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Viral Infection of the Pregnant Cervix Predisposes to Ascending Bacterial Infection

Karen Racicot,* Ingrid Cardenas,† Vera Wünsche,* Paulomi Aldo,* Seth Guller,* Robert E. Means,‡ Roberto Romero,§ and Gil Mor*

Preterm birth is the major cause of neonatal mortality and morbidity, and bacterial infections that ascend from the lower female reproductive tract are the most common route of uterine infection leading to preterm birth. The uterus and growing fetus are protected from ascending infection by the cervix, which controls and limits microbial access by the production of mucus, cytokines, and antimicrobial peptides. If this barrier is compromised, bacteria may enter the uterine cavity, leading to preterm birth. Using a mouse model, we demonstrate, to our knowledge for the first time, that viral infection of the cervix during pregnancy reduces the capacity of the female reproductive tract to prevent bacterial infection of the uterus. This is due to differences in susceptibility of the cervix to infection by virus during pregnancy and the associated changes in TLR and antimicrobial peptide expression and function. We suggest that preterm labor is a polymicrobial disease, which requires a multifactorial approach for its prevention and treatment. The Journal of Immunology, 2013, 191: 934–941.

Preterm birth is the major cause of neonatal mortality and morbidity worldwide, yet the underlying etiologies remain poorly understood (1, 2). Preterm labor is a syndrome diagnosed in the presence of increased uterine contractility, cervical ripening, and/or membrane rupture or activation, which may occur in response to multiple pathological processes (1, 2). Newborns born with a fetal inflammatory response are more likely to develop short- and long-term complications above those expected for the gestational age at birth (3–5).

Numerous studies suggest that intrauterine infection is an important mechanism leading to preterm labor and may account for 40% of preterm births (6–8). However, this number may be higher because many infections are likely to be subclinical and the pathogenesis is not detected due to the lack of sensitivity of conventional culture techniques (9, 10). Furthermore, our understanding of the normal flora of the female reproductive tract (FRT) is insufficient and we have limited knowledge of the mechanisms controlling pathogenic bacteria and their relationship with intrauterine infection and preterm labor (11, 12).

All women have microorganisms in the lower genital tract (vulva, vagina, and cervix); however, most studies indicate that amniotic fluid is normally sterile and does not contain microbial products such as endotoxin. During pregnancy, intrauterine infections begin in the decidua, extend to the amnion and chorion, and finally reach the amniotic cavity and the fetus (3). Bacteria gain access to gestational tissues through one of three major routes: ascending into the uterus from the lower tract, descending into the uterus from the peritoneal cavity, or via the maternal circulation (6, 13). Bacterial infections that ascend from the lower FRT are the most common route of uterine infection, and it is not known why some women suffer such infections that threaten pregnancies and fetal survival (14). In a healthy pregnancy, the uterus and growing fetus are protected from ascending infection by the cervix (12, 15, 16). The cervix has a unique role in the FRT in that it actively controls and limits microbial access by the production of mucus, inflammatory cytokines, and antimicrobial peptides (AMP) (16, 17). In nonpregnant women, the cervical mucus is a viscous fluid in the endocervical canal; however, after conception the endocervical canal develops a structure called the cervical mucus plug, which is an anatomical and immunological barrier against ascending infection (18). Indeed, analysis of the composition and antimicrobial activity of the cervical mucus plug revealed the presence of AMPs with potent antimicrobial activity (19). Also expressed in the cervical epithelia are the pattern recognition receptors such as TLRs capable of sensing the presence of microorganisms and eliciting an innate immune response characterized by the production of cytokines and AMPs (20–23). Collectively, the cervix plays a key role in the protection against ascending intra-amniotic infection. If the mucus plug is expelled or cervical length is short, the risk of ascending uterine infection increases.

Herpesviruses are the most common cause of viral-related perinatal neurologic injury in the United States (24). However, HSV-1, HSV-2, and CMV (25) are among the eight known human herpesviruses reported to induce adverse pregnancy and neonatal outcomes and have been found in the amnion, placenta, and even the lower reproductive tract of pregnant women (26). Murine gammaherpesvirus 68 (MHV68; Murid herpesvirus 4 [NC_001826.2]) is a gamma-herpesvirus of rodents that shares significant genomic colinearity with two human pathogens, EBV and Kaposi’s sarcoma-associated herpesvirus (27).

