

Mouse Strain-Dependent Differences in Susceptibility to *Neisseria gonorrhoeae* Infection and Induction of Innate Immune Responses[∇]

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Acute gonorrhea in women is characterized by a mucopurulent exudate that contains polymorphonuclear leukocytes (PMNs) with intracellular gonococci. Asymptomatic infections are also common. Information on the innate response to *Neisseria gonorrhoeae* in women is limited to studies with cultured cells, isolated immune cells, and analyses of cervicovaginal fluids. 17 β -Estradiol-treated BALB/c mice can be experimentally infected with *N. gonorrhoeae*, and a vaginal PMN influx occurs in 50 to 80% of mice. Here, we compared the colonization loads and proinflammatory responses of BALB/c, C57BL/6 and C3H/HeN mice to *N. gonorrhoeae*. BALB/c and C57BL/6 mice were colonized at similar levels following inoculation with 10⁶ CFU of *N. gonorrhoeae*. BALB/c, but not C57BL/6, mice exhibited a marked vaginal PMN influx. Tumor necrosis factor alpha, interleukin-6, macrophage inflammatory protein 2 (MIP-2), and keratinocyte-derived chemokine were elevated in vaginal secretions from infected BALB/c mice, but not in those from C57BL/6 mice. MIP-2 levels positively correlated with a vaginal PMN influx. In contrast to BALB/c and C57BL/6 mice, C3H/HeN mice were resistant to infection, and there was no evidence of an inflammatory response. We conclude that *N. gonorrhoeae* causes a productive infection in BALB/c mice that is characterized by the induction of proinflammatory cytokines and chemokines and the recruitment of PMNs. Infection of C57BL/6 mice, in contrast, is more similar to asymptomatic infection. C3H/HeN mice are inherently resistant to *N. gonorrhoeae* infection, and this resistance is not due to an overwhelming inflammatory response to infection. Host genetic factors can therefore impact susceptibility and the immune response to *N. gonorrhoeae*.

Uncomplicated gonorrhea is most commonly an infection of the urethra in men and the cervix. The female urethra may also be infected, and rectal and pharyngeal infection can occur in either sex. The hallmark of symptomatic gonococcal infection is the presence of a purulent exudate containing numerous polymorphonuclear leukocytes (PMNs), many of which contain intracellular gonococci. Asymptomatic infections are also common, particularly in females. Epithelial cells that line the genital mucosal surface are the first line of defense against this human-specific pathogen, and *Neisseria gonorrhoeae* produces a robust proinflammatory cytokine and chemokine response when incubated with cultured human vaginal, endocervical, ectocervical (12, 33), urethral (17), endometrial (3), and fallopian tube (31) tissue culture cells. Similarly, studies using the complex fallopian tube organ culture model suggest that *N. gonorrhoeae* induces the proinflammatory cytokines interleukin-1 α (IL-1 α) and tumor necrosis factor alpha (TNF- α) (29). Signaling through cellular receptors on epithelial cells results in the activation and recruitment of phagocytic cells, including

PMNs and macrophages. Primary macrophages and peripheral blood mononuclear cells also elicit a proinflammatory response when incubated with *N. gonorrhoeae* (30, 34, 39). These innate immune cells further contribute to the array of proinflammatory cytokines and antimicrobial factors.

Due to the multiple cell types that contribute to the host innate response to infection, it is important that whole model systems be utilized to measure the impact of *N. gonorrhoeae* infection on the host immune response. Experimental urethral colonization in male volunteers with *N. gonorrhoeae* evokes a strong innate response that is characterized by the production of proinflammatory cytokines (37, 38). Similar studies with female subjects are not feasible due to the risk of complications of gonococcal infection in women. Therefore, features of the innate response to *N. gonorrhoeae* in the female genital tract are predicted solely from tissue culture systems and the analysis of clinical samples. It is unclear whether women elicit a cytokine response to gonococcal infection. The reason why some infections are asymptomatic is also not known. Hedges et al. (18, 19) were unable to detect local proinflammatory cytokines in cervicovaginal secretions from infected women and detected a low anti-gonococcal antibody response. Based on these observations, it was proposed that *N. gonorrhoeae* fails to induce host inflammatory responses or is actively immunosuppressive. This finding is in marked contrast with the robust induction of proinflammatory cytokines observed from in vitro cell lines that constitute the female genital tract. The absence of various other cell types in tissue culture cell models could

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influence the cytokine response to infection; alternatively, the timing of sample collection from infected subjects may also influence the data. Therefore, a systematic analysis of cytokine induction over the course of infection in a female animal model is needed.

The 17 β -estradiol-treated mouse model is the only small-animal model available for studying the immune response to *N. gonorrhoeae* genital tract infection. While the mechanism by which estradiol promotes long-term colonization in female mice is not known, it is likely that promotion of an estrus-like state is beneficial for the gonococcus based on the fact that untreated mice can be transiently colonized with *N. gonorrhoeae* provided they are inoculated in the proestrus stage of the reproductive cycle (7, 46). The 17 β -estradiol-treated mouse model has been a useful system for studying many aspects of gonococcal infection, including gonococcal evasion of PMN killing (43, 49) and antimicrobial peptides (23, 48), antigenic variation in vivo (41), and interactions between *N. gonorrhoeae* and commensal flora (32). This model is based on the use of BALB/c mice. Approximately 50 to 80% of infected BALB/c mice that are treated with a slow-release estradiol pellet exhibit a significant vaginal PMN response following inoculation with *N. gonorrhoeae* strain FA1090 based on examination of stained vaginal smears (21, 22, 43), and PMNs and macrophages are also found in vaginal and cervical tissue samples from infected mice (44). Gonococci are localized within vaginal and cervical tissue, and similar to that which occurs in humans, an insignificant and transient humoral response to *N. gonorrhoeae* occurs which was not protective against reinfection with the same strain (44). A recent modification of the model utilizes water-soluble estradiol to reduce the length of time that mice are exposed to nonphysiological concentrations of estradiol. A vaginal PMN influx also occurs during infection of mice treated with water-soluble estradiol, and as with pelleted mice, infection persists despite the presence of PMNs (44).

