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Misoprostol Impairs Female Reproductive Tract Innate Immunity against Clostridium sordellii

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Fatal cases of acute shock complicating Clostridium sordellii endometritis following medical abortion with mifepristone (also known as RU-486) used with misoprostol were reported. The pathogenesis of this unexpected complication remains enigmatic. Misoprostol is a pharmacomimetic of PGE2, an endogenous suppressor of innate immunity. Clinical C. sordellii infections were associated with intravaginal misoprostol administration, suggesting that high misoprostol concentrations within the uterus impair immune responses against C. sordellii. We modeled C. sordellii endometritis in rats to test this hypothesis. The intrauterine but not the intragastric delivery of misoprostol significantly worsened mortality from C. sordellii uterine infection, and impaired bacterial clearance in vivo. Misoprostol also reduced TNF-α production within the uterus during infection. The intrauterine injection of misoprostol did not enhance mortality from infection by the vaginal commensal bacterium Lactobacillus crispatus. In vitro, misoprostol suppressed macrophage TNF-α and chemokine generation following C. sordellii or peptidoglycan challenge, impaired leukocyte phagocytosis of C. sordellii, and inhibited uterine epithelial cell human β-defensin expression. These immunosuppressive effects of misoprostol, which were not shared by mifepristone, correlated with the activation of the Gs protein-coupled E prostaglandin (EP) receptors EP2 and EP4 (macrophages) or EP4 alone (uterine epithelial cells). Our data provide a novel explanation for postabortion sepsis leading to death and also suggest that PGE2, in which production is exaggerated within the reproductive tract during pregnancy, might be an important causal determinant in the pathogenesis of more common infections of the gravid uterus. The Journal of Immunology, 2008, 180: 8222–8230.

The combination oral regimen of the progesterone/glucocorticoid receptor antagonist mifepristone (also known as RU-486) and the PGE1 analog misoprostol received approval by the U.S. Food and Drug Administration in September 2000 for use in the termination of pregnancy of \(<=49\) days duration. Soon after, there were five reported cases of otherwise healthy women who developed (and died from) an acute “toxic shock” syndrome complicating Clostridium sordellii endometritis within days of undergoing medical abortion with these agents (1, 2). These five cases were associated with the off-label administration of an increased dose of misoprostol (800 \(\mu\)g) applied directly into the vagina, as opposed to the approved oral use. More recently, an additional three cases of medical abortion-associated clostridial endometritis have been reported (3), with two cases involving the intravaginal administration of misoprostol. Although much consideration has been given to the potential role of mifepristone in the pathogenesis of this infection (4), a suppressive effect of misoprostol on innate immune responses has not received attention.

Misoprostol, a synthetic structural analog of PGE1, binds to and activates each of the four heptahelical G protein-coupled E prostaglandin (EP)3 receptors normally ligated by the endogenous lipid mediator PGE2. Because misoprostol, unlike PGE2, does not activate the I prostaglandin receptor of prostacyclin (5), it is described functionally as a PGE2 pharmacomimetic. PGE2 suppresses innate immune responses both in vitro and in vivo (6, 7) via its ability to increase intracellular levels of cAMP upon ligation of the EP2 and EP4 receptors. The association of high-dose, intravaginal misoprostol administration with clostridial endometritis prompted the hypothesis that this PGE2 mimetic suppressed local innate immune responses to C. sordellii, thereby predisposing to the development or progression of C. sordellii infection of the uterus.

To test this hypothesis, an animal model of C. sordellii endometritis was developed in nonpregnant rats and used to explore the influence of misoprostol on survival and bacterial clearance in vivo and to examine whether the route of administration of misoprostol (i.e., intrauterine (i.u.) vs intragastric (i.g.) injection) is an important determinant of its actions in this setting. In vitro studies were conducted to investigate the regulation by misoprostol of key cellular participants in female reproductive tract innate immunity (uterine macrophages (UMs) and uterine epithelial cells (UECs)) (8). This combined in vivo/in vitro approach revealed that misoprostol, not mifepristone, might be a causal risk factor in clostridial uterine infections following medical abortion, particularly when

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the drug is delivered at high doses directly into the reproductive tract.

