Male Rodent Genital Tract Infection With Chlamydia Muridarum: Persistence in the Prostate Gland That Triggers Self-Immune Reactions in Genetically Susceptible Hosts

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Abbreviations and Acronyms

dpi = days after inoculation

EAP = experimental autoimmune prostatitis

ELISA = enzyme-linked immunosorbent assay

MBP = maltose binding protein

MNC = mononuclear cell

MOMP = major outer membrane protein

NOD = nonobese diabetic

OMP2 = outer membrane protein 2

PAP = prostatic acid phosphatase

PCR = polymerase chain reaction

PE = prostate extract

PMN = polymorphonuclear

PSBP = prostate steroid binding protein

SI = stimulation index

Purpose: We investigated Chlamydia trachomatis infection and its pathogenic consequences in the male rodent genital tract.

Materials and Methods: Male rats were inoculated in the meatal urethra with Chlamydia muridarum. We sought bacterial DNA at early and late times after inoculation in different parts of the male genital tract. Histological alterations and the immune response against prostate antigens were analyzed.

Results: Male rats showed ascending infection with wide dissemination of bacteria in the genital tract at an early time point after inoculation. At later stages bacteria persisted only in some parts of the genital tract and in the prostate gland. C. muridarum was also detected in semen in a high proportion of rats irrespective of an acute or chronic stage of infection. Histological alterations that accompanied C. muridarum were especially observed in the prostate and mainly composed of CD3+ cell infiltration. Positive humoral and cellular responses against prostate antigens were noted in a considerable number of infected rats. NOD mice, an autoimmune, prostatitis prone strain, showed a similar pattern with C. muridarum in the prostate of 100% of infected mice, which was again accompanied by mononuclear cell infiltration and antibodies against prostate antigens at early and late times after inoculation.

Conclusions: Results reveal that C. muridarum infects the male rodent genitourinary tract with special persistence in the prostate gland, where it causes chronic inflammation that in turn may act as a trigger factor for self-immune reactions in susceptible hosts.

Key Words: prostate, Chlamydia trachomatis, prostatitis, autoimmunity, Rodentia

CHLAMYDIA trachomatis, the most prevalent sexually transmitted bacterial infection that affects humans, is associated with reproductive dysfunction.[1] Although antibiotics are used to treat symptomatic urogenital infections, most C. trachomatis infections are asymptomatic. Thus, an increased fraction of patients remains undetected, enabling further spread of the infection. The prevalence of chlamydial infection is similar in males and females but current research and screening strategies are mainly focused on females due to the well-known sequelae and infertility associated with female genital tract infections.[2] Human studies and experimental female genital

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Submitted for publication November 9, 2010.

Study received institutional review board approval.

Supported by Grant PICT 2005 05-38069 from the Agencia Nacional de Promoción Científica y Tecnológica and a grant from Secretaria de Ciencia y Técnica de a Universidad Nacional de Córdoba (VER).

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0022-5347/11/1863-1100/0
THE JOURNAL OF UROLOGY®

Vol. 186, 1100-1106, September 2011
Printed in U.S.A.
DOI:10.1016/j.juro.2011.04.086

tract infection models of C. muridarum, a murine pathogen closely related to C. trachomatis, have allowed a significant advance in understanding the pathophysiology underlying chlamydia associated female infertility.[3]

On the other hand, with a similar prevalence and an extensively accepted role for chlamydia in the development of male urethritis, epididymitis and orchitis,[4,5] data on male genitourinary tract infections are limited.[6] The primary male site of infection is the penile urethra and C. trachomatis is a major cause of male urethritis.[7] In contrast, although many groups have explored the relationship between C. trachomatis and prostatitis, there is still no direct evidence indicating that C. trachomatis is a cause of prostatitis.[6,8–10] However, the prevalence of C. trachomatis infection in patients with chronic prostatitis was already documented in several reports and differences among studies are related to the type of samples analyzed, such as swab, first voided urine, semen or expressed prostate secretions.[6] The characterization of chlamydial infections in males and possible fertility sequelae is also limited. To a certain extent this is due to the lack of appropriate animal models in which to study the course of infection, the elicited immune response and the pathophysiology of male chlamydial infection. Thus, the importance of this pathogen in male genital tract pathology has been neglected.