Viral survival depends on their capacity to disable host defenses, especially the innate immune system, establish latency, and secure mechanism for reactivation. One way in which viruses could un-
dermine host immunity is through the manipulation of innate immune receptors such as TLRs. We have previously reported the use of MHV68 as a murine model to determine whether a subclinical viral infection sensitized the mother to other microorganisms and induced preterm birth (15). Our published data suggested that bacteria or virus alone was not enough to evoke preterm labor, but the combination was a threat. HSV and CMV have latency periods and can be reactivated via TLR signaling (28); therefore, it can be postulated that they could affect pregnant women the way MHV68 affects pregnant mice in our model.

We have now tested the hypothesis that a viral infection reduces the ability of the pregnant cervix to protect against ascending bacterial infection. In this study, we show a dramatic difference in the capacity of the cervix to prevent ascending intrauterine bacterial infection in nonpregnant and pregnant mice, and furthermore, we demonstrate that viral infection compromises the nature of the innate immune response of the pregnant cervix predisposing to ascending intrauterine infection. Our results may explain the differential sensitivity observed in pregnant women to ascending bacterial infections.

Materials and Methods

Animals and treatments

C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME); adult mice (8–12 wk of age) with vaginal plugs were infected i.p. at embryonic day 8.5 postconception with either 1 × 10^6 PFU MHV68 in 200 μl or DMEM (vehicle). To develop the ascending bacterial model, Escherichia coli [BL21] expressing red fluorescent protein (RFP) induced by isopropyl β-D-thiogalactoside and arabinose (pZS2, Addgene plasmid 26598; Dr. M. Elowitz, University of Pennsylvania, Philadelphia, PA) were collected after reaching an OD of 0.6, and resuspended gently in 40 μl PBS. This was delivered in the vagina of mice seated with isoflurane using a 200-μl gel tip, and imaging was performed after 24 h using the Carestream In Vivo Imaging System FX PRO (Bruker). Lymphoid aggregates were dissected from the stratum basalis after gently removing the implantation site. For experiments determining gene expression and MHV68 titers in cervix without hormone treatments, mice were sacrificed 7 d postinfection, and organs were removed and stored at −80°C. In experiments comparing nonpregnant and pregnant mice, nonpregnant mice in diestrus were used. This was determined by morphology of the reproductive tract at time of sacrifice. For experiments determining the effect of systemic hormones, mice were ovariectomized, and after 21 d treated with progesterone (500 μg) and estrogen (500 ng) or vehicle, s.c. for 3 d. Both treatment groups then received injections of MHV68 and continued to get hormone or vehicle every 2 d for 7 d. Cervix and spleen were then collected, and MHV68 infection was determined by quantitative PCR (qPCR). All animals were maintained in the Yale University School of Medicine Animal Facility under specific pathogen-free conditions, and all procedures reported in this manuscript were approved by the Yale University Institutional Animal Care and Use Committee. All in vivo experiments used between three and six mice in at least two separate independent experiments.

Mycoplasma

Ureaplasma urealyticum was purchased (27618) and reconstituted in American Type Culture Collection media 2616, special modified formulation, as indicated by American Type Culture Collection. Serial dilutions of bacteria were made and incubated at 37°C until medium changed color, indicating growth. At time of color change, individual tubes were stored at 4°C until remaining dilutions exhibited growth. After 12 h, bacteria were gently pelleted and resuspended in growth medium. A small aliquot was then used to determine CFUs (1 × 10^5/ml), whereas the remaining bacteria were injected intravaginally into mice at pregnancy E16.5 with or without MHV68 infection (MHV68 at day 8.5).

Cell culture

Immortalized human ectocervical cells (ECT1; American Type Culture Collection, CRL-2614) were cultured in keratinocyte serum-free medium (17005-042; Life Technologies, Grand Island, NY) with bovine pituitary extract and human epidermal growth factor supplementation, as recommended by American Type Culture Collection, under 5% CO₂ at 37°C. In blocking studies, 500 μg fibronectin (Life Technologies) was added to ECT1 cells for 30 min and removed. Cells were then infected with MHV68 for 30 min, washed, and maintained in medium for 24 h. To block integrin α₁, the same protocol was used, but with blocking Abs for α₁ (P1B5; Millipore); all in vitro experiments were repeated three times.

