diseases.

References

1. Schachter, J.; Dawson, C. Human chlamydial infections. Littleton: PSG Publishing Co; 1978.
2. Stamm, W.; Holmes, K. Chlamydia trachomatis infections of the adult. In: Holmes, KKPM.; Sparling, PF.; Wiesner, PJ., editors. Sexually transmitted diseases. New York: McGrawHill Book Co; 1999.
3. Halberstaedter L, von Prowazek S. Zur atiologie des trachoms. Dtsch Med Wochenschr. 1907; 33:1285–7.
4. Lindner K. Zur atiologie der gonokokkenfreien urethritis. Wien Klin Wochenschr. 1910; 23:283–4.
5. Jones BR. Genital Trachoma. Rev Int Trach. 1964; 41:425–38. [PubMed: 14248813]
6. Miller WC, Ford CA, Morris M, Handcock MS, Schmitz JL, Hobbs MM, et al. Prevalence of chlamydial and gonococcal infections among young adults in the United States. Jama. 2004 May 12; 291(18):2229–36. [PubMed: 15138245]
7. Schachter, J. Infection and disease epidemiology. In: Stephens, R., editor. Chlamydia: intracellular biology, pathogenesis and immunity. Washington: ASM; 1999. p. 139-70.
8. Adler MW. Sexual health--health of the nation. Sex Transm Infect. 2003 Apr; 79(2):85–7. [PubMed: 12690123]

9. Hiltunen-Back E, Haikala O, Kautiainen H, Ruutu P, Paavonen J, Reunala T. Nationwide increase of Chlamydia trachomatis infection in Finland: highest rise among adolescent women and men. Sex Transm Dis. 2003 Oct; 30(10):737–41. [PubMed: 14520170]
10. Massari V, Dorleans Y, Flahault A. Persistent increase in the incidence of acute male urethritis diagnosed in general practices in France. Br J Gen Pract. 2006 Feb; 56(523):110–4. [PubMed: 16464324]
11. Massari V, Retel O, Flahault A. A recent increase in the incidence of male urethritis in France. Sex Transm Dis. 2002 Jun; 29(6):319–23. [PubMed: 12035020]
12. Berger RE, Alexander ER, Monda GD, Ansell J, McCormick G, Holmes KK. Chlamydia trachomatis as a cause of acute "idiopathic" epididymitis. N Engl J Med. 1978 Feb 9; 298(6):301–4. [PubMed: 622087]
13. Berger RE, Alexander ER, Harnisch JP, Paulsen CA, Monda GD, Ansell J, et al. Etiology, manifestations and therapy of acute epididymitis: prospective study of 50 cases. J Urol. 1979 Jun; 121(6):750–4. [PubMed: 379366]
14. Hori S, Tsutsumi Y. Histological differentiation between chlamydial and bacterial epididymitis: nondestructive and proliferative versus destructive and abscess forming--immunohistochemical and clinicopathological findings. Hum Pathol. 1995 Apr; 26(4):402–7. [PubMed: 7705819]
15. Cunningham KA, Beagley KW. Male genital tract chlamydial infection: implications for pathology and infertility. Biol Reprod. 2008 Aug; 79(2):180–9. [PubMed: 18480466]
16. Hosseinzadeh S, Brewis IA, Pacey AA, Moore HD, Eley A. Coincubation of human spermatozoa with Chlamydia trachomatis in vitro causes increased tyrosine phosphorylation of sperm proteins. Infect Immun. 2000 Sep; 68(9):4872–6. [PubMed: 10948099]
17. Quinn TC, Goodell SE, Mkrtichian E, Schuffler MD, Wang SP, Stamm WE, et al. Chlamydia trachomatis proctitis. N Engl J Med. 1981 Jul 23; 305(4):195–200. [PubMed: 7017409]
18. Pal S, Peterson EM, de la Maza LM. New murine model for the study of Chlamydia trachomatis genitourinary tract infections in males. Infect Immun. 2004 Jul; 72(7):4210–6. [PubMed: 15213165]
19. Nigg C. An Unidentified Virus Which Produces Pneumonia and Systemic Infection in Mice. Science. 1942 Jan 9; 95(2454):49–50. [PubMed: 17773453]
20. Nigg C, Eaton MD. Isolation from Normal Mice of a Pneumotropic Virus Which Forms Elementary Bodies. J Exp Med. 1944 May 1; 79(5):497–510. [PubMed: 19871384]
21. Swenson CE, Schachter J. Infertility as a consequence of chlamydial infection of the upper genital tract in female mice. Sex Transm Dis. 1984 Apr–Jun; 11(2):64–7. [PubMed: 6463810]
22. de la Maza LM, Pal S, Khamesipour A, Peterson EM. Intravaginal inoculation of mice with the Chlamydia trachomatis mouse pneumonitis biovar results in infertility. Infect Immun. 1994 May; 62(5):2094–7. [PubMed: 8168974]
23. Pal S, Fielder TJ, Peterson EM, de la Maza LM. Protection against infertility in a BALB/c mouse salpingitis model by intranasal immunization with the mouse pneumonitis biovar of Chlamydia trachomatis. Infect Immun. 1994 Aug; 62(8):3354–62. [PubMed: 8039906]
24. Caldwell HD, Kromhout J, Schachter J. Purification and partial characterization of the major outer membrane protein of Chlamydia trachomatis. Infect Immun. 1981 Mar; 31(3):1161–76. [PubMed: 7228399]
25. Pal S, Davis HL, Peterson EM, de la Maza LM. Immunization with the Chlamydia trachomatis mouse pneumonitis major outer membrane protein by use of CpG oligodeoxynucleotides as an adjuvant induces a protective immune response against an intranasal chlamydial challenge. Infect Immun. 2002 Sep; 70(9):4812–7. [PubMed: 12183524]
26. Schagger H, von Jagow G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem. 1987 Nov 1; 166(2): 368–79. [PubMed: 2449095]
27. Digiacomo RF, Gale JL, Wang SP, Kiviat MD. Chlamydial infection of the male baboon urethra. Br J Vener Dis. 1975 Oct; 51(5):310–3. [PubMed: 811318]
28. Taylor-Robinson D, Purcell RH, London WT, Sly DL, Thomas BJ, Evans RT. Microbiological, serological, and histopathological features of experimental Chlamydia trachomatis urethritis in chimpanzees. Br J Vener Dis. 1981 Feb; 57(1):36–40. [PubMed: 7470833]