The main goal of the current study was to establish an experimental model of male genital tract chlamydial infection that imitates the natural route of infection to study the pathophysiology of chlamydial male urogenital infection and its consequences in autoimmune susceptible hosts.

MATERIALS AND METHODS

Chlamydia Strain

The C. trachomatis MoPn strain, now C. muridarum MoPn, was propagated in LCCMK2 cells, as previously described.[11]

Animal Inoculation

Included in our study were 28, 2-month-old male Wistar rats and 20 male NOD mice. Animals were maintained in a 16-hour light/8-hour dark cycle with free access to food and water under specific pathogen-free conditions. The experimental protocol was reviewed and approved by the institutional review board.

Animals were infected with 10^7 IFU C. muridarum in 20 μl sucrose-phosphate-glutamate buffer 3 days consecutively. The animals were anesthetized and placed supine. The prepuce was pulled back and the inoculum was placed on the meatal urethra. The animals remained supine until the inoculum was observed to reflux into the urethra. A control group of animals was sham infected during 3 days with 20 μl sucrose-phosphate-glutamate buffer. The last day of inoculation was considered day 0 of the schedule.

Semen Collection

Semen samples were obtained as previously described[12] to assay for C. muridarum by PCR on days 15 and 80.

C. Muridarum Detection by PCR

The presence of C. muridarum was analyzed in semen and tissue samples from the urethra, bladder, prostate, seminal vesicles, epididymis and testes. Positive results were considered tissue that showed positivity for heat shock protein 60 and MOMP genes. DNA was extracted from semen and tissues by phenol-chloroform extraction and PCR was performed, as previously described by Berry et al.[13]

Antibodies Against Chlamydial Antigens

We used recombinant MBP-MOMP fusion protein and plasmid coding for OMP2.[14] Recombinant OMP2 protein was obtained after bacterial expression induced by isopropyl-β-d-thiogalactosidase and purification using $NiSO_4$ charged HiTrap® chelating Sepharose® columns according to preestablished protocols.[14]

Microtiter plate wells were coated with MBP-MOMP and OMP2 by adding 100 μl 5 μg/ml MBP-MOMP and 10 μg/ml OMP2 solutions in 0.1 M bicarbonate buffer, pH 9.5. After overnight incubation at 4C microwells were washed twice and blocked with 3% bovine serum albumin in PBS for 2 hours at 37C, rinsed once with PBS-Tween 20 at 0.05% and filled with 50 μl serum diluted 2-fold from 1:100 to 1:5,000 in PBS-3% bovine serum albumin for 2 hours at 37C. To detect specific IgG, IgA, IgM, IgG1, IgG2a or IgG2b the plates were washed again and incubated with horseradish peroxidase conjugated goat anti-rat IgG, IgA, IgM, IgG1, IgG2a or IgG2b, respectively (Sigma®) for 1 hour at 37C. The reaction was developed with 3,3',5,5'-tetramethylbenzidine-H_2O_2 (Sigma). Absorbance was measured at 450 nm in a plate reader (Bio-Rad®). Serum reactivity is expressed in titer inversed values.

Histopathology and Immune Histochemical Assays

The animals were sacrificed. Genitourinary tract organs were processed for paraffin embedding, 5 μm sectioning, and hematoxylin and eosin staining. Slides were analyzed for qualitative changes, infiltrating cells and epithelial desquamation.[15] We analyzed 5, 10 μm sections of each organ per animal. For immunohistochemical assays slides were incubated for 30 minutes in 10% normal rabbit serum to block nonspecific binding, followed by 30-minute incubation with monoclonal antibodies to CD11b, CD3, CD4 and CD8 (BD Pharmingen™) at room temperature with an autostainer (Dako, Glostrup, Denmark). Sections were then incubated with biotinylated secondary antibody and peroxidase labeled streptavidin using an LASB+ kit (Dako). Diaminobenzidine (Dako) served as the chromogenic substrate and activated hematoxylin served as the counterstain.