MHV68 production and quantification

MHV68 expressing GFP was passaged in NIH 3T3 cells with DMEM plus 10% FBS. After lysis, supernatants were harvested, filtered (0.45 μm pore), and tiered by 2-fold serial dilutions. To detect viral titers in mice, tissues were homogenized, and 25 μg tissue from the reproductive tract or 10 μg spleen was cut into small pieces and added to lysis buffer supplemented with proteinase K. Samples were incubated at 56°C with shaking for 4–6 h, as recommended for the DNeasy blood and tissue kit (Qiagen, Valencia, CA). Cells from culture were lysed with the same buffer and vortexed at room temperature. All samples were then processed according to the DNeasy protocol. DNA concentration and purity were assessed using spectrophotometric analyses of 260/280 and 260/230 ratios. A quantity amounting to 100 ng total DNA was then assayed using primers directed against MHV68 open reading frame (ORF)53 and compared with a standard curve created using serial dilutions of purified virus. Results are reported as copies per 100 ng DNA.

RNA synthesis, cDNA synthesis, and qPCR

RNA was extracted using the TRIzol method (Invitrogen, Carlsbad, CA). RNA concentration and purity were assessed using spectrophotometric analyses of 260/280 ratios, and only samples with values of 1.7 or higher were used for PCR analysis. For quantitative analysis of mRNA, 1 μg RNA was reverse transcribed for each sample using oligo(dT) primer and Verso cDNA kit, which includes a DNase enzyme (Invitrogen). Syber Green master mix (KAPA Biosystems) and gene-specific primers were added to the reverse-transcriptase reactions that were diluted 1:10 with nuclease-free water and run on the CF960, C1000 system qPCR machine (Bio-Rad). No reverse-transcriptase controls were used to confirm that values did not represent amplification of genomic DNA, and no template controls were used to confirm lack of contamination by any reagents. Values represented were normalized to β-actin and were calculated using the ∆∆ cycle threshold (CT) method, as follows: [β-actin Ct − β-actin Ct treated − β-actin Ct control] × 2^(−Δ Ct) for negative ∆ Ct values or −Δ(Δ Ct) for positive ∆ Ct values.

Cytokines

Cytokine concentrations were determined using cytokine multiplex assays from Bio-Rad. Briefly, wells were either loaded with 50 μl prepared standard or 50 μl cell-free supernatant and incubated on an orbital shaker at 500 rpm for 2 h in the dark at room temperature. Wells were washed three times with Bio-Rad wash reagent, and samples were then incubated with 25 μl biotinylated detection Ab for 30 min, washed, and incubated with 50 μl streptavidin-PE for 10 min. After final wash, samples were resuspended in assay buffer and measured using LUMINEX 200 (LUMINEX, Austin, TX). Cytokines included in this assay are as follows: IL-1β, IL-10, GM-CSF, IFN-γ, TNF-α, IL-1α, IL-6, IL-12-p40, IL-12-p70, G-CSF, KC, MIP-1α, RANTES, MCP-1, and MIP-1b.

Western blot analysis

Tissues were homogenized in phospho-cell lysis buffer (Cell Signaling, Danver, MA), and total protein concentrations were quantified using bicinchoninic acid assay (Pierce, Rockford, IL). Twenty micrograms of total proteins were dissolved in 1× sample buffer, boiled for 5 min, and separated on a 12% SDS-PAGE gel with 6% stacking gel in 1× electrode buffer at a constant current of 70 mA for ∼2 h. The proteins were transferred to nitrocellulose membranes (Protran, 0.2 μm; Schleicher & Schuell, Keene, NH) in a Mini-Protean II Cell apparatus (Bio-Rad Laboratories, Hercules, CA) at a constant 70 V for 90 min with an ice pack. Nonfat milk (5%) was used as a blocker (Fisher Scientific, Pittsburgh, PA), and immunoblotting was performed with a 1:50 dilution of primary Abs in 2% nonfat milk at 4°C overnight. Abs were Cell Signaling CS4749S (β integrin) and Millipore AB1920 (α₁ integrin). Membranes were washed, and a 1:10,000 dilution of goat anti-rabbit or goat anti-mouse IgG-HRP conjugate (Pierce) was added, as appropriate. Membranes were washed and incubated with Western Lighting Plus (PerkinElmer, Waltham, MA) to detect immunoreactive proteins.

Statistics

Differences between means (three groups or more) were determined using ANOVA, and differences between two groups were analyzed using independent t test functions of GraphPad inSTAT statistical software (La Jolla, CA).
CA). A p value ≤ 0.05 was considered significant, and data are presented as mean ± SEM.