29. Moller BR, Mardh PA. Experimental epididymitis and urethritis in grivet monkeys provoked by Chlamydia trachomatis. Fertil Steril. 1980 Sep; 34(3):275–9. [PubMed: 7409251]
30. Rank R, Batteiger B, Soderberg L. Susceptibility to reinfection after a primary chlamydial genital infection. Infect Immun. 1988; 56:2243–9. [PubMed: 2457553]
31. Patterson TL, Rank RG. Immunity to reinfection and immunization of male guinea pigs against urethral infection with the agent of guinea pig inclusion conjunctivitis. Sex Transm Dis. 1996 Mar–Apr; 23(2):145–50. [PubMed: 8919742]
32. Howard LV, O'Leary MP, Nichols RL. Animal model studies of genital chlamydial infections. Immunity to re-infection with guinea-pig inclusion conjunctivitis agent in the urethra and eye of male guinea-pigs. Br J Vener Dis. 1976 Aug; 52(4):261–5. [PubMed: 963491]
33. Morrison RP, Caldwell HD. Immunity to murine chlamydial genital infection. Infect Immun. 2002 Jun; 70(6):2741–51. [PubMed: 12010958]
34. Casadevall A, Pirofski LA. Exploiting the redundancy in the immune system: vaccines can mediate protection by eliciting 'unnatural' immunity. J Exp Med. 2003 Jun 2; 197(11):1401–4. [PubMed: 12782708]
35. Igietseme JU, Uriri IM, Kumar SN, Ananaba GA, Ojior OO, Momodu IA, et al. Route of infection that induces a high intensity of gamma interferon-secreting T cells in the genital tract produces optimal protection against Chlamydia trachomatis infection in mice. Infect Immun. 1998 Sep; 66(9):4030–5. [PubMed: 9712743]
36. Kelly KA, Robinson EA, Rank RG. Initial route of antigen administration alters the T-cell cytokine profile produced in response to the mouse pneumonitis biovar of Chlamydia trachomatis following genital infection. Infect Immun. 1996 Dec; 64(12):4976–83. [PubMed: 8945535]
37. Pate MS, Hedges SR, Sibley DA, Russell MW, Hook EW 3rd, Mestecky J. Urethral cytokine and immune responses in Chlamydia trachomatis-infected males. Infect Immun. 2001 Nov; 69(11): 7178–81. [PubMed: 11598098]
38. Pudney J, Anderson DJ. Immunobiology of the human penile urethra. Am J Pathol. 1995 Jul; 147(1):155–65. [PubMed: 7604877]
39. Mestecky J, Russell MW. Induction of mucosal immune responses in the human genital tract. FEMS Immunol Med Microbiol. 2000 Apr; 27(4):351–5. [PubMed: 10727891]
40. Igietseme JU, He Q, Joseph K, Eko FO, Lyn D, Ananaba G, et al. Role of T lymphocytes in the pathogenesis of Chlamydia disease. J Infect Dis. 2009 Sep 15; 200(6):926–34. [PubMed: 19656067]
41. Stanberry LR, Spruance SL, Cunningham AL, Bernstein DI, Mindel A, Sacks S, et al. Glycoprotein-D-adjuvant vaccine to prevent genital herpes. N Engl J Med. 2002 Nov 21; 347(21): 1652–61. [PubMed: 12444179]
42. Rank RG, White HJ, Soloff BL, Barron AL. Cystitis associated with chlamydial infection of the genital tract in male guinea pigs. Sex Transm Dis. 1981 Jul–Sep; 8(3):203–10. [PubMed: 7292213]