Antibodies and Cellular Proliferative Response to Prostate Antigens

To detect antibodies against PE, the purified prostate antigen PSBP and PAP (Sigma) we performed conventional ELISA, as previously described.[15] PE and PSBP were obtained according to procedures previously described by our group.[16] Serum reactivity is shown as optical density values for each animal serum.

To analyze the lymph node cellular response against PSBP we performed a T-cell proliferation assay, as previously described.[15] The response is expressed as SI, defined as cpm incorporated in antigen pulsed cultures/cpm incorporated in cultures with medium. Values above the mean SI + 3 SDs of the SI observed in the control group were considered positive.

Statistics

Statistical analysis was done using the Wilcoxon paired, paired t and Fisher exact tests, as appropriate, with $p < 0.05$ considered statistically significant

RESULTS

C. Muridarum in Urogenital Tract of Infected Male Rats

To analyze whether chlamydial infection follows an ascending pattern in Wistar rats sham treated and inoculated animals were sacrificed at 15 and 80 dpi, and urethra, bladder, seminal vesicle, prostate, epididymis and testis tissue samples were screened for C. muridarum by PCR. At 15 dpi C. muridarum DNA was detected in 66% and 50% of urethras and bladders, respectively, of infected animals (fig. 1). Prostate and seminal vesicle samples were positive for C. muridarum DNA in 33% of the cases. When epididymis and testis samples were assayed, none revealed C. muridarum DNA.

In contrast, C. muridarum at 80 dpi, more than 11 weeks after inoculation, showed a different pattern. At this later time point C. muridarum was no longer detectable in the urethra, bladder, epididymis or testes. However, it was present in 62% and 37% of prostate and seminal vesicle samples, respectively. Sham treated rats did not show C. muridarum DNA at 15 or 80 dpi in any sample analyzed (data not shown).

Considering that semen is a biological sample normally used for microbiological screening for clinical diagnosis, we then tested for C. muridarum DNA in semen samples from experimental rats. It was detected in 83% and 62% of semen samples from infected animals at 15 and 80 dpi, respectively (fig. 1). As expected, negative results were obtained in semen samples from sham treated rats at each time point (data not shown).

These results suggest that at late stages of C. muridarum infection bacteria were sustained in the male rat genital tract, especially in the prostate gland.

Humoral Immune Response to Chlamydia

Serum antibodies against MOMP and OMP2 antigens were analyzed at 15 and 80 dpi. All infected animals showed positive levels of these antibodies. As expected, high levels of MOMP specific IgM were observed at early stages of chlamydial infection (mean titer 1/1,000 on 15 dpi), which then decreased throughout the experimental schedule (mean titer 1/170 at 80 dpi). Conversely MOMP specific IgG was detected at 15 dpi at a mean titer of 1/500, which then significantly increased to 1/2,000 at 80 dpi. When OMP2 specific antibodies were screened, similar results were observed. A similar pattern of response was also noted for specific IgA. In addition, levels of IgG2a and IgG2b paralleled those of total IgG with a mean titer of 1/2,100 and 1/2,200 at 80 dpi. However, when specific IgG1 levels were