One advantage of using inbred mouse strains for studies of infectious diseases is that environmental and genetic components can be controlled. Interestingly, the susceptibility to infectious agents can often vary with the genetic background of the mouse. One example in the area of sexually transmitted infections is that genetically controlled differences in the development of infertility in inbred mouse strains following inoculation with chlamydia have been reported, with pregnancy rates following infection of C3H/HeN mice being significantly lower than those of C57BL/6 mice (10). Darville and colleagues (8) found similar results, and their data suggested that an earlier and more severe acute inflammatory response in the C57BL/6 strain may lead to earlier eradication of the infection, thus protecting the upper tract from disease. Numerous examples of vulnerability have been found for other infectious agents, including *Leishmania major*, *Listeria monocytogenes*, *Salmonella enterica* serovar Typhimurium, *Plasmodium chabaudi*, *Legionella pneumophila*, and *Mycobacterium tuberculosis* (reviewed by Kramnik and Boyartchuk [27]).

In this study, we sought to characterize in greater detail the cytokine and inflammatory response to genital tract infection with *N. gonorrhoeae* in 17 β -estradiol-treated BALB/c mice and to determine if susceptibility to colonization and the host inflammatory response to infection vary between inbred mouse strains. Our data demonstrate that BALB/c, C57BL/6, and

C3H/HeN mouse strains differ widely in their response to infection. While both BALB/c and C57BL/6 strains support colonization with the gonococcus, only the BALB/c strain appears to mount an inflammatory response. In contrast, the C3H/HeN strain appears to be resistant to colonization with the gonococcus. These data demonstrate significant divergence among inbred mouse strains in terms of susceptibility and inflammatory response to gonococcal infection, and they suggest that future studies can be designed to correlate genetic markers with the host response.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *N. gonorrhoeae* strain FA1090 (*porB1b*, AHU [an auxotype for arginine, hypoxanthine, and uracil], and serum resistant) was originally isolated from a female with disseminated gonococcal infection and has been extensively tested in male volunteers (4). Frozen stocks of FA1090 bacteria were clonally passaged on solid GC agar containing Kellogg's supplement 1 (24) and 12 μ M Fe(NO₃)₃ and incubated at 37°C in a humidified 7% CO₂ incubator. GC agar with antibiotic selection (vancomycin, colistin, nystatin, trimethoprim sulfate, and streptomycin [VCNTS]) and heart infusion agar were used to isolate *N. gonorrhoeae* and facultatively anaerobic commensal flora from murine vaginal mucus as described previously (21). All media were from Difco.

Experimental infection of mice. Female C57BL/6J (*H-2^b*), BALB/cJ (*H-2^d*), and C3H/HeN (*H-2^k*) mice (4 to 6 weeks old) were purchased from the National Cancer Institute, Bethesda, MD, and treated with water-soluble 17 β -estradiol (Sigma) to promote long-term susceptibility to *N. gonorrhoeae* as described previously (21). Mice were also given streptomycin sulfate and vancomycin (0.24 mg and 0.04 mg, respectively, intraperitoneally, twice daily) and trimethoprim sulfate (0.04 g/100 ml of drinking water) to inhibit the overgrowth of commensal flora that occurs under the influence of estradiol as described previously (21). Two days after the first dose of estradiol, groups of BALB/c ($n = 10$), C57BL/6 ($n = 9$) and C3H/HeN ($n = 10$) mice were vaginally inoculated with 1×10^6 CFU of *N. gonorrhoeae* strain FA1090 as described previously (21). Control groups consisted of mice of each background ($n = 5$) that were inoculated with phosphate-buffered saline (PBS). Vaginal mucus was collected from all mice daily for 10 days with a sterile swab, and samples of undiluted and diluted swab contents were cultured on GC-VCNTS agar. The percentage of PMNs among 100 vaginal cells was determined by microscopic examination of stained vaginal smears.