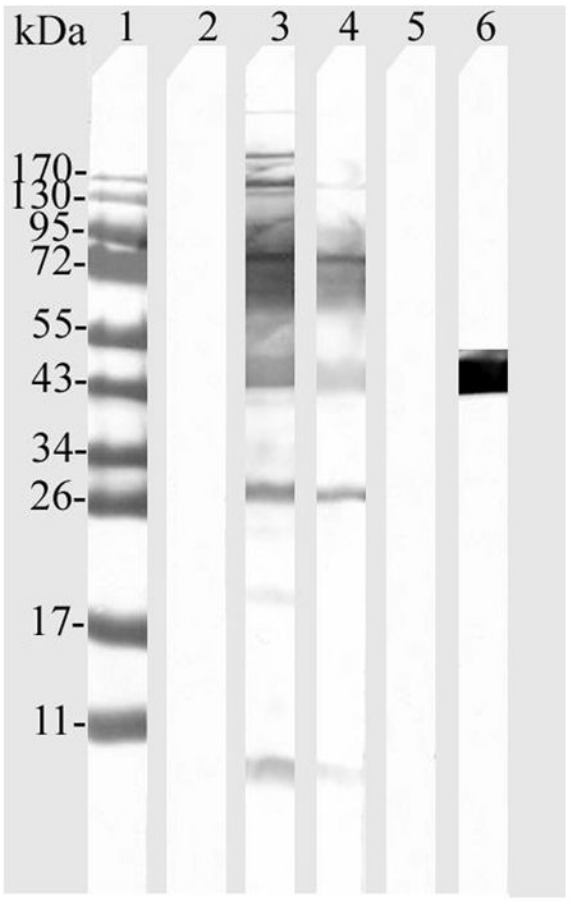

Figure 1.
Western blot of *C. trachomatis* MoPn EB probed with serum samples collected from immunized mice. Lanes: 1) Molecular weight standards; 2) Pre-immune serum; 3) Serum from mice immunized i.n. with EB; 4) Serum from animals immunized i.u. with EB; 6) Serum from sham-immunized animals; 6) mAb MoPn-40 used as a control that binds to MOMP.

Table 1

Antibody response the day before the challenge

	C. trachomatis MoPn-specific ELISA antibody titer							In vitro
Group	**IgM**	**IgG**	**IgG1**	**IgG2a**	**IgG2b**	**IgG3**	**IgA**	**Neutralization titer**
i.u. immunized	<100	3,200	800	6,400	6,400	6,400	400	1,250
i.n.immunized	<100	12,800	1,600	12,800	6,400	6,400	800	1,250
Sham immunized	<100	<100	<100	<100	<100	<100	<100	<50

Vaccine. Author manuscript; available in PMC 2011 November 10.

Table 2

T cell response the day before challenge[a]

	T cell proliferation to (cpm ± 1SD)			Cytokine secretion in response to EB[a] (pg/ml ± 1 SD)		
Group	**EB[b]**	**Con A[c]**	**Medium**	**IFN-γ**	**TNF-α**	**IL-4**
i.u. immunized	12,058±1,306[d]	41,529±10,111	225±143	10,685±301[d]	222±5[d]	<3.7
i.n. immunized	9,511±4,964[d]	67,786±20,166	163±113	10,975±0[d]	205±12[d]	<3.7
Sham immunized	730±612	67,876±16,100	99±27	297±188	60±7	<3.7

[a] Results are means for triplicate cultures (± 1 standard deviation). Data correspond to one of the experiments representative of duplicate separate experiments.

[b] UV-inactivated *C. trachomatis* MoPn EB were added at a 10:1 ratio to the T cells.

[c] Concanavalin A (Con A) was added at a concentration of 5 μg/ml.

[d] P<0.05 by the Student's *t* test compared to the sham immunized group.