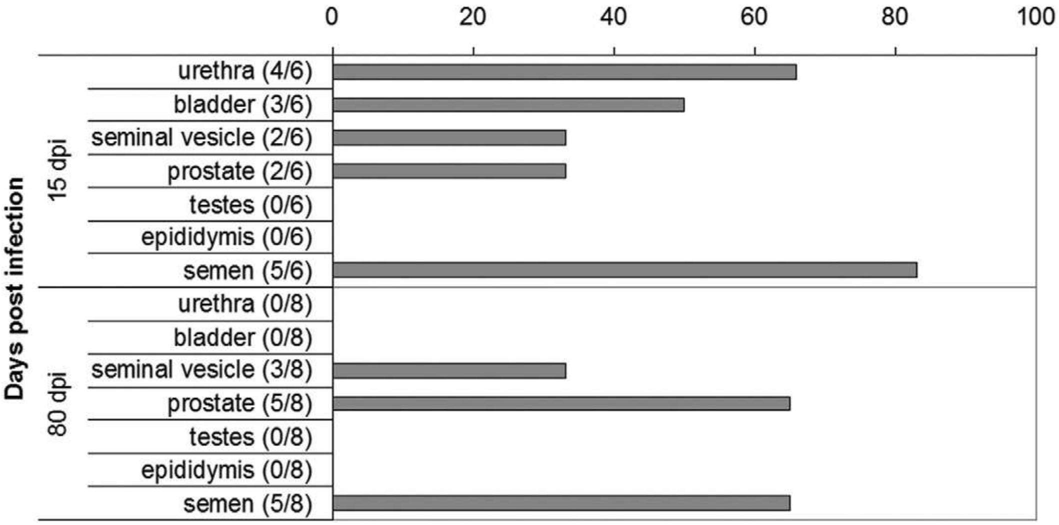

Figure 1. PCR revealed C. muridarum DNA in urethra, bladder, prostate, seminal vesicles, epididymis, testes and semen of 6 and 8 infected animals at 15 and 80 dpi, respectively. Data represent percent of animals per group with positive PCR.

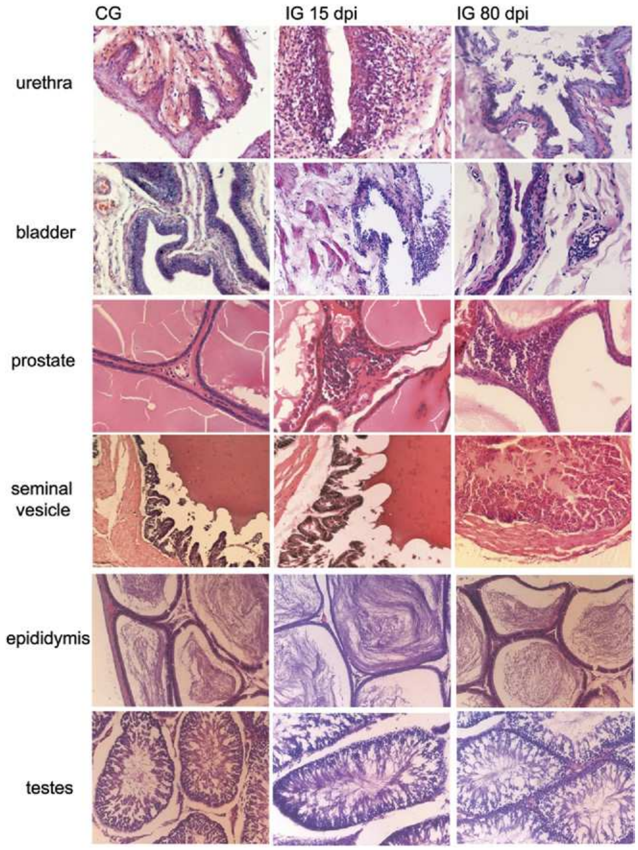

Figure 2. Representative tissue sections show histopathology of urethra, bladder, prostate, seminal vesicles, epididymis and testes of control (*CG*) and C. muridarum infected (*IG*) sacrificed rats at 15 and 80 dpi. Tissues were removed, fixed, processed for histology and stained with hematoxylin and eosin. Note inflammatory cells in urethra, bladder and prostate of infected rats. Reduced from ×200 (*CG* and *IG 15 dpi*) and ×400 (*IG 80 dpi*).

analyzed, no differences were found between sham treated and infected animals.

These results indicate that C. muridarum infection stimulates the production of a specific immune response that increases throughout the course of the infection.

Histopathological and Histochemical Analyses

Histological analysis was performed in the urethra, bladder, prostate, seminal vesicles, epididymis and testes of infected and sham treated rats (fig. 2). At 15 dpi the urethra from 50% of infected rats showed focal areas with acute interstitial and epithelium inflammatory infiltrates, mainly consisting of PMN neutrophils. The bladder from 33% and 16% of infected rats showed focal acute interstitial inflammatory and epithelial desquamation, respectively. At 15 dpi prostate sections from infected rats showed important alterations with interstitial inflammatory infiltration in 83%. Inflammatory infiltration was mainly composed of MNCs with PMN neutrophils. Prostate epithelial desquamation and inflammatory exudates were consistently observed in a significant fraction of animals. Although C. muridarum was detected in 33% of seminal vesicle samples at 15 dpi, we did not observe any morphological change or alteration in this tissue. Also, no significant morphological changes were detected in the epididymis or testes of infected rats at 15 dpi.