Materials and Methods

Animals

The 125- to 150-g female Wistar rat and CBA/J mice were from Charles River Breeding Laboratories and treated per National Institutes of Health guidelines for the use of experimental animals with the approval of the University of Michigan Animal Care and Use Committee.

Reagents

RPMI 1640 and DMEM/F12 mediums, and penicillin/streptomycin/amphotericin B solution were from Life Technologies-Invitrogen. FBS and charcoal-stripped PBS were from HyClone Laboratories. Reinforced clostridial medium was from BD Biosciences. AH-6809, butaprost free acid, mifepristone, misoprostol free acid, PGE2, and sulprostone were from Cayman Chemicals. The nonspecific phosphodiesterase inhibitor IBMX (3-isobutyl-1-methylxanthine) was purchased from EMD Biosciences. Ono-AEI-329 was a gift from Ono Pharmaceutical. Cytochalasin D, FITC, LPS of Escherichia coli strain O55:B5, peptidoglycan (PGN) from Bacillus subtilis, and trypan blue were from Sigma-Aldrich. Both LPS and PGN were prepared as stock solutions in endotoxin-free water (Sigma-Aldrich). Recombinant human β-defensins (hβd)-1 (36 aa) and hβd-2 (41 aa), recombinant mouse GM-CSF, human IL-1β, and human TNF-α were purchased from PeproTech.

Bacteria

C. sordellii strain 9714 and Lactobacillus crispatus strain 33820 were from the American Type Culture Collection (ATCC) and were grown in broth cultures. For infection, strain 9714 was grown in 37°C in reinforced clostridial medium within GasPak anaerobic canisters (BD). The OD of bacterial cultures to light at 600 nm was used to estimate bacterial concentrations from a previously determined standard curve of CFU vs OD600. Estimated bacterial concentrations were confirmed by serial 10-fold dilutions on solid agar composed of reinforced clostridial medium containing 1.5% (w/w) agar, incubated anaerobically. For some experiments, heat-killed C. sordellii were prepared by heating to 121°C for 30 min. E. coli strain BL21 from Amersham Biosciences was cultured under aerobic conditions at 37°C (5% CO2) in tryptic soy broth. Bacterial concentrations were estimated using an OD600 for C. sordellii, with confirmation of CFU estimates using serial 10-fold dilutions on solid tryptic soy agar plates.

A model of i.u. infection

The model of i.u. infection was designed on a previously published protocol (9). In anesthetized animals, a low 2.0- to 2.5-cm midline abdominal incision exposed the right uterine horn, which was ligated at the cervical junction to prevent loss of the inoculum. A total of 100 μL of PBS containing the desired CFU of bacteria (with or without misoprostol or DMSO vehicle) was injected into the right uterine horn. When indicated, misoprostol was directly injected into the lumen of the stomach during surgery, under direct visualization. To determine bacterial loads, rats were euthanized 18 h after infection (before death from infection) and the uterus removed en bloc. Uterine tissues were homogenized, and serial dilutions were cultured to enumerate CFU. The median LD50 was calculated using the Reed-Muench method (10).

Peripheral blood cell counts

Following euthanasia 18 h after infection, blood was removed via cardiac puncture into EDTA-containing sterile tubes. Determination of peripheral blood white blood cell counts and hematocrit levels was performed by the University of Michigan Unit for Laboratory Animal Medicine’s Animal Diagnostic Laboratory using a Hemavet cell analyzer (Drew Scientific).

Cells

ECC-1 human UECs were provided by Dr. B. Lessey (Greenville Hospital System, Greenville, SC) (11). RL 95-2 human UECs were from the ATCC. Human blood monocytes and neutrophils were isolated as described (12). Monocytes were cultured for 14 days to allow differentiation into macrophage-like cells. Resident rat peritoneal macrophages were isolated and adherence-purified from rats according to a published protocol (13). UECs were exposed to 5 μg/ml recombinant CD68 (Ald awarded) for 1 h. UEA 1-human UECs were adhered to chamber slides, fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.1% Triton X-100 in PBS with 0.1% BSA, then blocked with 1% BSA in PBS containing nonimmune goat serum (Pierce). Samples from each well were then serially 10-fold diluted in sterile, deionized, and distilled H2O in duplicate in 96-well U-bottom plates and 1 μL of each dilution (104, 105, and 106) was plated onto agar (reinforced clostridial medium broth plus 1.5% agar for C. sordellii and Luria-Bertani broth plus 2% agar for E. coli) and incubated anaerobically (C. sordellii) or aerobically (E. coli) at 37°C overnight. CFU were enumerated under direct visualization. To determine bacterial loads, rats were euthanized 18 h after infection (before death from infection) and the uterus removed en bloc. Uterine tissues were homogenized, and serial dilutions were cultured to enumerate CFU. The median LD50 was calculated using the Reed-Muench method (10).