Results
Development of a murine model of ascending bacterial infection

Our first objective was to determine whether a nonpathogenic form of *E. coli* could ascend through the FRT in pregnant and nonpregnant mice. Consequently, we inoculated genetically engineered bacteria, *E. coli* expressing RFP (RFP-*E. coli*), into the vagina of healthy nonpregnant and pregnant mice and monitored their location using an imaging system. In nonpregnant animals, bacteria were clearly detected in the cervix and endometrial cavity within 24 h (Fig. 1A). In contrast, bacteria were not detectable in the cervix or uterine cavity of pregnant animals (Fig. 1B), indicating a dramatic resistance to microbial invasion of the uterine cavity during pregnancy. Bacteria were not observed at earlier time points in pregnant animals, suggesting there was no access, and not that the infection was there, but rapidly eliminated (data not shown).

To confirm the specificity of the signal and time course of ascending microbial invasion of the endometrial cavity, mice were inoculated in the vagina with the same *E. coli*, but expressing GFP as the reporter gene. Animals were euthanized after 12, 24, 48, and 72 h. Imaging of the genital tract was performed, and, as observed with RFP-*E. coli*, GFP-*E. coli* ascended through the uterus of the nonpregnant mice; furthermore, we observed an increase in fluorescent signal as a function of time (Fig. 1C). That was not the case in the FRT of pregnant mice, in which there was no signal at any time (data not shown). These observations indicate a dramatic difference in the permissiveness of the nonpregnant and pregnant uterus to ascending infection from the lower genital tract.

Movement of pathogenic bacteria through the mouse female reproductive tract

Because we found that the pregnant FRT is capable of preventing an ascending *E. coli* bacterial infection, we investigated whether this protection is operative with the bacteria most frequently found in the amniotic fluid of pregnant women with preterm labor, *U. urealyticum* (29–31). *U. urealyticum* was inoculated intravaginally on E15.5, and, after 24 h, decidua and lymphoid aggregates were harvested to determine whether *U. urealyticum* was present in these tissues. Similar as observed with *E. coli*, the pregnant FRT was able to prevent the migration of *U. urealyticum* toward the endometrial cavity (Fig. 2B).

Because we observed that the normal FRT, during pregnancy, is highly protective of bacterial infection, we evaluated potential factors that would alter this protection and consequently would lead to an ascending bacterial infection as observed in pregnancy complications. Having previously established a mouse model of viral infection during pregnancy using a MHV68, which causes mice to be more susceptible to bacterial products (bacterial endotoxin or LPS) (14), we tested whether a systemic viral infection in pregnant animals could alter the resistance to microbial invasion of the uterine cavity. MHV68 was injected i.p. on E8.5 of pregnancy, and *U. urealyticum* was inoculated intravaginally on E15.5 (Fig. 2A). Twenty-four hours after bacterial inoculation, we harvested decidua and lymphoid aggregates to determine whether *U. urealyticum* bacteria was present in these tissues as determined using a specific PCR assay for *U. urealyticum*. *U. urealyticum* signal was significantly higher in the decidua and lymphoid aggregates in MHV68-infected mice compared with mice that had not been infected with MHV68 virus (Fig. 2B) or a control group of pregnant animals not exposed to bacteria or virus (data not shown). These data suggest that a viral infection during pregnancy alters the capacity of the FRT to control ascending bacterial infection.

Expression of antimicrobial peptides and TLR by the uterine cervix after viral infection with MHV68

The cervix has a unique role functioning as an interface between the upper and lower FRT, providing protection to the uterine cavity against ascending intrauterine infection from the lower genital tract. During pregnancy, epithelial cells of the cervix are responsible for the formation of the mucus plug that provides a mechanical and biochemical barrier to ascending infection (19). Because we observed that a viral infection resulted in dramatic

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**FIGURE 1.** Murine model of ascending bacterial infection. Nonpregnant (A) and pregnant (B) mice were visualized 24 h after receiving intravaginal injections of *E. coli* labeled with RFP. Legend indicates that red color in nonpregnant animal has been ascending bacterial infection in utero. NT = no bacterial injection; Bacteria = intravaginal bacterial injection; *n* = 6. (C) Time course of murine model of ascending bacterial infection. Nonpregnant mice were visualized 12, 24, 48, and 72 h after receiving intravaginal injections of *E. coli* labeled with GFP. Bacteria are visualized in the vagina and cervix at 12 h, found throughout the reproductive tract at 24 and 48 h, and are diminishing 72 h postinfection.