RNA isolation from vaginal washes and relative real-time PCR. Groups of estradiol-treated BALB/c and C57BL/6 mice were inoculated with strain FA1090 (test mice; $n = 7$) or PBS (control mice; $n = 4$) as described above. Dacron swabs were inserted into the vagina of mice on days 1, 3, 5, and 7 postinoculation. The swab contents were serially diluted and cultured for *N. gonorrhoeae*, and a sample was stained for visualization of PMNs. A second swab was collected and suspended in 100 μ l of PBS and centrifuged for cytokine analysis by real-time PCR. The resultant pellets were resuspended in 500 μ l of RNA-later (Ambion) reagent, and samples were stored at -80°C. The experiment was repeated three times to test reproducibility. For each experiment, total RNA was extracted from stored samples by using Qiagen mini-RNAeasy isolation kits according to the manufacturer's instructions. Total extracted RNA was then treated with Turbo DNase (Ambion) to eliminate contaminating DNA. cDNA was synthesized using reverse transcriptase SuperScript III RNaseH (Invitrogen, Carlsbad, CA) by following the manufacturer's instructions. Briefly, 100 ng to 1 μ g of RNA treated with DNase I (DNase Free Turbo; Ambion) in a 10- μ l volume was mixed with 1 μ l (300 ng) of random hexamers (Invitrogen) and 1 μ l (10 mM) deoxynucleoside triphosphate mix (Promega). This mixture was incubated at 65°C for 5 min and quickly chilled on ice. A cocktail containing 4 μ l of 5 \times first-strand buffer, 2 μ l of 0.1 M dithiothreitol, and 1 μ l of RNaseOut (Invitrogen) was then added to each tube. After a brief centrifugation, the tubes were incubated at room temperature for 10 min. The reaction mixtures were preincubated at 50°C for 2 min, before adding 1 μ l of SuperScript III. cDNA synthesis was allowed to proceed at 50°C for 50 min, followed by incubation at 70°C for 15 min to inactivate the reaction. Nuclease-free water (1 μ l) was added to reaction mixtures in place of Superscript for controls. A SYBR green master mix kit (ABI) was used to perform real-time PCR assays. cDNA reaction mixtures (20 μ l) were diluted to a final volume of 100 μ l with nuclease-free double-distilled water. Five microliters of diluted cDNA template was then subjected to PCR amplification in a 7500 sequence detector (ABI) in a total volume of 25 μ l containing 12.5 μ l SYBR green master mix, 1 μ l of each primer (10 μ M) and 5.5 μ l double-distilled water. The reactions were performed according to the following parameters: 10 min at 95°C, then 40

TABLE 1. Oligonucleotide primers used in this study

Gene	Primer (5'-3')	
	Forward	Reverse
IL-1 α gene	AGACCATCCAACCCAGATCA	TGACAAACTTCTGCCTGACG
IL-6 gene	CCGGAGAGGAGACTTCACAG	TCCACGATTTCCAGAGAAC
TNF- α gene	ACGGCATGGATCTCAAAGAC	GTGGGTGAGGAGCACGTAGT
KC gene	GCACCCAAACCGAAGTCATA	TGGGGACACCTTTTAGCATC
MIP-2 gene	TCCAGAGCTTGAGTGTGACG	TTCAGGGTCAAGGCAAACCT
β -Actin gene	GCGCAAGTACTCTGTGTGGA	CATCGTACTCCTGCTTGCTG
18 sRNA gene	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT

cycles at 95°C for 15 s and at 60°C for 1 min. Data were analyzed using the Sequence Detector v.1.7a software (ABI). The cycle threshold (C_T) was defined as the cycle number corresponding to the point at which the amplification plot of all samples is linear. We used the comparative C_T method ($\Delta\Delta C_T$) for relative quantification of cytokine and chemokine gene expression, and constitutively expressed β -actin served as the active reference control (normalizer). The expression of genes of interest in each sample was measured by normalizing to the constitutively expressed β -actin gene. The calculation used included the difference between the C_T values of the normalizer (β -actin) and the C_T values of cytokines and chemokines (C&C) in individual samples, as follows: $\Delta C_{T(\text{infected or uninfected})} = C_{T(\beta\text{-actin})} - C_{T(\text{C\&C})}$. The relative difference in gene expression between the infected and uninfected group on each day was determined by the difference of normalized gene expression levels, as follows: $2^{\Delta\Delta C_T} = \Delta C_{T(\text{uninfected})} - \Delta C_{T(\text{infected})}$. The sequences of the oligonucleotide primers used for this analysis are shown in Table 1

Cytokine enzyme-linked immunosorbent assay (ELISA). Groups of estradiol-treated BALB/c and C57BL/6 mice were inoculated with strain FA1090 (test mice; $n = 5$) or PBS (control mice; $n = 5$) as described above. Dacron swabs were inserted into the vagina of mice on days 1 to 10 postinoculation. The swab contents were serially diluted and cultured for *N. gonorrhoeae*, and a sample was stained for visualization of PMNs. On days 1, 3, 5, and 7, vaginal mucus samples were also collected an hour after the cultures were taken by pipetting 50 μ l PBS in and out of the vaginas of control and test mice 20 times. The lavage fluid was then centrifuged at $13,000 \times g$ for 3 min. The supernatant was frozen immediately and stored at -70°C until further analyzed. Analysis was performed with the Bio-Plex system (Bio-Rad, Hercules, CA) as described previously (5).

Statistical analysis and animal assurances. The average durations of recovery and colonization loads over time were compared between groups by an unpaired t test and repeated measure of variance, respectively. Cytokine/chemokine levels as measured by ELISA and reverse transcription-PCR (RT-PCR) were compared by an unpaired t test. Pearson's correlation was applied to determine correlation coefficients between chemokine levels and PMN influx. GraphPad software was used for all statistical analyses. All animal experiments were conducted at the Uniformed Services University of the Health Sciences, a facility fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, under a protocol that was approved by the university's Institutional Animal Care and Use Committee.