Phagocytosis assay

Assay of phagocytosis of unopsonized C. sordellii by rat peritoneal macrophages and human polymorphonuclear neutrophils was modeled after our protocol for FITC-labeled, IgG-opsonized E. coli (6). Heat-killed, FITC-labeled C. sordellii were prepared according to a published method (16). Experiments using FITC-labeled E. coli opsonized with polyclonal IgG (both from Molecular Probes) were conducted in a similar manner as previously published (6).

Cytokine and cAMP determination

Uterine homogenate and serum TNF-α levels were determined by a commercially available enzyme immunoassay (EIA) kit (R&D Systems). Macrophages were exposed to mifepristone or vehicle for 24 h and then for 1 h to eicosanoid compounds, followed by an 18-h incubation with LPS (100 ng/ml), PNG (10 μg/ml), or heat-killed C. sordellii (multiplicity of infection at 100:1). Cell-free supernatants were stored at −80°C and TNF-α and MIP-1α levels were quantified later by commercially available EIA kits (R&D Systems). For cAMP determination, rat peritoneal macrophages or UECs were incubated for 30 min with IBMX (250 μM), followed by 15 min in the presence or absence of compounds of interest. Intracellular cAMP levels were determined by EIA according to the manufacturer (Assay Designs).

Real-time PCR analysis of β-defensins

UECs were pretreated with misoprostol for 1 h and incubated with cytokes (IL-1β, TNF-α) or vehicle for 24 h. Total RNA was extracted and RT-PCR performed using the following primer sequences: hβd-1 5'TCG TGT TTA CTC TCT GCT TAC T-3 and 5'-TTGGG CGG ATG TCC ACG TCA-3'; hβd-2 5'-GGA GCC CTT TCT GAA TCC GCA-3' and 5'-TTGGG CGG ATG TCC ACG TCA-3'; hβd-2 5'-CCCA GTG AGC ATG GCC GTT TC-3 and 5'-TTGGG CGG ATG TCC ACG TCA-3'; and hβd-2 5'-CCCA GTG AGC ATG GCC GTT TC-3 and 5'-TTGGG CGG ATG TCC ACG TCA-3'. cDNA was generated and analyzed for hβd-1, hβd-2, and β-actin expression by quantitative RT-PCR on an ABI Prism 7000 ThermoCycler (Applied Biosystems).

Antimicrobial activity assays for hβd-1 and hβd-2

C. sordellii or E. coli (105 CFU) were treated with varying concentrations of either hβd-1 or hβd-2 (as indicated in each experiment; range 0–20 μg/ml) for 3 h at 37°C under anaerobic (C. sordellii) or aerobic (E. coli) conditions in triplicate in a 96-well plate. For time-course experiments, C. sordellii were exposed to 20 μg/ml hβd-2 for various times as indicated in each experiment. Assays were performed in a total well volume of 50 μl containing 105 CFU of bacteria suspended in 10 mM sodium phosphate buffer (pH 7.4) containing 0.01% BSA and the defense of interest (or vehicle). Samples from each well were then serially 10-fold diluted in sterile, deionized, and distilled H2O in duplicate in 96-well U-bottom plates and 1 μl of each dilution (104, 105, and 106) was plated onto agar (reinforced clostridial medium broth plus 1.5% agar for C. sordellii and Luria-Bertani broth plus 2% agar for E. coli) and incubated aerobically (C. sordellii) or aerobically (E. coli) at 37°C overnight. CFU were enumerated the next day. Due to interprotocol variability in CFUs, data were expressed as a percentage of bacterial survival relative to untreated bacteria.