**FIGURE 2.** *U. urealyticum* ascends into the uterus in MHV68-infected animals. Mice were infected with MHV68 or DMEM (control) at E8.5 of pregnancy, received an intravaginal injection of pathogenic *U. urealyticum* at E15.5, and were sacrificed (Sac) at E16.5 (A). NT = mice with no MHV68; MHV68 = virus-treated animals. Both groups received *U. urealyticum*; decidua and lymphoid aggregates are represented as fold difference from the same tissue from mice with no viral infection. Mice with MHV68 had higher concentrations of bacteria in the decidua and lymphoid aggregates (Lymph Agg) compared with mice without viral infection (B). *n* = 5, *p* = 0.05.
susceptibility to microbial invasion of the uterine cavity in pregnant animals, we explored whether this could be due to a change in the capacity of the cervix to control microorganisms due to alteration in the expression and function of pattern recognition receptors (TLRs), responsible for sensing microorganisms. The uterine cervix from nonpregnant and pregnant mice was analyzed for changes in TLR mRNA expression in the presence or absence of systemic MHV68 infection. Systemic MHV68 viral infection, in the nonpregnant mice, either did not change or increased the expression of specific TLRs (Fig. 3A). In contrast, the cervix of pregnant mice that had been exposed to MHV68 showed a substantial decrease in the expression of specific TLRs (Fig. 3B).

TLRs play a central role in maintaining the control of microorganisms in the cervix by sensing microbial products and eliciting an innate immune response. To determine whether these changes in TLR gene expression resulted in functional differences in the capacity of the cervix to respond to bacterial infection, pregnant mice were exposed to either MHV68 or vehicle at pregnancy E8.5, followed by an intravaginal challenge of E. coli bacteria at E15.5 of pregnancy. Because TLR ligation induces changes in cytokine/chemokine expression and AMP secretion, we characterized the cervical cytokine/chemokine profile and AMP mRNA expression. We observed a robust proinflammatory cytokine response to bacteria in the cervix of pregnant mice not exposed to MHV68 infections characterized by high tissue concentration of IL-1β, IL-6, KC, MCP-1, MIP-1α/β, IL-12, and RANTES (Fig. 4A–C). This cytokine/chemokine profile is stereotypic for a strong proinflammatory antibacterial response. A dramatically different profile was observed in MHV68-infected pregnant animals. The presence of bacteria in the lower genital tract in MHV68-infected mice was not able to elicit a similar inflammatory response as the one observed in mice, which received the same type of bacteria (Fig. 4A–C). Indeed, the cervical protein concentration of IL-12, MIP-1α and β, RANTES, and IL-6 of mice with MHV68 viral infection and bacteria was similar to those of the control group (not exposed to either bacteria or virus).

In addition, AMP mRNA expression was lower in the pregnant cervix of MHV68-infected mice than in that of pregnant animals not exposed to the virus (Fig. 4D). These observations suggest that the decreased TLR gene expression in the cervix of MHV68-infected mice is associated with a lack of response to bacteria, as demonstrated by the absence of proinflammatory cytokines and decreased expression of AMPs. These changes in the MHV68-infected pregnant mice may be functionally linked to the increased susceptibility to ascending bacterial infection.

**FIGURE 3.** MHV68 infection decreases TLR expression in the pregnant cervix. NT = no MHV68; MHV68 = mice infected with virus. TLR mRNA expression was higher or did not change in the cervix of nonpregnant MHV68-infected animals relative to noninfected controls (A), but specific TLR mRNA was significantly decreased in the cervix of MHV68-infected pregnant animals (B). Significance as denoted by bars is *p < 0.05, n = 5.

**Systemic administration of MHV68 results in viral infection of the cervix of pregnant mice**

The differences in the cervical response to viral and bacterial infections of pregnant and nonpregnant mice could be attributed to variations in the systemic response to the virus or changes in cervical viral infection. To test this postulate, we sought to determine whether the virus was directly infecting the cervix. Pregnant and nonpregnant mice received i.p. injections with MHV68, as described above; cervix and spleen samples were collected 7 d after injection and analyzed for MHV68 infection using qPCR for MHV68 ORF53. MHV68 viral infection was observed at similar concentrations in the spleen of pregnant and nonpregnant mice (Fig. 5A), but, surprisingly, MHV68 viral infection was observed in the cervix of pregnant mice, but was absent in the cervix of nonpregnant mice (Fig. 5B). These results indicate pregnancy renders the uterine cervix susceptible to a systemic viral infection.