At 80 dpi histopathological analysis of the urethra and bladder from infected rats showed weak interstitial infiltration in 14% and 28% of samples, respectively. However, severe alterations were seen in prostate and seminal vesicles, which were more prominent in the prostate (fig. 2). Prostate alterations mainly consisted of MNC infiltrates in 37.5% of samples, accompanied by epithelial cell desquamation. Seminal vesicle tissue showed alterations, consisting of epithelial cell desquamation in 60% of infected rats and PMN cell infiltration in 12%. No morphological changes were noted in the epididymis or testes.

When the infiltration was characterized, few CD11b+, CD3+, CD4+ and CD8+ cells were detected in the prostate of sham treated animals. In contrast, prostate infiltrates in infected rats were composed of abundant CD3+ cells with high staining for CD8+ and CD4+ cells (fig. 3). No differences were noted between infected and control glands when CD11b+ cells were analyzed (data not shown).

These results indicate that histological alterations accompanied C. muridarum infection. These alterations were especially present in the prostate gland after C. muridarum inoculation and were mainly composed of CD3+ cell infiltration.

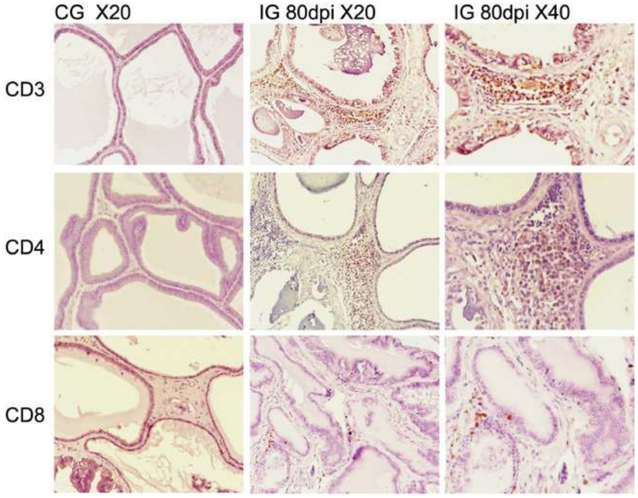

Figure 3. Immunohistochemical analysis of CD3, CD4 and CD8 in prostate of C. muridarum infected (*IG*) and control (*CG*) rats at day 80pi.

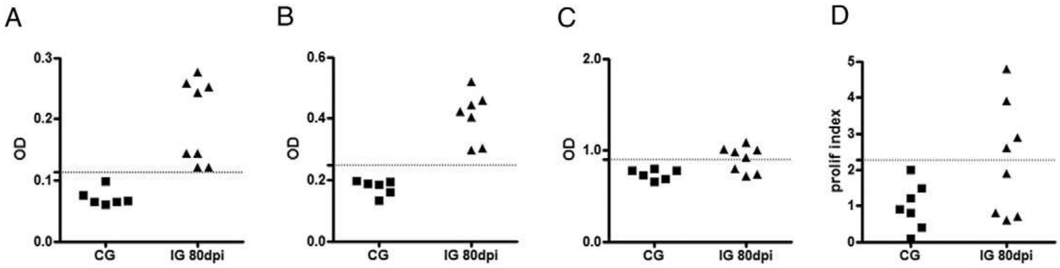

Figure 4. Autoreactive humoral and cellular immune response against prostate antigens in control and infected rats. *A* to *C*, ELISA reveals serum antibody levels against prostate homogenate, PSBP and PAP at 80 dpi. *A*, IgG against PSBP. *B*, IgG against PE. *C*, IgG against PAP. Serum was diluted 1:100 for ELISA. *D*, lymphoproliferative index of lymph node MNCs cultured with PSBP. Dotted lines indicate mean +3 SD. *CG*, control. *IG*, infected.