Immunocytostaining

Macrophages, infected with heat-killed FITC-labeled C. sordellii or left uninfected, were adhered to chamber slides, fixed with 4% parafomaldehyde for 30 min, permeabilized with 0.1% Triton X-100 in PBS with 0.1% BSA, then blocked with 1% BSA in PBS containing nonimmune goat serum. Primary Ab against CD68 (Ald awarded) was prepared in 1% BSA in PBS (1/200) and applied for 1 h at 37°C. Mounts were washed three times with 1% BSA in PBS and FITC-conjugated goat anti-rabbit secondary (1/250) was added for 1 h at 37°C. After washing three times, preparations were mounted using VectaShield mounting medium containing 4',6-diamidino-2-phenylindole (Vector Laboratories). Fluorescence was visualized with a Nikon Labophot 2 microscope equipped for epifluorescence.

Statistical analyses

Data were analyzed with Prism 4.0 (GraphPad Software). Comparisons between two groups were performed with Student’s t test. Comparisons

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The i.u. injection of misoprostol (MISO) worsens C. sordellii uterine infection. A. Nonpregnant rats (n = 5–7 per group) were inoculated with C. sordellii in the right uterine horn on day 0 and monitored for survival. PBS vehicle-treated rats (negative control) did not die (n = 5; data not shown). B. Rats were infected with C. sordellii (1 × 10^5 CFU) into the right uterine horn as in A, with the administration of misoprostol (MISO, 300 μg/kg) injected into the ipsilateral uterine horn (i.u.) or stomach (i.g.). Rats per group (n = 10, i.g.) and (n = 15, i.u.) are shown. C. Rats were infected as in B with the concomitant administration of misoprostol 0–600 μg/kg injected i.u. into the ipsilateral uterine horn and survival was determined at 72 h after inoculation (n = 5–7 rats per group). D. Rats were infected with L. crispatus (1 × 10^8 CFU) into the right uterine horn, with the administration of vehicle or misoprostol 300 μg/kg injected i.u. into the ipsilateral uterine horn. Rats per group (n = 5) are shown.

Results
The i.u. injection of misoprostol enhances lethality of C. sordellii uterine infection

The lack of an animal model has hampered progress in understanding C. sordellii infections, and motivated us to develop a new model of i.u. infection in nonpregnant Wistar rats. The i.u. inoculation of C. sordellii induced a rapidly lethal infection that was inoculum dose-dependent (Fig. 1A). Rats became noticeably ill with typical sickness behaviors (e.g., bradycardia, piloerection, ocular dryness, tachypnea) within 16–24 h of infection and the majority of deaths occurred within 24–48 h of inoculation. The estimated LD₅₀ for this model was 5 × 10^4 CFU. We compared two routes of administration of misoprostol (i.g. vs i.u.), given at the time of infection, using a dose of misoprostol known to induce abortion in rats (300 μg/kg) (17). As illustrated in Fig. 1B, the mortality of rats given 1 × 10^6 CFU C. sordellii was unaltered by misoprostol administered i.g. However, the mortality of rats given misoprostol i.u. (mimicking intravaginal delivery in humans) concomitant with C. sordellii exhibited a dramatic increase in mortality (~80% dead within 4 days). This effect was dose-dependent (Fig. 1C). Moreover, the i.u. administration of misoprostol (300 μg/kg) increased the 7-day mortality of rats infected with a non-lethal inoculum of 1 × 10^7 CFU (Fig. 1A) to 50% (n = 10 rats, p < 0.05; data not shown). Thus, a 300-μg/kg dose of misoprostol reduced the LD₅₀ from C. sordellii 50-fold, from 5 × 10^8 to 1 × 10^6 CFU. Misoprostol i.u. administered to uninfected rats did not elicit sickness behavior or mortality and misoprostol did not impair the growth of C. sordellii in vitro (data not shown).

To determine whether the suppressive effects of misoprostol on the host defense against C. sordellii were potent enough to confer enhanced virulence to relatively nonpathogenic bacteria, rats were infected with L. crispatus, a commensal member of the human vaginal microbiota. Inoculation of 1 × 10^5 CFU of L. crispatus did not induce sickness behavior (data not shown) or death (Fig. 1D), and the coadministration of misoprostol had no effect on either of these endpoints. These data support the hypothesis that misoprostol impairs host defense against pathogenic C. sordellii when local (reproductive tract) concentrations of the drug are high.