**Sex hormones increase the susceptibility of the cervix to viral infection**

Estrogen and progesterone are major factors associated with morphologic and functional changes of the cervix, during pregnancy (18). Because we found that virus was only infecting the cervix of pregnant mice, we hypothesized high hormone concentrations during pregnancy induced modifications in the epithelium of the cervix that increase its susceptibility to viral infection. To test this premise, we ovariectomized mice and they received hormone treatment (estradiol [500 ng] and progesterone [500 μg]) or placebo (vehicle) 21 d postsurgery for 3 d (Fig. 6A), mimicking the hormonal status during pregnancy. Both treatment groups then received injections of MHV68 i.p. and continued to get hormone therapy or vehicle every 2 d for 7 d (Fig. 6A). Afterward, the cervix was collected and it was determined whether sex hormones made the cervix more susceptible to MHV68 infection. As suspected, MHV68 was detected in the cervix of animals receiving hormone therapy, but not in ovariectomized mice receiving only vehicle (Fig. 6B). These data suggest that hormonal changes associated with pregnancy induced modifications of the cervix, leading to increased susceptibility to viral infection, and this could in turn affect the role of the uterine cervix in protecting against ascending bacterial infection.

**Integrins are increased in the cervix of pregnant mice and have a role in viral entry**

Having determined that the high levels of sex hormones found during pregnancy might increase the susceptibility of the cervix to viral infection, we hypothesized that this was due to increased expression of a protein or proteins on cervical cells that may act as a receptor for viral entry. Although the specific receptor for MHV68 entry is unknown, integrins are common entry receptors for related viruses (31, 32). To test the possibility that integrins are responsible for MHV68 infection of cervical cells, we established an in vitro model using human ectocervical cells clone ECT1 that are permissive to MHV68 infection. Fibronectin is a high m.w. glycoprotein present in the extracellular matrix that binds to integrins (33); therefore, we...
Postulated that fibronectin would bind to integrins present in cervical cells and block viral entry. To test this premise, we incubated human cervical cells with fibronectin (500 μg/ml), followed by MHV68 infection. We observed a significant decrease in MHV68 infection in cells pretreated with fibronectin compared with the nonfibronectin-treated control, suggesting an integrin might be involved in viral entry into cervical cells (Fig. 7A). We then examined integrins in the cervix of nonpregnant and pregnant mice to determine whether their expression was correlated with MHV68 infectivity. Our data showed integrin α3 and β1 expression was positively correlated with MHV68 infection in the cervix of infected pregnant (high expression) and nonpregnant (low expression) mice (Fig. 7B). This was not the case for integrin α4, which was equally expressed in nonpregnant and pregnant cervices (Fig. 7B). Finally, to determine whether integrin α3, specifically, had a role in the susceptibility of MHV68 infection in the cervix, we evaluated whether specific blocking Abs for this integrin could

**FIGURE 4.** MHV68 infection reduces cytokine response to bacteria in the cervix. Cervical cytokines were measured using luminex technology; “NT” are cytokines from the cervix from animals that did not receive MHV68 or bacteria. “Bacteria” are cytokines from the cervix from animals with an intravaginal bacteria injection, but no MHV68. “MHV + Bacteria” are cytokines from the cervix of animals that received an intravaginal injection of bacteria while having a MHV68 infection. A number of cytokines and chemokines (A–C) were upregulated in the cervix of animals with artificial intravaginal bacterial infection, whereas this response was significantly reduced in animals with MHV68 infection. β defensin 1 (BD1), β defensin 3 (BD3), and β defensin 14 (BD14) mRNA expression was also reduced in cervix from MHV68-infected animals (D). Significance is denoted by bars and is *p < 0.05. n = 5.

**FIGURE 5.** MHV68 infects the cervix of pregnant mice. Both pregnant and nonpregnant mice had similar systemic infections, as determined by MHV68 ORF53 in the spleen (A), but pregnant mice also had high MHV68 titers in the cervix, whereas nonpregnant mice had no cervical infection (B). n = 4, *p < 0.001.

**FIGURE 6.** Sex hormones play a role in MHV68 infection of cervix tissue. Mice were overiectomized (OVX) and, after 3 wk, given either P4 + E2 or vehicle for 3 d; they were then injected with MHV68 and remained on either hormone treatments or vehicle for 7 d (A). Seven days postinfection, MHV68 was detected in the cervix of mice treated with hormones, but absent in vehicle-treated controls (B). n = 4, *p < 0.05.
prevent MHV68 viral infection, in vitro. As shown in Fig. 7C, we found that there was a significant decrease in the MHV68 titers in human cervical cells pretreated with α3, blocking Ab (Fig. 7C), but not with the control IgG. Collectively, these data suggest a possible mechanism whereby pregnancy can increase the susceptibility of the cervix to viral infection with MHV68 (Fig. 8).