RESULTS

Inbred mouse strains differ in their ability to support vaginal colonization with *N. gonorrhoeae*. Our previous studies with the 17 β -estradiol-treated mouse model have relied on the use of the BALB/c strain. In order to compare the susceptibility and infection kinetics of gonococcal infection in mice of different genetic backgrounds, in two separate experiments, we intravaginally inoculated groups of 17 β -estradiol-treated BALB/c, C57BL/6, and C3H/HeN mice with 1×10^6 CFU of *N. gonorrhoeae* strain FA1090. Mice of the same background received PBS as a control. BALB/c and C57BL/6 mice were similarly susceptible to experimental gonococcal infection, with 80% of mice of each strain colonized on day 3 and high numbers of gonococci recovered from the vaginas through the entire 10-day course of the experiment (Fig. 1A). In contrast,

C3H/HeN mice were resistant to colonization, as evidenced by the recovery of low numbers of gonococci from only 2 of 10 mice on days 1 to 3 postinoculation. The average duration of colonization in C3H/HeN mice was significantly shorter (mean, 0.75 days; range, 0 to 3 days) than that of BALB/c and C57BL/6 mice ($P < 0.01$), from which gonococci were recovered for an average of 6.6 days and 6.25 days, respectively (range, 0 to 10 days for both mouse strains). The resistance to colonization in the C3H/HeN mice did not appear to be dose related, as they were also not susceptible to *N. gonorrhoeae* infection following inoculation with a 10-fold higher inoculum (1×10^7 CFU) of FA1090 bacteria (data not shown). The majority of mice had negative heart infusion agar cultures throughout the experiment; lactobacilli and a few gram-positive cocci were isolated from some of the mice, but there was no correlation between these commensal bacteria and colonization. All mouse strains responded to the estradiol, based on the presence of mostly squamous epithelial cells in stained vaginal smears from PBS control mice over the course of the 10-day experiment.

Inbred mouse strains differ in their inflammatory response to infection. As reported previously, *N. gonorrhoeae* induces a PMN influx in BALB/c mice that is detected in tissue on day 2 postinoculation and in vaginal smears on day 5 (44). Consistent with these reports, a higher percentage of PMNs was detected in vaginal smears from BALB/c mice on days 5 to 9 than that detected in those from the PBS-treated BALB/c mouse control group (Fig. 1B). Five of 10 (50%) BALB/c mice showed a PMN influx of at least 15%, which is higher than that of the average number recovered from placebo controls (Fig. 1B). Infection persisted in 4/5 mice that had a PMN influx. In contrast, only a slight increase in the percentage of PMNs was observed in stained vaginal smears from C57BL/6 test mice compared to that observed in those from PBS-treated C57BL/6 mice (Fig. 1C). With the exception of one test mouse on day 1, very few samples from C3H/HeN test mice showed vaginal PMNs relative to those from PBS-treated mice of the same background (Fig. 1D). While the absence of a PMN influx in C3H/HeN mice was not surprising since they were relatively resistant to gonococcal colonization, the lack of an inflammatory response in the C57BL/6 mice was unexpected given that they had similar gonococcal colonization levels as the BALB/c mice.

To better assess the basis for the observed difference in PMN influx in BALB/c versus C57BL/6 mice, we next used real-time RT-PCR to measure the expression kinetics of proinflammatory cytokines (IL-1 α , TNF- α , and IL-6) and chemokines (keratinocyte-derived chemokine [KC] and macrophage inflammatory protein 2 [MIP-2]) compared to those of unin-

