Published in final edited form as:

Vaccine. 2008 October 23; 26(45): 5741–5751. doi:10.1016/j.vaccine.2008.08.020.

Local and humoral immune responses against primary and repeat *Neisseria gonorrhoeae* genital tract infections of 17 β -estradiol-treated mice

Wenxia Song^{a,*}, Sara Condrón^a, Brian T. Mocca^b, Sandra J. Veit^b, Dawn Hill^a, Asima Abbas^a, and Ann E. Jerse^{b,**}

^aDepartment of Cell Biology and Molecular Genetics, University of Maryland, College Park, Maryland 20742, United States

^bDepartment of Microbiology and Immunology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, Maryland 20814, United States

Abstract

The 17 β -estradiol-treated mouse model is the only small animal model of gonococcal genital tract infection. Here we show gonococci localized within vaginal and cervical tissue, including the lamina propria, and high numbers of neutrophils and macrophages in genital tissue from infected mice. Infection did not induce a substantial or sustained increase in total or gonococcal-specific antibodies. Mice could be reinfected with the same strain and repeat infection did not boost the antibody response. However, intravaginal immunization of estradiol-treated mice induced gonococcal-specific primary and secondary serum antibody responses. We conclude that similar to human infection, experimental murine infection induces local inflammation but not an acquired immune response or immunological memory.

Keywords

Gonorrhea; Mouse model; Humoral response

1. Introduction

Gonorrhea ranks high in public health importance among sexually transmitted infections with an estimated 62 million cases worldwide [1] and ca. 350,000 reported cases in the United States each year [2]. Uncomplicated lower urogenital tract infections involve the urethra or cervix. Other commonly infected mucosal sites include the rectum and pharynx. Ascended gonococcal infections frequently occur, particularly in women, and the most significant morbidity and mortality caused by *Neisseria gonorrhoeae* is due to pelvic inflammatory disease and the post-infection complications associated with scarring of the fallopian tubes. Dissemination of *N. gonorrhoeae* to the skin and joints via the bloodstream occurs in 0.5–3% of urogenital tract infections [3]. Concern over the high incidence of gonorrhea is intensified by the rapid emergence of antibiotic resistant strains [4], which threatens current control strategies and the fact that gonorrhea is a co-factor in the spread of human immunodeficiency virus [5].

The development of a gonorrhea vaccine is challenged by the antigenic variability of the gonococcal surface and a lack of understanding of the immune response that is required to effectively block or attenuate infection. The hallmark of symptomatic *N. gonorrhoeae* urogenital infections is an acute purulent discharge characterized by numerous polymorphonuclear leukocytes (PMNs) that contain intracellular diplococci, extracellular gonococci, and desquamated epithelial cells [6]. Asymptomatic infections are also common, with approximately 50% of infections in women silent [3]. The host immune response to *N. gonorrhoeae* infection is not well defined, and while gonococcal-specific antibodies are detected in patients with uncomplicated gonococcal infections, titers in general are low and a high percentage of subjects do not have detectable antibodies [7–13]. Natural infections do not induce a protective response, even with the same strain [14] or serovar [15–17] although there is evidence that repeated infections may induce partial strain-specific immunity [18,19].

Mechanistic studies on the host response to *N. gonorrhoeae* have been hindered by the lack of an animal model with which one can manipulate the host response and perform controlled studies with defined strains. Although several host restrictions limit the use of laboratory mice as a surrogate host for human infection, female mice are transiently susceptible to *N. gonorrhoeae* colonization during the early proliferative stage of the estrous cycle when estradiol levels are high and commensal flora are low [20–22]. Prolonged colonization can be obtained through the administration of exogenous 17 β -estradiol and the use of germ-free mice [23] or antibiotics [24] to reduce the overgrowth of commensal flora that occurs under the influence of estrogen. An influx of vaginal PMNs occurs in ca. 50% of mice infected with *N. gonorrhoeae* based on cytological differentiation of stained vaginal smears, and high numbers of gonococci are recovered from vaginal mucus during periods of inflammation [24,25]. Here we analyzed the localization of bacteria within genital tract tissues and the immune responses to primary and repeat infections to further define the usefulness of the 17 β -estradiol-treated mouse model in pathogenesis and host response studies. Similar to that which occurs during human infections, we demonstrated that mice were susceptible to repeat infection by the same strain and that repeat infection failed to induce a significant secondary antibody response.

2. Materials and methods

2.1. Bacterial strains and culture conditions

N. gonorrhoeae strain FA1090 is a serum-resistant PorB.1B, streptomycin resistant (Sm^R) strain [26] and the only gonococcal strain for which a complete genome sequence is currently available. An OpaB-expressing variant of strain FA1090 (var. A30) with piliated colony morphology was used in all experiments, the frozen stock of which was prepared from a subculture of a single urine isolate from a male volunteer who was experimentally infected with strain FA1090 [27]. *N. gonorrhoeae* was cultured on GC agar with Kellogg's supplement I and 12 μ M Fe(NO₃)₃ at 37 °C in 7% CO₂. GC-VCNTS agar [24] and heart infusion agar were used to isolate *N. gonorrhoeae* and facultatively anaerobic commensal flora from vaginal mucus, respectively. All media and antibiotics were from Difco.

2.2. Experimental murine infection

Female BALB/c mice (6–8 weeks old) (NCI, Bethesda, Maryland) in the diestrus or anestrus stages of the estrous cycle were treated with 17 β -estradiol to promote long-term susceptibility to *N. gonorrhoeae*. Two estradiol treatment regimens were used, the first of which was implantation of a 5mg 21 day slow-release 17 β -estradiol pellet (Innovative Research of America) under the skin as described [24]. Two days after pellet implantation, mice were inoculated intra-vaginally with 10⁶ colony forming units (CFU) of *N.*

gonorrhoeae suspended in 20 μ l of phosphate buffered saline (PBS) (test group) or PBS alone (control group). A modified protocol was also used in which 0.5 mg of a water soluble form of estradiol, 17 β -estradiol_{ws} (Sigma) was delivered subcutaneously on days -2, 0, and +2 with respect to the day of bacterial challenge (day 0). Mice were inoculated intravaginally with 10⁶CFU of *N. gonorrhoeae* or PBS (control) as above. For both protocols, antibiotics were administered to prevent commensal flora overgrowth as described [28]. Vaginal mucus from test mice was quantitatively cultured on GC-VCNTS agar every day for 10–12 consecutive days; vaginal mucus from test and control mice was also smeared onto glass slides, stained, and the number of PMNs among 100 vaginal cells was determined. Inoculum preparation, quantitative culture for *N. gonorrhoeae*, and the monitoring of facultative anaerobic commensal flora were as described [24,28]. Occasional mice developed an overgrowth of inhibitory commensal flora during the experiments (i.e. members of the *Enterobacteriaceae* or *Pseudomonas aeruginosa*); these mice were not used in data analysis as their immune response may have been unrelated to gonococcal infection.