Discussion

In the current study, we demonstrate, to our knowledge for the first time, that a viral infection of the cervix during pregnancy reduces the capacity of the lower reproductive tract to prevent bacterial infection of the pregnant uterus. Furthermore, we report that pregnancy increases the sensitivity of the cervix to viral infection, leading to changes in the expression and function of TLRs and antimicrobial products. This sensitivity is partially caused by hormonal regulation of proteins in the cervix that cause it to be permissive to virus.

Approximately 30% of all neonatal death is directly caused by preterm birth, and 50% of premature births are idiopathic (1, 34, 35). Because preterm birth represents a major health problem, it is, therefore, of the greatest importance to determine its causes, which then will allow us to develop novel ways for prevention and treatment. A strong body of evidence suggests that a majority of intrauterine bacterial infections during pregnancy result from bacteria ascending from the lower reproductive tract (2, 6). The amniotic cavity is normally sterile, and a major transition during life is the emergence from a sterile to a nonsterile environment at the time of birth. Microbial invasion of the amniotic cavity can lead to preterm labor with intact membranes, preterm premature rupture of membranes, cervical insufficiency, a short cervix, fetal sepsis, and preterm delivery. The presence of organisms in the amniotic cavity elicits an intense inflammatory response, which may evolve into a fetal inflammatory response syndrome. The latter predisposes preterm neonates to short- and long-term consequences, such as neonatal sepsis, bronchopulmonary dysplasia, and cerebral palsy. It has been estimated that one of every three preterm neonates is born to a mother with intra-amniotic infection, identified with either cultivation or molecular microbiologic techniques (36).

Two major events need to occur for bacteria to induce preterm labor, as follows: 1) bacteria need to breach the cervix and reach the decidua, amnion, and potentially the fetus; 2) bacterial numbers must reach a threshold that will trigger an inflammatory response that in turn induces preterm labor (37). A major question is how bacteria can breach the cervical barrier and reach the maternal–fetal interface. All women have microorganisms in the lower genital tract, yet these microorganisms do not gain access to the amniotic cavity during normal pregnancy. What are the mechanisms responsible for preventing ascending intrauterine infection in most pregnant women? It is widely believed that the cervical mucus plug plays an important role in host defense against microbial invasion of the amniotic cavity (19, 38). Indeed, the cervical mucus plug has antimicrobial activity (38), contains antimicrobial peptides, as well as Igs and cellular components of the innate immune system (macrophages, neutrophils, etc.) (39). Therefore, alterations of the composition or formation of the cervical plug may affect the protection against ascending bacteria.

In this study, we first evaluated the regulation of bacteria in the mouse FRT. We provide novel insights into the regulation of bacterial movement through the reproductive tract and reveal major differences depending on the reproductive stage. We observed, in our murine model, that bacteria delivered in the lower FRT is able to ascend to the uterine cavity, confirming new findings in women, showing that the nonpregnant uterus has a normal flora (2, 40). In contrast, pregnant mice were able to prevent ascending bacteria from the lower FRT. These findings could sug-

FIGURE 7. Integrins play a role in MHV68 entry into cervical cells. Human ectocervical cells (ECT1) were pretreated with fibronectin to block integrin–ligand interactions and then infected with MHV68; 24 h postinfection, MHV68 was found to be partially blocked from cells treated with fibronectin compared with cells treated with MHV68, but no fibronectin (NT) (three independent experiments) (A). Western blot analysis showed integrin α3 and β1 were upregulated in the pregnant cervix of infected mice (B), n = 3. Abs blocking integrin α3 (MHV68 + anti-integrin α3 Ab) partially blocked MHV68 infection, whereas treatment with nonspecific IgG (MHV68 + IgG) did not block MHV68 infection as compared with cells only treated with MHV68 (MHV68 only) (representative of three independent experiments with a minimum of three animals per group) (C). Significance is denoted by bars and is *p < 0.05.
gest that bacteria in the upper FRT may not be a result of changes of the type of vaginal bacteria, but of the capacity of the cervix to regulate its ascension (41). These differences correlate with the biochemical and physical differences between the pregnant and nonpregnant cervix (19, 41).

A puzzling question has been why some pregnant women develop an ascending intra-amniotic infection and others do not. In pregnancies complicated with bacterial infection, we propose that it is due to alteration in the capacity of the cervix (lower FRT) to control bacteria and not in the type of bacteria present in the vagina. In the current study, we demonstrate that, under normal conditions, U. urealyticum, the most common microorganism isolated in tissues from the maternal–fetal interface (6, 29, 42), is protected from the upper FRT by the cervix; however, if the cervix is infected with virus, the protection is decreased and U. urealyticum is able to reach the pregnant uterus. We propose that this reduced protection is due to the decrease in TLRs, antimicrobials, and inflammatory cytokines in the cervix infected with virus.