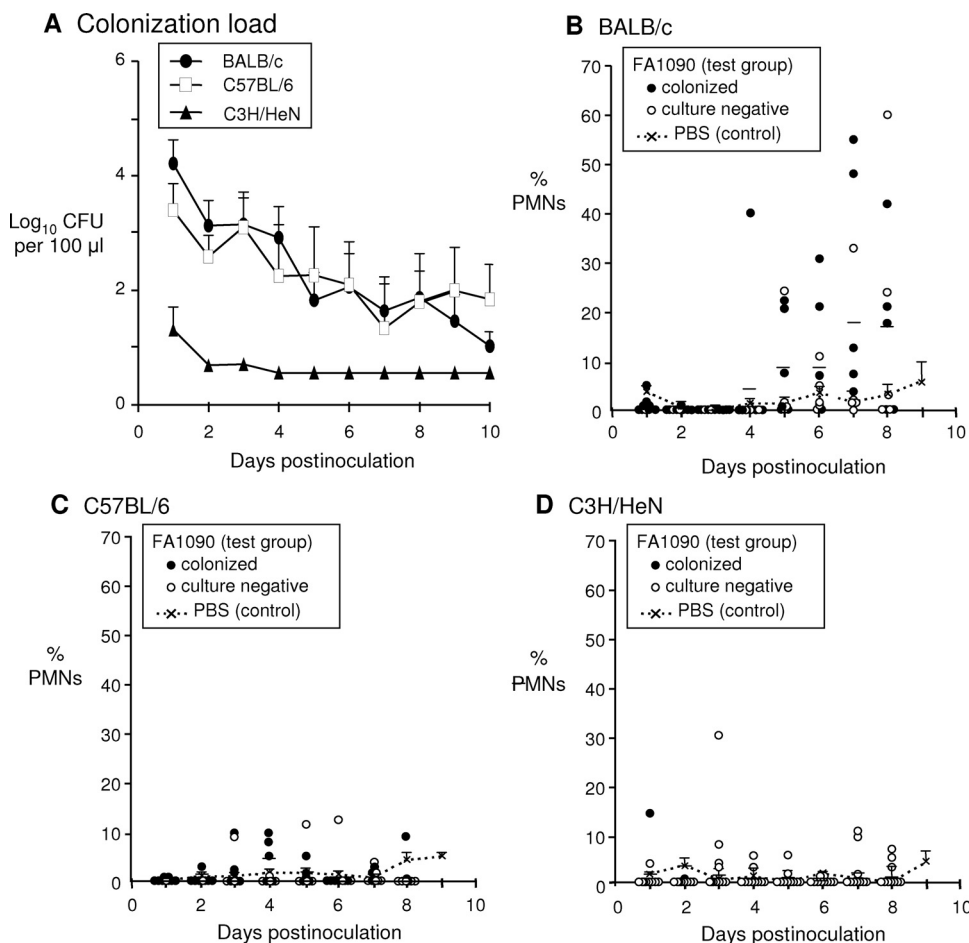


FIG. 1. Kinetics of gonococcal infection in BALB/c, C57BL/6, and C3H/HeN mice. Groups of BALB/c, C57BL/6, and C3H/HeN mice (9 to 10 mice per group) were inoculated vaginally with 1×10^6 CFU of strain FA1090 as described in Materials and Methods, which is the 80% infective dose for this strain. Control mice of each background ($n = 5$ per group) were inoculated with PBS. (A) The average number of gonococci (\log_{10} CFU) recovered from each group (\pm standard error) is shown over time. BALB/c and C57BL/6 mice were colonized with *N. gonorrhoeae* to similar levels, but C3H/HeN mice were resistant to infection. The limit of detection (4 CFU/100 μ l vaginal swab suspension) was used for mice that were culture negative. (B to D) The number of PMNs among 100 vaginal cells over time. Each data point corresponds to an individual mouse, and the horizontal lines indicate the mean percentage. Solid circles indicate mice that were culture positive at that time point; open circles correspond to culture-negative mice. The average percentage of PMNs detected in vaginal smears from in PBS controls was used as a baseline and is represented by a dotted line on each graph with standard error bars shown. A marked influx of PMNs into the vagina above baseline levels was observed only in BALB/c mice.

ected controls in both mouse backgrounds. As shown in Fig. 2, BALB/c mice showed a slight elevation (~ 2 -fold) of IL-1 α in response to FA1090 on days 1 through 5, which then dropped on day 7 (Fig. 2A). TNF- α and IL-6 transcript levels were increased on day 1 and day 3 (~ 2 -fold), peaked on day 5 (~ 6 - to 8-fold) and decreased on day 7 (~ 2 -fold). The expression patterns for chemokines KC and MIP-2 were similar to those of TNF- α and IL-6, but the peak responses of KC and MIP-2 were of a higher magnitude (~ 20 - to 30-fold) on day 5. C57BL/6 mice demonstrated a remarkable difference in the expression of proinflammatory cytokines and chemokines in response to FA1090. Expression of IL-1 α , TNF- α , IL-6, KC, and MIP-2 was minimally elevated and remained constant from days 1 through 7 (Fig. 2B). There was no evidence of a peak in the expression of these cytokines/chemokines in C57BL/6 mice as was observed in BALB/c mice on day 5.

Vaginal MIP-2 levels correlate with vaginal PMNs. To confirm the real-time RT-PCR results, we repeated the experi-

ment in BALB/c mice and collected vaginal wash samples on day 5 for ELISA, which, as in the RT-PCR experiments, is when a PMN influx was first observed. As before, an influx of vaginal PMNs was observed on day 5 (Fig. 3A). As with the RT-PCR results, we detected a slight elevation in IL-1 α (~ 2 -fold) that was not statistically significant (Fig. 3B). An ~ 8 -fold increase in IL-6 was detected (Fig. 3D), and significant increases were also observed for TNF- α , which was detected only in the infected group (Fig. 3C), and the chemokines KC and MIP-2, which were elevated ~ 2 to 3-fold and ~ 10 to 15-fold, respectively (Fig. 3E and F). We conclude that *N. gonorrhoeae* induces a classic proinflammatory cytokine and chemokine response in BALB/c mice and that the degree of induction in BALB/c and C57BL/6 mice mirrors the vaginal PMN response to *N. gonorrhoeae*. Interestingly, we also found that MIP-2 but not KC transcript and protein levels correlated with the degree of PMN influx in BALB/c mice. Mice that produced larger amounts of MIP-2 had larger percentages of vaginal PMNs, but a similar