2.3. Immunohistochemistry

At days 5 and 10 post-inoculation, the entire genital tracts were surgically removed from test and control mice and either embedded in paraffin after fixation in 10% neutral buffered formalin or in TissueTek OCT compound (Sakura Finetek USA, Torrence, CA) followed by snap freezing. Tissue sections (5–7 μ m) were processed as follows. Paraffin sections were mounted on slides, washed with xylene, and rehydrated with descending grades of ethanol. Cryosections were air dried at room temperature and fixed in acetone. Endogenous peroxidase activity was blocked with 0.3–0.6% H₂O₂ in methanol for 30 min. Nonspecific binding was minimized by blocking the sections with 0.1% bovine serum albumin (BSA) and 1% fetal bovine serum (FBS) in PBS. The sections were then incubated with monoclonal antibody (mAb) H5, which is specific for FA1090 porin (kindly provided by Janne Cannon, University of North Carolina), anti-F4/80 mAb (Accurate Chemical and Scientific Co., Westbury, NY) for macrophages, anti-Gr-1 mAb (BD Pharmingen, San Diego, CA) for PMNs, or anti-mouse IgG, IgM or IgA antibodies (Southern Biotechnology Associates, Birmingham, AL) followed by the corresponding horse radish peroxidase (HRP)-conjugated secondary antibodies. Antibody staining was visualized using HRP substrate 3,3'-diaminobenzidine. For background controls, primary antibodies were either omitted or substituted with isotype control antibodies. No significant background staining was detected. Sections were analyzed under a light microscope and positively stained cells from at least five randomly selected fields viewed at 100 $\times$ magnification were counted. The mean values of cell numbers for each experimental group were calculated.

2.4. Analyses of serum and mucosal antibodies

Venous blood was collected from mice on days 5 and 10 post-inoculation with bacteria or PBS, and serum was separated and stored at -20 °C. Vaginal washes were collected at the same time points by pipetting 50 μ l PBS in and out of the vagina twice and pooling the two samples from each mouse. Vaginal washes were centrifuged to remove debris, and the protease inhibitor phenyl-methyl-sulfonyl-fluoride (10 mM final concentration) was added to prevent protein degradation. Gonococcal-specific and total IgG, IgM, and IgA in sera and vaginal washes were measured by ELISA. Briefly, 96-well plates (Nalgene Nunc, Rochester, NY) were coated either with affinity-purified goat anti-mouse IgG, IgM, or IgA antibody (Southern Biotech) or outer membranes (OMs) from *N. gonorrhoeae* FA1090 (1 μ g/ml) in coating buffer (50 mM sodium carbonate/bicarbonate buffer, pH 9.6) overnight at 4°C. OMs were prepared essentially as described [29,30]. After washing, the sera or vaginal wash samples were added in triplicate wells and known concentrations of affinity-purified mouse IgG, IgM, IgA or the H5 mouse monoclonal antibody were used to establish standard curves. Bound antibodies were detected by HRP-conjugated goat anti-mouse IgG or IgM

antibodies and the HRP substrate 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma). Absorbance was read at 405 nm on a 96-well plate reader (BioRad). For immunization studies, the titers of gonococcal-specific antibodies in serum or vaginal washes were measured via a standard ELISA protocol. Briefly, 96-well plates were coated with OM (0.5 µg), and incubated in PBS containing 0.05% Tween 20 and 15% FBS to block nonspecific binding of antibody. Sera and vaginal washes were serially diluted and added to each well. HRP-conjugated polyclonal goat anti-mouse IgG + M (Fitzgerald Industries International, Concord, MA) or HRP-conjugated goat polyclonal anti-mouse IgA (α chain-specific) (Sigma) were used as secondary antibodies. Sera, vaginal washes and all antibodies were diluted in PBS with 0.05% Tween 20. The addition of HRP substrate was as described above. The reactions were stopped with an equal volume of 1N H₂SO₄ and plates were read at 405 nm. Titers were defined as the highest dilution of a sample that gave a value that was ≥3 standard deviations above the average A₄₀₅ reading of the secondary antibody control wells.

2.5. Reinfection studies

For reinfection studies, 17β-estradiol_{ws}-treated mice were inoculated with *N. gonorrhoeae* (test group) or saline as described above (round one). Vaginal mucus was cultured from test mice every day for 10 consecutive days. After the day 10 culture, all mice were treated with ceftriaxone (300µg in a 100 µl volume, intraperitoneally) and vaginal mucus was cultured the following day to confirm clearance of infection. Four weeks after ceftriaxone treatment, mice in the diestrus or anestrus stages of the cycle were identified in each group and given three doses of 0.57 mg of 17β-estradiol_{ws} at the time points that correspond to days -2, 0, and +2 of the round two infection protocol. The dose of estradiol was increased for round two infections to account for the average weight gain between challenges. On day 0, test mice (previously infected) and control mice (naïve) were inoculated with 10⁷ CFU from the same stock of FA1090 bacteria that was used in the first round of infection. Vaginal mucus from all mice was cultured for *N. gonorrhoeae* for 10 consecutive days to determine the average duration of recovery and colonization load. The percentage of PMNs among 100 vaginal cells in stained vaginal mucus from test and control mice in each round of infection was determined. Blood and vaginal washes were collected for antibody measurement from test and control mice on days 5 and 10 as described above. All experiments were performed at least twice and the results were reproducible.

2.6. Immunization of estradiol-treated and untreated mice

Female BALB/c mice were given 0.5 mg 17β-estradiol_{ws} three times and antibiotics as described above and immunized intranasally as described [30] or intravaginally with FA1090 OMs (20 µg of protein) after the second dose of estradiol. This time point corresponds to the day of bacterial challenge for the infection studies. Groups of 4–5 untreated mice (no estradiol) of the same age were immunized in parallel as controls. All mice were treated with ceftriaxone 10 days after the first immunization to mimic the reinfection protocol. Four weeks later, mice were treated with antibiotics and 0.57 mg of 17β-estradiol_{ws} three times as above or left untreated, and re-immunized on the day that corresponded to the second estradiol injection via the same delivery route as that used in the primary immunizations. Sera and vaginal washes were collected 10 days after each of the two immunizations for detection of OM-specific antibodies by ELISA.