Pregnant women are more susceptible to the effects of viral infection than nonpregnant women. The mortality rate of pregnant women infected by influenza in the 1918 pandemic and in the most recent H1N1 epidemic was significantly greater than that of nonpregnant women (43). This has been attributed to either an exaggerated inflammatory response (cytokine storm caused by the prime state of activation of the innate immune system during normal pregnancy) or secondary bacterial infections (44). The latter has been a subject of interest for many years, and Dr. Luis Cruveilhier (45) is cited to have stated that “flu condemns, an additional infection executes.” Compelling evidence suggests that more than one microorganism causes many infectious diseases of humans and animals (46). Polymicrobial diseases are defined as pathologies caused by the synergistic or sequential action of infectious agents from either the same or different kingdoms (46, 47). Among the best known examples is the relationship between influenza and bacterial pneumonia. It is noteworthy that viral infections of the lower genital tract are relatively common (e.g., papilloma virus, herpes virus, HIV, etc.). Yet, there is a paucity of knowledge about the effect of such localized infections on mucosal immunity of the lower genital tract during pregnancy (48). In this study, to our knowledge, we show for the first time that a viral infection could interfere in the normal antimicrobial function of the cervix during pregnancy.

In previous studies, we used a murine γ-herpesvirus, MHV68 (15). This is a DNA virus of the same family as CMV and the human herpes viruses, which have been found in the amnion, placenta, and even the lower reproductive tract of pregnant women. These viruses all have latency periods and produce immunosuppressive factors, and many can be reactivated by regulating TLR function and expression. Naturally, MHV68 infects the nasopharyngeal tissue, leading to lytic infection and expansion in lung epithelial cells, followed by infection of B cells, macrophages, and dendritic cells, and viral latency (49). Interestingly, in our study, we found that it also infected the cervical cells of the pregnant mice. In humans, the most common viral infection of the cervix is human papillomavirus (HPV), and a handful of studies has shown that HPV infection of the placenta is associated with adverse pregnancy outcomes (50, 51). Even more recently, HPV infection of the cervix was related to placental abnormalities and preterm birth (52).

To understand why the cervix protection was compromised, we first characterized the molecular response to viral infection. One of the main characteristics of the pregnant cervix is the formation of the cervical plug, which is normally enriched with natural anti-microbial peptides such as α and β defensins that can kill bacteria by damaging their outer barriers (19) and recruit immune cells to the site of bacterial invasion. The epithelial cells further help to protect against pathogens by the appropriate expression of TLRs, secretion of cytokines, and antimicrobial peptides (53, 54). We demonstrate that MHV68 infection of the cervix decreases TLR expression and consequently dampens the antimicrobial and inflammatory response to bacteria. This effect could be part of the viral mechanism of evading host immune recognition. In contrast, TLR3, TLR9, and TLR2 are upregulated in the cervix of nonpregnant animals, potentially for heightened protection against viral infection. These results show that systemic infection regulates specific TLRs in mucosal epithelia quite differently, as compared with direct infection.

The pregnant state requires dramatic remodeling of the uterus. Sex steroid hormones and, in particular, estrogen and progesterone have a powerful effect in remodeling the uterine cervix and the myometrium. Because only the pregnant cervix was infected by MHV68, this suggested that the changes of the cervix are associated with the hormonal profile characteristic of pregnancy. This was confirmed with the observation that ovariotomized mice receiving estrogen and progesterone were more susceptible to viral infection of the cervix, suggesting that a systemic endocrine milieu can create conditions favoring viral invasion and multiplication. We were able to establish that integrin α3 plays a role in the permissiveness of the cervix to MHV68 infection, although additional factors may also be involved because the blocking studies did not completely diminish MHV68 infection.

Based on these findings, we propose the following model: commensal bacteria are located in the lower reproductive tract, and, during healthy pregnancy, the uterine cervix provides protection against bacteria ascending into the upper reproductive tract (Fig. 8A). If the protection provided by the uterine cervix is jeopardized, bacteria can ascend from the lower tract, to the decidua and amnion, leading to inflammation and pregnancy complications such as preterm birth (Fig. 8B). In summary, to our knowledge, these experiments are the first to describe a viral infection during pregnancy that alters the physiologic protection of the cervix against intrauterine infection. These studies also provide evidence that such changes are potentially mediated by altered components of the innate immune response. Together our results suggest that preterm labor is a polymicrobial disease, which requires a multifactorial approach for its prevention and treatment.

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Disclosures

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References