Self-Reactivity to Prostate in C. Muridarum Infected Male Animals

EAP can be induced in Wistar but not Sprague-Dawley rats, showing the importance of the genetic background for autoimmune prostatitis.[15–18] Also, NOD mice are susceptible to EAP, showing more severe disease.[17,18]

Since persistent C. muridarum infection in prostate tissue caused marked infiltration, similar to that reported for EAP,[17] we then determined whether infected and sham treated animals of susceptible strains (Wistar rats and NOD mice) showed evidence of immune reactivity against the prostate.

We noted higher levels of autoantibodies against different prostate autoantigens, such as PSBP, PE and PAP, in infected rats (fig. 4). Positive PSBP and PE specific antibody levels were found in 100% of infected rats and PAP specific IgG was found in 62.5%. When we analyzed the lymphoproliferative response against PSBP, which is the major autoantigen described in EAP, 50% of infected rats showed positive results (fig. 4, *D*). Those with the most severe histological alterations also showed higher cellular and humoral autoreactivity to prostate antigens.

We also investigated whether the prostate alterations observed in infected rats would be detected in NOD mice. Mice were also infected in the meatal urethra with C. muridarum and bacteria were searched for by PCR. At early and late experimental time points (15 and 60 dpi) C. muridarum was detected in 40% and 100% of prostate samples, respectively (data not shown). Histological analysis revealed higher histological alterations, mainly composed of MNC infiltration (fig. 5). As expected, sham treated mice did not show C. muridarum DNA or histological alterations in the prostate. In addition, PSBP specific antibodies were detected in serum from infected mice at early and late dpi while sham treated mice showed no reactivity (fig. 6).

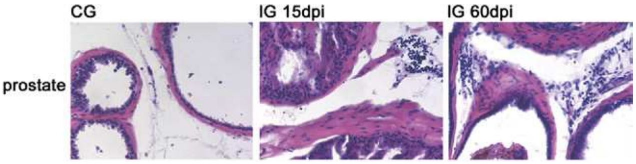

Figure 5. Representative tissue sections from infected (*IG*) and control (*CG*) NOD mice show prostate histopathology after C. muridarum infection. Prostate was removed, fixed and processed for histology. Note inflammatory cells in prostate of infected mouse. H & E, reduced from ×40.

Together these results represent evidence that C. muridarum infects the rodent male genitourinary tract and persists in the prostate gland, where it causes chronic inflammation. This in turn may promote self-immune reactions in susceptible hosts.

DISCUSSION

Although most chlamydial associated morbidity has been described in women, men provide a reservoir for continued transmission of new and recurrent

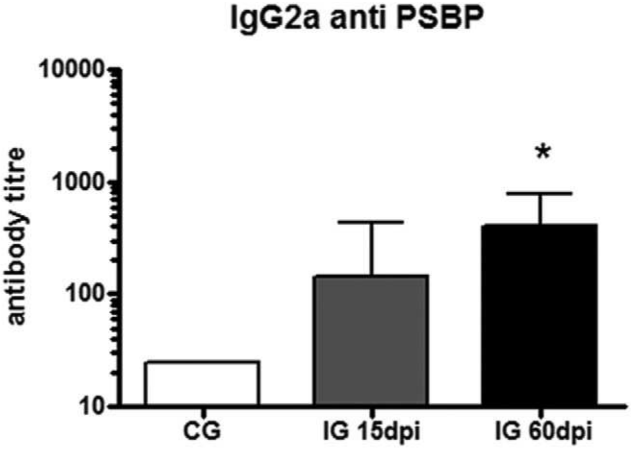

Figure 6. ELISA reveals serum antibody levels against purified antigen PSBP in control (*CG*) and infected (*IG*) NOD mice at 15 and 60 dpi. Serum was diluted 1:100 for ELISA. Asterisk indicates $p < 0.05$ vs control.

infections among women. Male genital tract organs are potential targets of chlamydial pathology that may alter male fertility.[6] Assessing the duration of infection in humans is difficult since the onset is generally unknown, re-exposure is common and bacterial clearance is rarely followed.[19,20] Experimental models of infection may provide answers to many existing questions but animal models of chlamydial male genitourinary infection are scarce[21–23] and to our knowledge there is no available bibliography on in vivo prostate chlamydial infection models.