2.7. Serum estradiol levels

Venous blood was collected via retro-orbital bleeding from uninfected mice treated with the 5 mg slow-release 17β-estradiol pellet or 17β-estradiol_{ws}. For pelleted mice, blood was collected 2, 8, 14, 28, and 36 days following pellet implantation (*n* = 5 mice per time point). For mice treated with 17β-estradiol_{ws}, blood was collected at 6 and 24 h after the second

dose of estradiol_{WS}, which corresponds to the time of bacterial challenge and day 1 of infection, respectively. Blood was also collected immediately before the third dose of estradiol_{WS} and at 6, 24, 48, 96, and 144 h afterwards ($n = 3$ mice per time point); these latter time points correspond to days 2, 3, 4, 6, and 8 of infection of the modified infection protocol. No mouse was used for two consecutive time points or more than twice in either experiment. Serum estradiol levels were measured by ELISA (IBL America, Fitzgerald Industries International) as per the manufacturer's instructions.

2.8. Statistical analysis and animal use assurances

An unpaired student t test was used to analyze differences in the average duration of infection, percent of PMNs in vaginal smears, number of PMNs and macrophages in tissue, and concentration of antibody in serum and vaginal washes. The average colonization load over time was analyzed by a repeated analysis of variance (ANOVA). All animal experiments were conducted in the laboratory animal facility at the Uniformed Services University, which is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care under a protocol approved by the University's Institutional Animal Care and Use Committee.

3. Results

3.1. Localization of gonococci within mouse genital tract tissue

We previously reported that gonococci are associated with squamous and columnar epithelial cells and within PMNs in stained vaginal smears from infected mice treated with a 5 mg, 21 day slow-release 17 β -estradiol pellet [24,25]. To further characterize this infection model, here we analyzed the distribution of bacteria within the genital tract tissue of infected mice. Groups of 6–10 estradiol-treated mice were inoculated with FA1090 bacteria or PBS and sacrificed on days 2 and 5 post-inoculation. The distribution of bacteria in genital tissue was examined by immunohistochemistry. Gonococci were detected within the vaginal lumen and in vaginal and cervical tissue where the bacteria appeared to be associated with epithelial cells and within PMNs (Fig. 1A–C). Occasional gonococci were also seen in the lamina propria (Fig. 1B and D). No bacteria were detected in the upper genital tract. Quantitation of the number of bacteria seen per 100 $\times$ oil immersion field confirmed that the majority of the gonococci were found in the vaginal lumen. However, significant numbers of gonococci were also observed within lower genital tract tissue at 2 and 5 days after intravaginal inoculation (Fig. 1E). These data confirm that intravaginally inoculated gonococci establish themselves within both vaginal and cervical tissues of the lower genital tract of estradiol-treated mice and beyond the association with mucous overlaying the epithelial cell layer.

3.2. Local inflammatory response to genital tract infection

To follow the local inflammatory response to *N. gonorrhoeae*, we determined the number of PMNs in vaginal smears and genital tract tissue. PMNs were first identified by their nuclear morphology, and based on this method, more PMNs were found in the genital tissues from infected mice compared to tissues from uninfected mice (Fig. 2). To further determine the types of leukocytes recruited to the infection sites, we stained neutrophils and macrophages with Gr-1- or F4/80-specific antibody, respectively. The numbers of Gr-1-positive cells were significantly increased in both the vaginal and cervical tissues of infected mice in comparison to uninfected mice (Fig. 3A–C). The number of F4/80-positive cells was also increased, but the difference between infected and uninfected mice was only significant in the vaginal lumen on day 5 post-infection (Fig. 3D–F). The demonstration that *N. gonorrhoeae* induced recruitment of PMNs to infection sites, particularly Gr-1-positive neutrophils, indicates the induction of an inflammatory response.

3.3. Humoral immune response to experimental *N. gonorrhoeae* infection

Mucosal antibody provides an essential immune protection against bacterial infection. To test whether gonococcal infection of the lower genital tract of female mice induces an antibody response, we determined the relative levels of gonococcus-specific and total IgG, IgM, and IgA in sera and vaginal washes of infected and uninfected mice. Gonococcal infection generally increased gonococcus-specific IgG in vaginal washes by two- to three-fold at 10 days post-inoculation (Fig. 4A, panel a), while gonococcus-specific IgA in vaginal washes was slightly increased at 5 days post-inoculation (Fig. 4A, panel c). The concentrations of IgM in vaginal washes were very low, and no significant increase in vaginal IgM was detected (Fig. 4A, panel b). Total IgG and IgA were increased in vaginal washes from infected mice compared to those of uninfected controls on 10 day post-inoculation but there was no difference in the level of total IgM in vaginal washes (data not shown).

Infected mice also demonstrated an increase in serum antibodies, with a two- to three-fold higher concentration of gonococcus-specific IgG and IgM (Fig. 4A, panels d and e) and total IgG and IgM (data not shown) detected 5 days post-inoculation. Interestingly, levels of gonococcus-specific and total IgG and IgM returned to control levels by 10 days post-inoculation (Fig. 4A, panels d and e). Consistent with ELISA results, immunohistochemical analysis on tissue harvested on day 5 post-inoculation showed a significant increase in total IgG, but no significant increase in total IgM in the genital tissue in comparison with those of uninfected mice (Fig. 4B). In summary, these data show that gonococcal infection of the murine lower genital tract increased gonococcus-specific antibody on both the mucosal surface and in sera; however, these increases were transient and unsubstantial.

3.4. Kinetics of infection in mice treated with 17 β -estradiol_{ws}

To study the antibody responses to repeat gonococcal infection, we modified the estradiol treatment to use a water soluble form of 17 β -estradiol so that the normal mouse reproductive cycle would resume more quickly and thus facilitate re-challenge studies. Three doses of 0.5 mg of 17 β -estradiol_{ws} were given every other day (total dose, 1.5 mg). Serum estradiol concentrations were 8- and 20-fold higher than normal proestrus-stage levels when measured 6 h after the second and third dose of 17 β -estradiol_{ws}, respectively. Consistent with its water-soluble nature, concentrations declined to physiological levels within 24 h after each injection and remained at normal levels from the time point that corresponds to day 3 of infection onwards (Fig. 5A). In contrast, use of the slow-release pellet (total dose, 5 mg) resulted in a sustained increase in serum estradiol levels. Estradiol levels were 10–15-fold higher than normal proestrus-stage concentrations until 10 days after pellet implantation, at which point the estradiol levels began to decline. Normal proestrous-stage levels were detected 28 days after pellet implantation (Fig. 5B).

The effects of estradiol treatment were further monitored by examining stained vaginal smears. Estradiol causes cellular proliferation, which results in a multilayered epithelial cell lining with cornified epithelial cells at the outermost layer. This histological state is reflected by a predominance of squamous epithelial cells in stained vaginal smears [31]. Consistent with the lower serum estradiol levels, the vaginal epithelia of mice subjected to the 17 β -estradiol_{ws} treatment appeared much less estrogenized than pelleted mice. This conclusion is based on the presence of few squamous epithelial cells and a predominance of nucleated epithelial cells and mucus strands in stained vaginal smears in estradiol_{ws}-treated mice at the time point that corresponds to the time of bacterial inoculation (day 0) and the first 4 days of infection. In contrast, vaginal smears from mice given the 17 β -estradiol slow-release pellet showed mostly squamous epithelial cells by day 2 or 3 after bacterial inoculation (data not shown).