Table 3

Protection of male BALB/c mice against an intraurethral challenge

Group	Daysp.c.	Median (range) no. of IFU recovered from organs (% + mice)								Mice+/ total mice (%+)[c]	Organ+/ total organ tested (%+)
		Urethra		Bladder		Epididymides		Testes			
		IFU	%	IFU	%	IFU	%	IFU	%		
i.u. immunized[a]	7	<20 (<20-1×10^6)[e]	(30)[d]	<20 (<20–30)[e]	(20)	<20 (<20–50)[e]	(10)[d]	<20[e]	(0)[d]	3/10 (30)[d]	6/40 (15)[g]
i.n. immunized[b]	7	0.07×10^6 (<20-0.4×10^6)	(90)	5 (<20–600)	(50)	<20 (<20–90)	(30)	<20 (<20–300)	(20)	9/10 (90)	19/40 (48)
Sham immunized	7	1.3×10^6 (<20-11.5×10^6)	(75)	30 (<20–47,495)	(55)	55 (<20–18,998)	(55)	<20 (<20–500)	(50)	17/20 (85)	47/80 (59)
i.u. immunized	14	<20 (<20–480)[e]	(10)[d]	<20 (<20–78,200)	(20)	<20[e]	(0)[d]	<20	(0)	2/10 (20)[f]	3/40 (8) [g]
i.n. immunized	14	<20 (<20–200)[e]	(10)[d]	<20 (<20–20)	(20)	<20[e]	(0)	<20	(0)	2/10 (20) [f]	3/40 (8)[g]
Sham immunized	14	1,342 (<20-2.4×10^6)	(60)	<20 (<20– 125,800)	(35)	<20 (<20–260)	(25)	<20 (<20–200)	(10)	12/20 (60)	26/80 (33)
i.u. immunized	21	<20[e]	(0)[d]	<20[e]	(0)[d]	<20	(0)	<20	(0)	0/10 (0)[d]	0/40 (0) [g]
i.n. immunized	21	<20[e]	(0)[d]	<20 (<20–20)	(10)	<20	(0)	<20	(0)	1/10 (10) [d]	1/40 (3) [g]
Sham immunized	21	<20 (<20–578)	(40)	<20 (<20–491,470)	(35)	<20 (<20–60)	(5)	<20	(0)	10/20 (50)	16/80 (20)
i.u. immunized	28	<20	(0)	<20[e]	(0)	<20	(0)	<20	(0)	0/10 (0)	0/40 (0) [g]
i.n. immunized	28	<20	(0)	<20 (<20–2,400)	(10)	<20 (<20–40)	(20)	<20	(0)	2/10 (20)	3/40 (8)
Sham immunized	28	<20 (<20–640)	(5)	<20 (<20–4,200)	(15)	<20 (<20–80)	(5)	<20 (<20–200)	(5)	4/20 (20)	6/80 (8)
i.u. immunized	35	<20	(0)	<20	(0)	<20	(0)	<20	(0)	0/10 (0)	0/40 (0)
i.n. immunized	35	<20	(0)	<20	(0)	<20	(0)	<20	(0)	0/10 (0)	0/40 (0)
Sham immunized	35	<20 (<20–140)	(20)	<20	(0)	<20	(0)	<20	(0)	4/20 (20)	4/80 (5)

[a] Mice were immunized in the meatus urethra with 1×10^6 IFU *C. trachomatis* MoPn.

[b] Mice were immunized intranasally with 10^4 IFU of *C. trachomatis* MoPn.

[c] Percent positive in at least in one organ.

[d] P<0.05 by Fisher's Exact test compared to the sham immunized group.

[e] P<0.05 by the Mann Whitney U test compared to the sham immunized group.

[f] P<0.10 by Fisher's Exact test compared to the sham immunized group.

[g] P<0.05 by the Chi square test compared to the sham immunized group.

Table 4

C. trachomatis MoPn IFU burden in four organs (urethra, bladder, epididymides, and testes) of the UGT

Group	Total mice/ group	Median (range) no. *C. trachomatis* IFU recovered from UGT per group					No. mice positive in 5 weeks/total mice (%+)
		D7	D14	D21	D28	D35	
i.u. immunized	50	<20 (<20–952,000)[a]	<20 (<20–78,200)[a]	<20[a]	<20	<20	5/50 (10)[b]
i.n. immunized	50	<20 (<20–425,400)	<20 (<20–200)[a]	<20 (<20–20)[a]	<20 (<20–2,400)	<20	14/50 (28)[b]
Sham immunized	100	25 (<20–11,556,700)	<20 (<20–2,482,000)	<20 (<20–491,470)	<20 (<20–4,200)	<20 (<20–140)	47/100 (47)

[a] P<0.05 by the Mann Whitney U test compared to the sham immunized group.

[b] P<0.05 by the Chi square test compared to the sham immunized group.