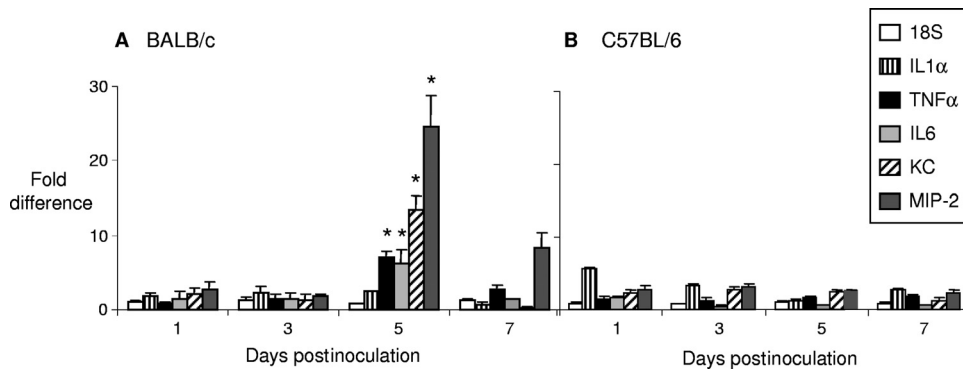


FIG. 2. Induction of proinflammatory cytokines and chemokines in BALB/c but not C57BL/6 mice in vaginal washes was isolated from vaginal swabs of BALB/c (A) and C57BL/6 (B) mice on days 1, 3, 5, and 7 and reverse transcribed into cDNA. Cytokine and chemokine levels were examined by relative RT-PCR in infected samples, normalized to β -actin levels, and then compared to those of normalized uninfected samples. The difference in cytokine expression levels is shown as an n -fold increase in arbitrary units in the levels of expression of cytokines and chemokines in the infected animals over uninfected controls (\pm standard error of the ratios). Proinflammatory cytokines and chemokines were induced greatly on day 5 in BALB/c but not in C57BL/6 mice. *, $P < 0.05$ (two-tailed unpaired t test).

correlation was absent when KC expression levels were compared to the degree of PMN influx (Fig. 4).

DISCUSSION

N. gonorrhoeae is a common sexually transmitted pathogen that is most often associated with infections of the lower genital tract. In women, the infection can be associated with acute

cervicitis; however, asymptomatic infections are not uncommon. The immunological events that lead to inflammation versus asymptomatic colonization are not defined. Here, we used a mouse infection model to characterize the induction of proinflammatory cytokines during lower-genital-tract infection of a female host, and consistent with the occurrence of inflammation, we found increased levels of TNF- α , IL-6, MIP-2, and KC in infected BALB/c mice. Increased levels of MIP-2, which

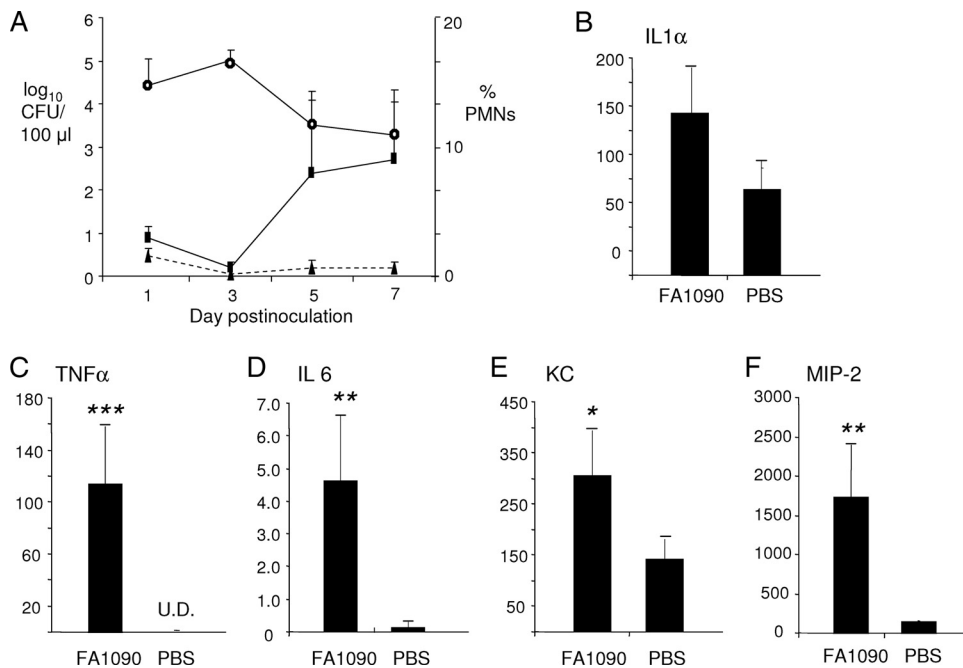


FIG. 3. Confirmation of the proinflammatory cytokine response in the BALB/c mice by ELISA. In a separate experiment from that shown in Fig. 2, BALB/c mice were inoculated with *N. gonorrhoeae* strain FA1090 (test mice) or PBS (control mice) ($n = 5$ mice per group). Vaginal mucus was cultured for *N. gonorrhoeae* and stained for PMNs, and vaginal washes were collected for cytokine analysis by ELISA as described in Materials and Methods. A PMN influx was observed on day 5 in test mice, and mice inoculated with strain FA1090 produced significant levels of proinflammatory cytokines and chemokines compared to control mice. (A) The recovery of gonococci from BALB/c over time is expressed as average \log_{10} CFU per 100 μ l of vaginal swab suspension (\pm standard error) (open circles). PMN influx was expressed as the average number of PMNs out of 100 vaginal cells in stained smears from test mice (rectangles, solid line) and control mice (triangles, broken line). (B to F) The average concentration of proinflammatory cytokines (IL-1 α , IL-6, and TNF- α) and chemokines (KC and MIP-2) in vaginal lavages from test and control mice. Single, double, and triple asterisks denote P values of <0.05 , <0.005 , and <0.0001 , respectively.