The kinetics of infection in mice treated with the 17 β -estradiol_{ws} was similar to that observed in mice implanted with a slow-release pellet [24]. Mice were colonized by *N. gonorrhoeae* for an average of 9.9 days (range 0–12 days) and high numbers of gonococci (10^3 – 10^5 CFU/100 μ l vaginal swab suspension) were recovered from a majority of mice each day for over 10 days in each of two experiments (Fig. 6A). A vaginal PMN influx occurred in mice inoculated with *N. gonorrhoeae* on days 5–8 post-bacterial inoculation that was significantly higher than that seen in 17 β -estradiol_{ws}-treated mice inoculated with saline (placebo control) (Fig. 6B). The late influx of PMNs seen on day 9 in the control group and clearance of gonococci in the test group are consistent with the effects of the estradiol wearing off and transition into the metestrus and diestrus stages of the estrous cycle. Gonococci were associated with nucleated epithelial cells (Fig. 6C) and within PMNs (Fig. 6D) in stained vaginal smears from infected mice. We conclude that *N. gonorrhoeae* establishes a productive infection in female mice treated with either form of estradiol, including estradiol that is not sustained at non-physiological levels over the course of infection. The fact that estradiol_{ws} treatment results in a less keratinized vaginal epithelial cell lining is during the first 4 days infection is an additional improvement over the previous model in that squamous epithelial cells are less relevant to human infection.

3.5. Reinfection fails to induce a secondary antibody response

Repeat gonococcal infections are common in humans. To test if a primary infection induces a protective immune response in the mouse model, reinfection studies were performed in which 17 β -estradiol_{ws}-treated mice were inoculated with 10^6 CFU of *N. gonorrhoeae* strain FA1090 (test group) or PBS (naïve controls), and then treated with ceftriaxone on day 10. Four weeks later, mice in both groups were re-treated with 17 β -estradiol_{ws} and challenged with 10^7 CFU of the same strain of *N. gonorrhoeae* used in the primary infection. We found no significant difference in either the duration of infection in the test and control groups (Fig. 7A) or the average number of gonococci recovered from mice in each group over time (Fig. 7B). We also found that the average duration of reinfection in each group was shorter than that seen with the primary infection. This observation is consistent with our general observation that older mice are less susceptible to *N. gonorrhoeae* and respond less well to estradiol.

The demonstration that the age-matched estradiol_{ws}-treated control group sustained colonization with *N. gonorrhoeae* for a similar length of time as previously infected mice suggests a primary lower genital tract infection fails to establish protective immunity and that repeat genital tract infection cannot mount an effective secondary immune response or memory response, which should be faster and more robust than a primary immune response. In support of this hypothesis, we found that repeat infection with the same strain of *N. gonorrhoeae* did not significantly increase gonococcus-specific IgG antibody in sera and vaginal washes (Fig. 7C). From these results we conclude that re-exposure to *N. gonorrhoeae* does not induce a secondary antibody response, which suggests the absence of humoral immune memory.

3.6. Effect of body site on humoral response to gonococcal OM

Russell and Mestecky reported a weak antibody response to immunogens delivered intravaginally compared to other routes [32]. To test if the infection site may contribute to the poor antibody response seen during *N. gonorrhoeae* lower genital tract infection of mice, we measured serum and vaginal gonococcal-specific antibodies in 17 β -estradiol_{ws}-treated mice that were immunized intravaginally or intranasally with gonococcal OMs on the day that they would normally be inoculated with viable *N. gonorrhoeae*. To mimic the reinfection studies, all mice were treated with ceftriaxone 10 days later, re-treated with 17 β -estradiol_{ws} 4 weeks later, and immunized a second time at the time point that corresponded

to a second bacterial challenge. Mice that were not treated with estradiol (untreated mice) were immunized in parallel to measure the affect of estradiol on antibody responses. Titers of OM-specific antibodies in sera and vaginal washes 10 days after the first and second immunizations were determined by ELISA. Intravaginal immunization of both estradiol-treated and untreated mice elicited OM-specific serum IgG/M that was boosted to a higher level after the second immunization (Table 1). The serum IgG/M titer in the estradiol-treated group was significantly lower than the untreated group after the first immunization, but there was no significant difference between the two groups after the second immunization. Intranasal immunization resulted in similar levels of serum IgG/M in both estradiol-treated and untreated mice. The serum titers of OM-specific IgG/M induced by intranasal immunization were significantly higher after the second immunization than that induced by intravaginal immunization. Furthermore, OM-specific vaginal IgG/M and IgA were only detected in vaginal washes of the intranasal groups, and not the intravaginal group. These results suggest that estradiol treatment may reduce the primary antibody response in the vagina. Additionally, the lower antibody titers in mice immunized intravaginally suggest the infection site is at least partially responsible for the poor immune response to *N. gonorrhoeae* observed during experimental murine infection. We cannot rule out the possibility that *N. gonorrhoeae* possesses mechanisms for actively evading induction of the adaptive immune response, however, based on our finding that intravaginal immunization with gonococcal OM, but not lower genital tract infection with live bacteria increased serum IgG/M.

4. Discussion

Gonorrhea is one of the most common sexually transmitted infections in the world. The success of this well adapted pathogen is facilitated by the high incidence of repeat infection. Information on the immune response to *N. gonorrhoeae* is currently based mainly on the analysis of clinical specimens and may be confounded by several variables. For example, it has been documented that gonococcal-specific IgA [13] and IgG [33] decline in infected individuals over time; therefore, variability in the length of time between the occurrence of infections and the collection of samples may affect the data. The influence of previous gonococcal infections or coinfection with other sexually transmitted pathogens can also not be controlled in clinical studies, and strain-specific differences might also influence the induction of an immune response. The immune responses of women to *N. gonorrhoeae* infections are further complicated by variability in the subjects' hormonal state. Studies with defined strains have been performed in male volunteers. In this human urethritis model, subjects showed a proinflammatory cytokine response to *N. gonorrhoeae* [34], which is consistent with the development of a urethral exudate that contains PMNs [26,35]. Schmidt et al. [36] showed no protection against reinfection of volunteers with the same strain. Pre-existing antibodies that recognized gonococcal proteins were detected in many subjects; however, a significant increase in antibody specific for lipooligosaccharide was observed post-infection, which was significantly associated with protection from reinfection.