Our results reveal that C. muridarum produces ascending infection from the rat meatal urethra with wide bacterial dissemination at early dpi. C. muridarum was detected in the urethra, bladder, prostate and seminal vesicles at 15 dpi. However, 11 weeks later bacteria only persisted in some parts of the male rat genital tract, especially the prostate gland. The presence of C. muridarum in the urethra and bladder was accompanied by focal inflammatory infiltrates and epithelial desquamation. These results are in agreement with those reported by Wang[21] and Pal[22] et al. In the latter study C. muridarum was inoculated in the meatal urethra of mice and detected in the urethra and bladder within 1 to 4 weeks after inoculation. C. muridarum was present in the epididymis and testes of some mice 2 weeks after inoculation. The bacteria cleared by 42 dpi in wild-type mice. Pal et al also reported the presence of specific antibodies, which were first detected in animal serum at 21 dpi and increased until 42 dpi. Unfortunately they did not analyze the presence of C. muridarum in the prostate and seminal vesicle. Other in vivo chlamydial genitourinary infection models have been reported but in these studies chlamydial prostate infection was not assessed.[24–26]

In our current series and in agreement with the study by Pal et al,[22] we noted that C. muridarum in the male rodent genital tract also stimulated the production of a specific immune response, which increased throughout the infection. C. muridarum persisted in the prostate gland, particularly late after infection. This presence was accompanied by significant histological alterations, such as MNC and PMN cell infiltration. The infiltration was composed mostly of CD3+ cells with high staining for CD8+ and CD4+ cells. This infiltration may have been a response to the chronic permanence of the bacteria and/or autoreactivity to the prostate gland.

Our group previously reported that prostate epithelial cells are susceptible to C. muridarum in vitro infection and respond with up-regulating chemokine and inflammatory cytokine genes,[11] which could be related to the infiltrates detected in the current in vivo study. Also, Al-Mously et al reported that human urethral epithelial cells and normal adult prostate epithelial cells are susceptible to C. trachomatis infection and secrete significant amounts of interleukin-8 and this effect was more evident for prostate cells.[27]

Our experimental model also allowed us to advance the study of the possible consequences of chlamydial infection on autoimmunity prone hosts. An important proportion of infected animals showed self-reactivity to prostate antigens after C. muridarum infection. We previously reported that Wistar rats and NOD mice had autoimmune prostatitis after immunization with prostate antigens plus adjuvants.[16,17] This fact implies that autoreactive lymphocytes are present in the immune repertoire of these animals and are kept under control in normal conditions. However, after immunization or possibly after chronic chlamydial infection accompanied by chronic inflammation the onset of an autoimmune process could be triggered.

Diverse human pathological conditions, including autoimmune components, are associated with previous infection.[28] Available information on chlamydia suggests that the concurrence of persistent chlamydial infection and heat shock protein 60 self-immune responses can promote autoimmune aggression toward stressed cells and the development of diseases such as autoimmune arthritis, multiple sclerosis, atherosclerosis, vasculitis, diabetes and thyroiditis, among others.[29,30] However, to our knowledge this is the first time that chlamydia has been associated with autoimmune prostatitis in a rodent model of chlamydial male urogenital infection. As is well known, autoimmune diseases are multifactorial, and different genetic (major histocompatibility complex related and nonmajor histocompatibility complex related), endocrine and environmental factors determine susceptibility. Several mechanisms that are often used to explain the association of autoimmunity and microbial infection include molecular mimicry, bystander activation with or without epitope spreading and microorganism persistence. In conclusion, our results prompt us to postulate that during the course of C. muridarum male rodent urogenital tract infection the special tropism of these bacteria for the prostate gland and its continuous presence together with cytokine and chemokine production would induce chronic inflammation, which would evolve into the onset of an autoimmune process in genetically susceptible hosts.

ACKNOWLEDGMENTS

C. muridarum MoPn was provided by K. H. Ramsey, United States of America. Recombinant MBP-MOMP fusion protein was provided by Kenneth W. Beagley, University of Newcastle, Newcastle, New South Wales, Australia. Plasmid coding for OMP2 was provided by Prof. J. S. H. Gaston, Addenbrooke's Hospital, Cambridge, United Kingdom.

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