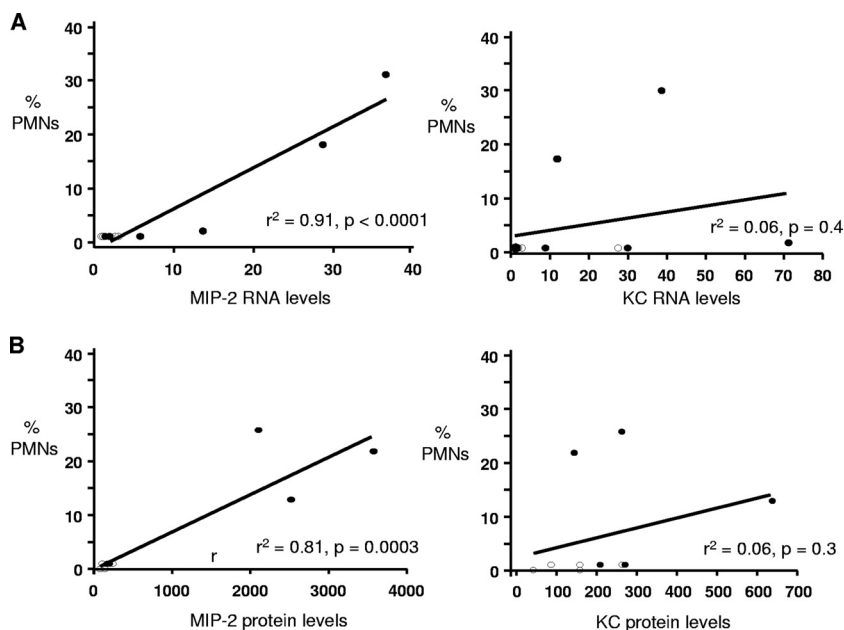


FIG. 4. Correlation between MIP-2 levels and PMN influx. The relationship between the number of PMNs and the levels of MIP-2 and KC was examined in two different experiments using Pearson's correlation. (A) The levels of MIP-2 and KC transcripts were measured on day 5 postinoculation in the test mice (closed circles; $n = 7$) and control mice (open circles; $n = 4$) used in the RT-PCR experiments shown in Fig. 2. Results are expressed as n -fold increase compared to uninfected mice. MIP-2, but not KC, production was significantly associated with an influx of vaginal PMNs ($P < 0.005$). (B) MIP-2 and KC protein levels were measured by ELISA in the test mice (closed circles; $n = 5$) and control mice (open circles; $n = 5$) used in the experiments shown in Fig. 3. Only MIP-2 protein levels correlated with a PMN influx in infected mice on day 5 of infection.

is the functional homologue of IL-8 in mice and has potent PMN-attracting properties (2, 25), correlated with a PMN influx into the genital tract. Initially, *N. gonorrhoeae* may induce proinflammatory cytokines via interactions with Toll-like receptor 2 (TLR-2) expressed on epithelial cells, as suggested by studies with cultured human vaginal and cervical cells (11). The recruitment of other cell types to the site of infection over time may further modulate the cytokine milieu by bringing into play additional innate immune receptors as well as negative regulatory mechanisms.

In the three experiments reported here, the influx of vaginal PMNs occurred in 5/10 (50%), 3/5 (60%), and 2/7 (29%) of infected mice. This range of "responders" is consistent with previous data from our laboratory. In studies in which a placebo control group was used to measure the baseline level of vaginal PMNs, an influx of PMNs occurred in ca. 50 to 80% of infected BALB/c mice that were treated with a slow-release estradiol pellet to promote susceptibility (21, 22, 43) and in 30% to 50% of BALB/c mice that were treated with water-soluble estradiol to promote susceptibility (B. T. Mocca and A. E. Jerse, unpublished data). In our model, proinflammatory cytokines and chemokines were significantly increased in test mice over those in placebo controls on day 3 of infection, with a peak response at day 5, followed by a decline to basal levels by day 7. The basis for the observed kinetics is not known. Estradiol may play an immunosuppressive role in cytokine production by epithelial cells and cytokine-producing innate immune cells (reviewed extensively by Straub [45]). We utilized water-soluble estradiol in this study, which returns to physiological levels by day 3 of infection (44). Therefore, the

initial delay in the induction of proinflammatory cytokines could be due to the administration of estradiol early during infection. The downregulation of proinflammatory cytokines and chemokines on day 7 without the eradication of *N. gonorrhoeae* suggests that, at later time points, *N. gonorrhoeae* infection may negatively regulate inflammation. A similar pattern of localized MIP-2 and IL-6 production occurs during chlamydial genital tract infection of progesterone-treated BALB/c mice (9), suggesting that this pattern of cytokine induction may reflect a dynamic that is inherent to the immune responses of the lower female genital tract.

Cytokines were measured in test mice that were colonized for 3 to 5 days or longer. Only MIP-2 correlated with PMN influx. The detection of PMNs in vaginal smears is probably less sensitive than are other measures of inflammation, and it is possible that low numbers of PMNs or other effectors of inflammation were present in the other mice. It is not known at this time whether the increase in IL-6 in *N. gonorrhoeae*-infected mice, which was significant but at low concentration, is biologically significant. Continued investigation of the murine response to *N. gonorrhoeae* by our laboratory and others may answer these questions.