Currently, the only small animal model of gonococcal genital tract infection is the 17 β -estradiol-treated mouse model. Here we defined the usefulness of this model for studying host responses to *N. gonorrhoeae* and addressing immunological questions in the context of a female mammalian host. Our goal was three-fold. First, we further characterized the mouse model in terms of localization of bacteria within genital tract tissue and the cellular response to infection. Gonococci were found deep within genital tract tissue including in the lamina propria, and the infected regions included both vaginal and cervical tissue. Infection induced inflammation in the vaginal and cervical tissue, which was characterized by mostly neutrophils. Based on these results, we conclude the 17 β -estradiol mouse model is a lower genital tract infection model rather than a vaginal colonization model. The mechanism by

which gonococci penetrate murine tissue is not yet known; the bacterial adhesins and host receptors responsible for gonococcal association with murine genital tract epithelial cells have also not been identified. Female mice are limited as surrogate hosts for gonorrhea by the absence of several adherence and invasion pathways identified using cultured human cells. These host-restricted pathways include pilus-mediated adherence, which may occur via the CD46 receptor [37], opacity protein-mediated adherence and invasion via binding to human carcinoembryonic cellular adhesion molecules (CEACAMs)[38], and entry via the human CR3 receptor via a cooperative interaction with LOS, pili, and porin [39]. The host specificity of other known adhesins has not been tested. For example, we recently reported that an *ompA* mutant of *N. gonorrhoeae* was attenuated for murine infection; this protein mediates adherence to and invasion of human cervical and endometrial tissue culture cells [40].

Our second objective was to characterize the humoral response to infection. Infection did not induce substantial antibody responses as indicated by a slight increase in gonococcus-specific IgG, IgM, and IgA in vaginal washes and/or sera. The unremarkable antibody response seen during murine infection is consistent human infection. Most studies with naturally infected patients report the detection of gonococcal-specific antibodies in a proportion (18–50%) of serum samples [8,11,12], urethral exudates [9,10,13], vaginal washes, and cervical secretions [7,8,10,13,41]. Hedges et al. [42], however, found no difference in the levels of gonococcal-specific antibodies in serum or cervical lavage fluid from infected women versus those in uninfected patients. The discrepancy between these data may be due to variability of patients, different target antigens, and different methods used to measure antibody levels.

The third goal of this study was to test whether repeat infections can establish protective immunological memory against *N. gonorrhoeae*. We demonstrated that mice have a similar susceptibility to reinfection with the same strain compared to age-matched, estradiol-treated controls. Furthermore, repeat infection one month after the initial infection did not boost the titer of gonococcal-specific IgG antibodies. These results indicate that repeat infections fail to generate a protective immune response and humoral immune memory. There are several possible explanations for the incapability of gonococci to induce protective immune responses in the female lower genital tract. First, female genital tract tissue may be generally immunosuppressed to accommodate semen and commensal bacteria. Toll-like receptors (TLRs) are a family of pattern recognition receptors that bind to surface molecules of microbial pathogens and initiate innate immune responses, and although recently controversial [43], TLRs are thought to serve as adjuvant for the acquired immune responses [44]. TLR1–6 can be detected in the human female genital tract by both RT-PCR and immunohistochemistry [45], and TLR1–9 was detected in the female mouse genital tract by RT-PCR [46,47]. However, a much lower level of TLR4, which binds to lipopolysaccharide, a major bacterial surface molecule, is present in tissues of the female lower genital tract compared to the upper genital tract [45,47]. A lesser amount of TLR4 would desensitize the genital mucosal surface from responding to colonization by Gram-negative bacteria. Recent studies suggest that regulatory T cells play an essential role in down-regulation of autoantigen-induced immune responses in the intestine [48], which may also provide a mechanism for suppressing the central immunity to response to bacterial genital infection.

Second, the lack of organized lymphoid tissues in the female genital tract like the intestinal Peyer patches could prevent effective induction of immune responses, especially the acquired immune response. Consistent with the absence of organized lymphoid tissues in the vagina, we did not detect vaginal IgA or IgG/M antibodies in mice immunized intravaginally with gonococcal OMs. In contrast, mice that received intranasal immunizations had detectable vaginal antibodies. Serum antibody levels were increased

following immunization by either delivery route. Third, hormonal fluctuations during the reproductive cycle could influence immune responses against *N. gonorrhoeae*. The use of exogenously administered estradiol to promote long-term colonization of mice could also alter the adaptive immune response to infection. With the exception of a lower serum IgG/M response in estradiol-treated versus untreated mice 10 days after the first intravaginal immunization, however, we did not find any evidence that the estradiol suppressed the antibody response to *N. gonorrhoeae*. Last, gonococci may interfere with host immune responses. Phase variation of surface molecules limits the recognition of gonococci by specific immunity. *N. gonorrhoeae* also inhibits the activation of T [49] and B lymphocytes [50] *in vitro*, including suppression of CD4⁺ T lymphocytes via signaling through CEACAM1 [51]. CEACAM interactions are thought to be host-restricted and thus should not play a role in the murine response to *N. gonorrhoeae*. In summary, multiple mechanisms potentially prevent the full induction of innate immune responses by *N. gonorrhoeae* in the female lower genital tract, leading to the failure of establishing adaptive immune responses and immunological memory.

In summary, the data presented here showed that a mild inflammatory and limited humoral immune response against *N. gonorrhoeae* occurs in the murine genital tract infection model, which are similar to the immune responses observed in women with gonorrhea. The lack of an adaptive humoral response and the capacity of *N. gonorrhoeae* to cause repeat infections are intriguing and challenge the development of an effective vaccine. The mouse infection model may therefore be a valuable tool for studying the interaction of gonococci with the immune system and to search for new strategies to effectively induce the immune responses against *N. gonorrhoeae*, especially on the genital mucosal surface. The human immune system has several similarities to that of mice in general. However, specific differences between humans and mice should be carefully considered. Of particular importance to host response studies are host restrictions in the complement cascade [52,53] that may increase the susceptibility of strains with a porin-mediated serum resistant phenotype to complement-mediated killing in the mouse model. The use of transgenic technology to provide humanized mice for future studies should improve this animal infection model.

Acknowledgments

Funding for this work was provided to A.E.J. by grant RO1-AI 42053 and U19 AI31496 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health. Funding to W.S. was provided by grant RO1-AI68888 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health. S.C. and D.H. were supported by undergraduate research fellowships from Howard Hughes Medical Institute.

We thank Lotisha E. Garvin for assistance with preparing this manuscript.

References

1. Gerbase AC, Rowley JT, Heymann DH, Berkley SF, Piot P. Global prevalence and incidence estimates of selected curable STDs. *Sex Transm Infect* 1998;74:S12–S16. [PubMed: 10023347]
2. McNabb SJ, Jajosky RA, Hall-Baker PA, Adams DA, Sharp P, Worshams C, et al. Summary of notifiable diseases—United States, 2006. *MMWR Morb Mortal Wkly Rep* 2008;55(March (53)):1–92. [PubMed: 18354375]
3. Hook, EW.; Handsfield, HH. Gonococcal Infections in the adult. In: Holmes, KKMP.; Sparling, PF.; Lemon, SM.; Stamm, WE.; Piot, P.; Wasserheit, JN., editors. Sexually transmitted diseases. New York: McGraw-Hill; 1999. p. 451–466.
4. Tapsall JW. Antibiotic resistance in *Neisseria gonorrhoeae*. *Clin Infect Dis* 2005;41:S263–S268. [PubMed: 16032562]
5. Cohen MS, Hoffman IF, Royce RA, Kazembe P, Dyer JR, Daly CC, et al. Reduction of concentration of HIV-1 in semen after treatment of urethritis: implications for prevention of sexual

- transmission of HIV-1. AIDSCAP Malawi Research Group. *Lancet* 1997;349(June (9069)):1868–1873. [PubMed: 9217758]
6. Veale DR, Goldner M, Penn CW, Ward J, Smith H. The intracellular survival and growth of gonococci in human phagocytes. *J Gen Microbiol* 1979;113(August (2)):383–393. [PubMed: 117078]
  7. O'Reilly RJ, Lee L, Welch BG. Secretory IgA antibody responses to *Neisseria gonorrhoeae* in the genital secretions of infected females. *J Infect Dis* 1976;133(February (2)):113–125. [PubMed: 812926]
  8. Ison CA, Hadfield SG, Bellinger CM, Dawson SG, Glynn AA. The specificity of serum and local antibodies in female gonorrhoea. *Clin Exp Immunol* 1986;65(July (1)):198–205. [PubMed: 3098473]
  9. Kearns DH, Seibert GB, O'Reilly R, Lee L, Logan L. Paradox of the immune response to uncomplicated gonococcal urethritis. *N Engl J Med* 1973;289(November (22)):1170–1174. [PubMed: 4201952]
  10. McMillan A, McNeillage G, Young H, Bain SR. Serum immunoglobulin response in uncomplicated gonorrhoea. *Br J Vener Dis* 1979;55(February (1)):5–9. [PubMed: 427515]
  11. Zak K, Diaz JL, Jackson D, Heckels JE. Antigenic variation during infection with *Neisseria gonorrhoeae*: detection of antibodies to surface proteins in sera of patients with gonorrhea. *J Infect Dis* 1984;149(February (2)):166–174. [PubMed: 6142073]
  12. Cohen IR. Natural and immune human antibodies reactive with antigens of virulent *Neisseria gonorrhoeae*: immunoglobulins G, M, and A. *J Bacteriol* 1967;94(July (1)):141–148. [PubMed: 4961630]
  13. Tramont EC. Inhibition of adherence of *Neisseria gonorrhoeae* by human genital secretions. *J Clin Invest* 1977;59(January (1)):117–124. [PubMed: 401507]
  14. Faruki H, Kohmescher RN, McKinney WP, Sparling PF. A community-based outbreak of infection with penicillin-resistant *Neisseria gonorrhoeae* not producing penicillinase (chromosomally mediated resistance). *N Engl J Med* 1985;313(September (10)):607–611. [PubMed: 3160949]
  15. Brooks GF, Darrow WW, Day JA. Repeated gonorrhea: an analysis of importance and risk factors. *J Infect Dis* 1978;137(February (2)):161–169. [PubMed: 627736]
  16. Fox KK, Thomas JC, Weiner DH, Davis RH, Sparling PF, Cohen MS. Longitudinal evaluation of serovar-specific immunity to *Neisseria gonorrhoeae*. *Am J Epidemiol* 1999;149(February (4)):353–358. [PubMed: 10025478]
  17. Hobbs MM, Alcorn TM, Davis RH, Fischer W, Thomas JC, Martin I, et al. Molecular typing of *Neisseria gonorrhoeae* causing repeated infections: evolution of porin during passage within a community. *J Infect Dis* 1999;179(February (2)):371–381. [PubMed: 9878021]
  18. Buchanan TM, Eschenbach DA, Knapp JS, Holmes KK. Gonococcal salpingitis is less likely to recur with *Neisseria gonorrhoeae* of the same principal outer membrane protein antigenic type. *Am J Obstet Gynecol* 1980;138(December (7 Pt 2)):978–980. [PubMed: 6781350]
  19. Plummer FA, Simonsen JN, Chubb H, Slaney L, Kimata J, Bosire M, et al. Epidemiologic evidence for the development of serovar-specific immunity after gonococcal infection. *J Clin Invest* 1989;83(May (5)):1472–1476. [PubMed: 2496142]
  20. Braude AI. Maxwell Finland lecture. Resistance to infection with the gonococcus. *J Infect Dis* 1982;145(May (5)):623–624. [PubMed: 6804574]
  21. Dalal SJ, Estep JS, Valentin-Bon IE, Jerse AE. Standardization of the Whitten effect to induce susceptibility to *Neisseria gonorrhoeae* in female mice. *Contemp Top Lab Anim Sci* 2001;40(March (2)):13–17. [PubMed: 11300681]
  22. Streeter PR, Corbeil LB. Gonococcal infection in endotoxin-resistant and endotoxin-susceptible mice. *Infect Immun* 1981;32(April (1)):105–110. [PubMed: 6783547]
  23. Taylor-Robinson D, Furr PM, Hetherington CM. *Neisseria gonorrhoeae* colonises the genital tract of oestradiol-treated germ-free female mice. *Microb Pathog* 1990;9(November (5)):369–373. [PubMed: 2129173]
  24. Jerse AE. Experimental gonococcal genital tract infection and opacity protein expression in estradiol-treated mice. *Infect Immun* 1999;67(November (11)):5699–5708. [PubMed: 10531218]