It is also not known whether phase-variable expression of gonococcal surface molecules during infection differentially triggers the innate response and thus contributes to the observed peak and subsequent decline in proinflammatory cytokines. Neither gonococcal pili nor the opacity (Opa) proteins are known to activate TLRs, and although phase variation of the glycosyltransferases genes used to build the oligosaccharide chain of gonococcal lipooligosaccharide (LOS) occurs in

vivo (40), different LOS variants induce the same proinflammatory cytokines in isolated human monocytes (35). Therefore, it seems unlikely that phase variation of pili, Opa, or the carbohydrate moieties of LOS during infection are responsible for changes in cytokines over time. Perhaps a more relevant question is how phase-variable expression of surface structures might allow the gonococcus to persist during periods of high versus low induction of proinflammatory cytokines and downstream factors. Interestingly, the recovery of Opa protein-expressing gonococci from mice has a similar pattern as we observed for the induction of proinflammatory cytokines. Opa-positive variants are recovered early during infection (days 1 to 3 postinoculation), followed by a predominance of Opa-negative variants and then a return of Opa-positive variants (41). Whether this cyclical selection for Opa protein expression is driven by immunological events is not yet known.

Another important finding of our study was the demonstration of three distinct outcomes following the inoculation of estradiol-treated BALB/c, C57BL/6, and C3H-HeN mice with *N. gonorrhoeae*. C57BL/6 mice, while colonized to the same degree as were BALB/c mice, did not exhibit a marked PMN influx or significant increases in cytokine or chemokine levels in response to infection. The upregulation of chemokine secretion and the subsequent influx of PMNs are most likely driven by the innate immune system. Since there is no obvious difference between the BALB/c and C57BL/6 mice in terms of innate immunity, the explanation for this observation remains unclear. Certainly, C57BL/6 mice are capable of mounting an inflammatory cytokine and PMN response following intravaginal inoculation with *Chlamydia muridarum* (9), so the innate immune response of the lower genital tract would appear to be intact in both strains. Thus, the genetic basis of the observed difference in inflammation in response to *N. gonorrhoeae* between the two strains remains unclear. One possible explanation could be the presence of high levels of secretory phospholipase A2 (sPLA₂) in BALB/c mice, while C57BL/6 mice are genetically deficient for this enzyme (26). sPLA₂ may play an important role in the generation and amplification of inflammation by the production of lipid mediators (28) and the induction of proinflammatory cytokines and chemokines from immune cells (15, 47). Moreover, sPLA₂ affects migration of human neutrophils in vitro (14) and could serve to explain the differential PMN influxes in these two diverse mouse backgrounds. Recently, it was reported that *N. gonorrhoeae* induces a T helper type 17 (Th17) response during murine infection (B. Feinen, S. L. Gaffen, A. E. Jerse, and M. W. Russell, submitted for publication), which may regulate the immune response by induction of suppressive regulatory T cells (Tregs) (20). Since polarization of immune responses is known to be biased in different mouse backgrounds, an investigation of these responses in BALB/c versus C57BL/6 mice might further elucidate the basis for genetic differences in the proinflammatory response to *N. gonorrhoeae*.

In addition to BALB/c and C57BL/6 mice, estradiol-treated SLC:ddY mice are also susceptible to infection, but in this early study, the development of a PMN influx in SLC:ddY mice cleared infection (21). Mice of the outbred strain CD1 have been successfully infected with *N. gonorrhoeae* for prolonged periods of time (A. E. Jerse, unpublished observation). Our demonstration that C3H/HeN mice, in contrast to these mouse

strains, is resistant to *N. gonorrhoeae* is important in that it suggests the existence of protective host factors. The resistance shown by C3H/HeN mice was observed within 1 day postinoculation, and therefore, we hypothesize that host factors present early during infection are responsible. We found no difference in the capacity of *N. gonorrhoeae* to survive in serum from C3H/HeN mice versus BALB/c mice (data not shown), and thus, the possibility that antimicrobial factors in serum are responsible seems unlikely. The C3H/HeN mouse strain appears to display resistance to infection with a number of intracellular mouse pathogens, including *Salmonella* serovar Typhimurium (36), *Leishmania donovani* (1), and *Mycobacterium bovis* BCG (16). The genetic basis for resistance against these pathogens has been attributed to the presence of natural resistance-associated macrophage protein (Nramp1), recently renamed SLC11A1, which transports divalent metals from the phagosome and thereby limits access to essential metals, such as iron and manganese (13). Whether Nramp1 plays any role in gonococcal pathogenesis is not known. However, gonococci survive and even replicate within human PMNs (6, 42), so a role for Nramp1 during this intracellular phase is not inconceivable. While PMNs are not generally seen in vaginal smears until day 5 of infection in BALB/c mice, they were detected in tissue within 2 days after inoculation with *N. gonorrhoeae* (44). Finally, differences in commensal flora among the different mouse strains tested could also explain or contribute to the observed strain-dependent differences in susceptibility and host response. We found no differences in number or type of facultative anaerobic bacteria recovered from vaginal mucus during infection; however, strict anaerobes or fastidious flora would not have been isolated in this study.

In summary, these data further detail the early innate immune response to lower-genital-tract infection of female mice with *N. gonorrhoeae* and correlate peak PMN influx with the chemokine MIP-2. Our observation that BALB/c, C57BL/6, and C3H/HeN mouse strains differ in their ability to support colonization with *N. gonorrhoeae* and mount an inflammatory response to *N. gonorrhoeae* in our model demonstrates a significant role for genetics in controlling host-pathogen interactions. Future comparisons between the innate response of BALB/c and C57BL/6 mice may facilitate studies of the complex interactions that promote symptomatic versus asymptomatic gonococcal infections, and the observed host differences in susceptibility may lead to the identification of factors that could be exploited to protect against gonococcal infection.

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