25. Soler-Garcia AA, Jerse AE. *Neisseria gonorrhoeae* catalase is not required for experimental genital tract infection despite the induction of a localized neutrophil response. *Infect Immun* 2007;75(May (5)):2225–2233. [PubMed: 17296753]
26. Cohen MS, Cannon JG, Jerse AE, Charniga LM, Isbey SF, Whicker LG. Human experimentation with *Neisseria gonorrhoeae*: rationale, methods, and implications for the biology of infection and vaccine development. *J Infect Dis* 1994;169(March (3)):532–537. [PubMed: 8158024]
27. Jerse AE, Cohen MS, Drown PM, Whicker LG, Isbey SF, Seifert HS, et al. Multiple gonococcal opacity proteins are expressed during experimental urethral infection in the male. *J Exp Med* 1994;179(March (3)):911–920. [PubMed: 8113683]
28. Jerse AE, Crow ET, Bordner AN, Rahman I, Cornelissen CN, Moench TR, et al. Growth of *Neisseria gonorrhoeae* in the female mouse genital tract does not require the gonococcal transferrin or hemoglobin receptors and may be enhanced by commensal lactobacilli. *Infect Immun* 2002;70(May (5)):2549–2558. [PubMed: 11953395]
29. Brodeur BR, Larose Y, Tsang P, Hamel J, Ashton F, Ryan A. Protection against infection with *Neisseria meningitidis* group B serotype 2b by passive immunization with serotype-specific monoclonal antibody. *Infect Immun* 1985;50(November (2)):510–516. [PubMed: 3932211]
30. Plante M, Jerse A, Hamel J, Couture F, Rioux CR, Brodeur BR, et al. Intranasal immunization with gonococcal outer membrane preparations reduces the duration of vaginal colonization of mice by *Neisseria gonorrhoeae*. *J Infect Dis* 2000;182(September (3)):848–855. [PubMed: 10950780]
31. Corbeil LB, Chatterjee A, Foresman L, Westfall JA. Ultrastructure of cyclic changes in the murine uterus, cervix, and vagina. *Tissue Cell* 1985;17(1):53–68. [PubMed: 4039853]
32. Mestecky J, Russell MW. Induction of mucosal immune responses in the human genital tract. *FEMS Immunol Med Microbiol* 2000;27(April (4)):351–355. [PubMed: 10727891]
33. Miettinen A, Hakkarainen K, Gronroos P, Heinonen P, Teisala K, Aine R, et al. Class specific antibody response to gonococcal infection. *J Clin Pathol* 1989;42(January (1)):72–76. [PubMed: 2564006]
34. Ramsey KH, Schneider H, Cross AS, Boslego JW, Hoover DL, Staley TL, et al. Inflammatory cytokines produced in response to experimental human gonorrhea. *J Infect Dis* 1995;172(July (1)):186–191. [PubMed: 7797909]
35. Schneider H, Griffiss JM, Boslego JW, Hitchcock PJ, Zahos KM, Apicella MA. Expression of paragloboside-like lipooligosaccharides may be a necessary component of gonococcal pathogenesis in men. *J Exp Med* 1991;174(December (6)):1601–1605. [PubMed: 1744587]
36. Schmidt KA, Schneider H, Lindstrom JA, Boslego JW, Warren RA, Van de Verg L, et al. Experimental gonococcal urethritis and reinfection with homologous gonococci in male volunteers. *Sex Transm Dis* 2001;28(October (10)):555–564. [PubMed: 11689753]
37. Kallstrom H, Liszewski MK, Atkinson JP, Jonsson AB. Membrane cofactor protein (MCP or CD46) is a cellular pilus receptor for pathogenic *Neisseria*. *Mol Microbiol* 1997;25(4):639–647. [PubMed: 9379894]
38. Dehio C, Gray-Owen SD, Meyer TF. The role of neisserial Opa proteins in interactions with host cells. *Trends Microbiol* 1998;6(December (12)):489–495. [PubMed: 10036728]
39. Edwards JL, Apicella MA. The molecular mechanisms used by *Neisseria gonorrhoeae* to initiate infection differ between men and women. *Clin Microbiol Rev* 2004;17(October (4)):965–981. [table of contents]. [PubMed: 15489357]
40. Serino L, Nesta B, Leuzzi R, Fontana MR, Monaci E, Mocca BT, et al. Identification of a new OmpA-like protein in *Neisseria gonorrhoeae* involved in the binding to human epithelial cells and in vivo colonization. *Mol Microbiol* 2007;64(June (5)):1391–1403. [PubMed: 17542928]
41. Kasper DL, Rice PA, McCormick WM. Bactericidal antibody in genital infection due to *Neisseria gonorrhoeae*. *J Infect Dis* 1977;135(February (2)):243–251. [PubMed: 402426]
42. Hedges SR, Mayo MS, Mestecky J, Hook EW III, Russell MW. Limited local and systemic antibody responses to *Neisseria gonorrhoeae* during uncomplicated genital infections. *Infect Immun* 1999;67(August (8)):3937–3946. [PubMed: 10417159]
43. Gavin AL, Hoebe K, Duong B, Ota T, Martin C, Beutler B, et al. Adjuvant-enhanced antibody responses in the absence of toll-like receptor signaling. *Science (New York, NY)* 2006;314(December (5807)):1936–1938.

44. Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol* 2001;2(August (8)):675–680. [PubMed: 11477402]
45. Fazeli A, Bruce C, Anumba DO. Characterization of Toll-like receptors in the female reproductive tract in humans. *Hum Reprod (Oxford, England)* 2005;20(May (5)):1372–1378.
46. Soboll G, Schaefer TM, Wira CR. Effect of toll-like receptor (TLR) agonists on TLR and microbicide expression in uterine and vaginal tissues of the mouse. *Am J Reprod Immunol* 2006;55(June (6)):434–446. [PubMed: 16674601]
47. Pioli PA, Amiel E, Schaefer TM, Connolly JE, Wira CR, Guyre PM. Differential expression of Toll-like receptors 2 and 4 in tissues of the human female reproductive tract. *Infect Immun* 2004;72(October (10)):5799–5806. [PubMed: 15385480]
48. Izcue A, Coombes JL, Powrie F. Regulatory T cells suppress systemic and mucosal immune activation to control intestinal inflammation. *Immunol Rev* 2006 August;(212):256–271. [PubMed: 16903919]
49. Boulton IC, Gray-Owen SD. Neisserial binding to CEACAM1 arrests the activation and proliferation of CD4+ T lymphocytes. *Nat Immunol* 2002;3(March (3)):229–236. [PubMed: 11850628]
50. Pantelic M, Kim YJ, Bolland S, Chen I, Shively J, Chen T. *Neisseria gonorrhoeae* kills carcinoembryonic antigen-related cellular adhesion molecule 1 (CD66a)-expressing human B cells and inhibits antibody production. *Infect Immun* 2005;73(July (7)):4171–4179. [PubMed: 15972507]
51. Lee HS, Boulton IC, Reddin K, Wong H, Halliwell D, Mandelboim O, et al. Neisserial outer membrane vesicles bind the coinhibitory receptor carcinoembryonic antigen-related cellular adhesion molecule 1 and suppress CD4+T lymphocyte function. *Infect Immun* 2007;75(September (9)):4449–4455. [PubMed: 17620353]
52. Ngampasutadol J, Ram S, Blom AM, Jarva H, Jerse AE, Lien E, et al. Human C4b-binding protein selectively interacts with *Neisseria gonorrhoeae* and results in species-specific infection. *Proc Natl Acad Sci USA* 2005;102(November (47)):17142–17147. [PubMed: 16275906]
53. Ngampasutadol J, Ram S, Gulati S, Agarwal S, Li C, Visintin A, et al. Human factor H interacts selectively with *Neisseria gonorrhoeae* and results in species-specific complement evasion. *J Immunol* 2008;180(March (5)):3426–3435. [PubMed: 18292569]

Fig. 1. Colonization of the murine lower genital tract by *N. gonorrhoeae*. Two days after estradiol pellet implantation and antibiotic treatment, mice were inoculated intravaginally with 1×10^6 CFU of *N. gonorrhoeae* strain FA1090 (infected) or the same volume of PBS (uninfected). Genital tissues were collected at days 2 and 5 post-inoculation, embedded in paraffin after fixation in 10% neutral buffered formalin. Tissue sections (5–7 μ m) were probed with mAb (H5), which is specific against gonococcal porin followed by HRP-conjugated secondary antibody, and visualized with HRP substrate 3,3-diaminobenzidine in the presence of hydrogen peroxide, followed by hematoxylin and eosin staining. Shown are represented images of H&E staining only (A and B) and antibody plus H&E staining (C and

D). Sections were analyzed under a light microscope using a 100× oil objective and numbers of antibody-positive gonococci in five randomly selected fields were estimated. The data are plotted as the average number of bacteria among 100 cells from five mice of each group (E).

Fig. 2. Increased PMNs in the genital tissues of infected mice. Paraffin embedded sections from tissues of (A and B) infected mice and (C and D) uninfected controls were stained with hematoxylin and eosin. Images were acquired using 100× oil objectives.

Fig. 3.

Recruitment of neutrophils and macrophages to genital tissues. Cryosections from tissues of infected and uninfected control mice were stained with (A and B) Gr-1- or (D and E) F4/80-specific antibody and HRP-conjugated secondary antibodies. The sections were further stained with hematoxylin. Images were acquired using 100 $\times$ oil objective. Control experiments where the primary antibody was omitted did not show significant staining. (C and F) The number of cells with positive staining on five randomly selected microscopic fields of each area in each mouse was counted. The data are plotted as the average number of positively stained cells among 100 mouse cells ($\pm$ S.D.) based on five mice in each group. * $P < 0.05$.

Fig. 4.

Humoral responses against gonococcal lower genital tract infection. (A) Vaginal washes and sera were collected from infected and control mice on days 5 and 10 post-inoculation. Protease inhibitors were added to prevent protein degradation. The concentration of gonococcal-specific IgG, IgM, and IgA were determined by ELISA. For gonococcal-specific IgG, purified mouse Mab H5 of known concentrations was used to establish a standard curve. Open circles represent data for individual mice and the averages are represented by horizontal bars. $*P < 0.05$. (B) Paraffin-embedded sections from tissues of infected and uninfected control mice were stained with HRP-conjugated goat-anti-mouse IgG or IgM antibody. The sections were further stained with hematoxylin. Images were acquired using

10× objectives. Control experiments using irrelevant goat antibodies that were conjugated to HRP did not show significant staining.

Fig. 5.

Serum estradiol levels in mice treated with a high dose, slow-release pellet versus a lower total dose of estradiol_{ws}. Groups of mice were either given (A) three 0.5 mg injections of estradiol_{ws} spaced two days apart or (B) a 5 mg, 21 days slow-release estradiol pellet. Serum estradiol levels were determined at the time points indicated. Mice were not challenged with *N. gonorrhoeae*. The day on which mice normally would be inoculated with gonococci is two days after estradiol pellet implantation or 6 h after the second dose of estradiol_{ws}. Normal serum estradiol levels range from 10 to 130 pg/ml over the course of the estrous cycle [21].

Fig. 6. Kinetics of colonization and PMN response in mice treated with 17β -estradiol_{ws}. BALB/c mice were treated with 17β -estradiol_{ws} and antibiotics and inoculated intravaginally with 1×10^6 CFU *N. gonorrhoeae* FA1090 (infected), or PBS (control) as described in the Materials and methods. (A) Recovery of *N. gonorrhoeae* over time expressed as $\log_{10}$ CFU within a 100 μ l of vaginal swab suspension [$\pm$ standard error (S.E.)]. (B) Average number of PMNs out of 100 vaginal cells in stained smears from test mice (diamonds) and mice inoculated with saline (squares) ($\pm$ S.E.). Representative images of stained vaginal smears from infected mice show (C) epithelial cell-associated gonococci on day 3 post-bacterial challenge and (D) gonococci within a PMN on day 5 of infection. The lesser impact of using 17β -estradiol_{ws}

on vaginal histology is reflected by a predominance of nucleated epithelial cells as opposed to mostly squamous epithelial cells in panel C.

Fig. 7.

Susceptibility of mice to repeat gonococcal infection. Test mice were inoculated with *N. gonorrhoeae* strain FA1090 (1×10^6 CFU) using the estradiol_{WS} protocol (round one, primary infection). Ten days after infection, mice were treated with ceftriaxone. Four weeks later mice were re-treated with 17 β -estradiol_{WS} and challenged with 10^7 CFU of the same strain of *N. gonorrhoeae* (round two, repeat infection). Age-matched, estradiol-treated mice were challenged with PBS (round one) followed by *N. gonorrhoeae* (primary infection, round two) in parallel to test mice, and served as naïve controls. Vaginal mucus was quantitatively cultured each day after bacterial challenge. (A) The duration of infection (last positive culture day) from individual test mice (rounds 1 and 2) and naïve control mice (round 2), and represents the combined results of two experiments, each of which were

similar. The mean duration of infection is indicated by the horizontal bars with the actual value indicated in parenthesis. (B) The average number ($\pm$ S.E.) of bacteria in vaginal specimens from five mice in one of the two experiments shown in panel A. (C) Vaginal washes and sera were collected from test and naïve control mice on days 5 and 10 of the second round of infection. Protease inhibitors were added to prevent protein degradation. The concentration of gonococcal-specific IgG were determined by ELISA. Purified mouse Mab H5 of known concentrations was used to establish a standard curve.

Table 1

Antibody responses induced by intranasal (in) or intravaginal (ivag) immunization of estradiol-treated or untreated mice

		Antibody titer ($\pm$ S.D.)			
		First immunization		Second immunization	
		Serum IgG/M	Vaginal IgG/M	Serum IgG/M	Vaginal IgA
Untreated	in	5,496 ($\pm$ 5,364)	<16	<16	190 ($\pm$ 120) ^c
	ivag	1,403 ($\pm$ 541) ^a	<16	<16	<16
Estradiol-treated	in	2,407 ($\pm$ 1,600)	<16	<16	83 ($\pm$ 32) ^c
	ivag	156 ($\pm$ 60)	<16	<16	<16

^a *p*-Value < 0.02 when compared to ivag, estradiol-treated mice.

^b *p*-Value < 0.001 when compared to ivag, untreated or estradiol-treated mice.

^c *p*-Value < 0.05 when compared to ivag, untreated or estradiol-treated mice.