The i.u. injection of misoprostol reduces bacterial clearance and TNF-α production in vivo

Notably, misoprostol (300 μg/kg) administered i.u. reduced the capacity of infected rats to control bacterial proliferation in vivo, as evidenced by the 3.2-fold increase in the uterine bacterial load observed in the presence of the drug compared with vehicle (18 h after infection, p < 0.05) (Fig. 2A). The use of i.u. misoprostol was also associated with significantly higher circulating white blood cell counts when compared with vehicle-treated animals (mean ± SE, 4.9 ± 0.44 × 10⁸ cells/μl vs 2.65 ± 0.44 × 10⁸ cells/μl, respectively, for n = 3 rats per group; p < 0.05). Human C. sordellii infection is associated with hemocencentration (18). The hematocrits of rats treated with misoprostol were significantly greater than those treated with vehicle (57.2 ± 0.65% vs 48.5 ± 3.13%, respectively, for n = 3 rats per group; p < 0.05).

Misoprostol (300 μg/kg) administered i.u. suppressed local TNF-α production in response to infection (Fig. 2B). Eighteen hours after infection, uterine TNF-α levels were 266.7 ± 59.2 pg/ml of uterine tissue in vehicle-treated animals and were 128.0 ± 24.8 pg/ml of uterine tissue in misoprostol-exposed rats (n = 6 rats per group; p < 0.05). The levels of TNF-α in the plasma at this same time point were not significantly different (mean plasma TNF-α level, 6.3 ± 1.5 pg/ml and 11.8 ± 4.3 pg/ml among three or more experimental groups were performed with ANOVA followed by the Bonferroni correction as indicated. Comparison of survival between two groups was performed using the log-rank test. Differences were considered significant for p ≤ 0.05.

Materials and Methods

FIGURE 1. The i.u. injection of misoprostol (MISO) impairs C. sordellii uterine infection. A. Rats were infected with C. sordellii (1 × 10^5 CFU) into the right uterine horn as in A, with the administration of misoprostol (MISO, 300 μg/kg) injected into the ipsilateral uterine horn (i.u.) or stomach (i.g.). Rats per group (n = 10, i.g.) and (n = 15, i.u.) are shown. C. Rats were infected as in B with the concomitant administration of misoprostol 0–600 μg/kg injected i.u. into the ipsilateral uterine horn and survival was determined at 72 h after inoculation (n = 5–7 rats per group). D. Rats were infected with L. crispatus (1 × 10^8 CFU) into the right uterine horn, with the administration of vehicle or misoprostol 300 μg/kg injected i.u. into the ipsilateral uterine horn. Rats per group (n = 5) are shown.
Misoprostol increases cAMP in macrophages

By their capacities for microbial ingestion and killing as well as the elaboration of proinflammatory cytokines and chemokines, macrophages play an important role in immune surveillance and pathogen clearance within the uterus, as they do in other organs. Previous studies demonstrate that immunosuppression of macrophage functions by PGE2 is driven by the activation of adenylate cyclase and subsequent triggering of cAMP-dependent signaling networks (6, 19, 20). Misoprostol (1–10 µM), like PGE2, increased cAMP production in primary rat peritoneal macrophages (Fig. 3A), providing a mechanistic basis for immunosuppressive effects of the drug on this leukocyte population. Misoprostol was not as potent as PGE2 on a molar basis, which may reflect its lower binding affinity for EP receptors (5). The highly specific EP2 and EP4 agonists butaprost free acid, or the EP4 agonist Ono-AE1-329, markedly inhibited macrophage TNF-α production by rat peritoneal macrophages challenged with PGN (Fig. 4C). That these effects were driven by cAMP-dependent signaling is suggested by the lack of ability of sulprostone to regulate TNF-α production (Fig. 4C). To confirm this result in a reproductive tract macrophage, rat UMs (expressing the macrophage cell surface marker CD68) were isolated. When challenged with PGN, both misoprostol and PGE2, but not sulprostone, extinguished TNF-α production by UMs (Fig. 4D). Misoprostol and PGE2 similarly blocked production of the chemokine MIP-1α by rat UMs (data not shown).

Macrophages were then challenged with intact bacteria. To reduce confounding effects of C. sordellii toxin production on macrophages in vitro, heat-killed bacteria were used. The production of TNF-α by rat macrophages activated with heat-killed C. sordellii was proportional to the duration of incubation with the microbes as well as the inoculum size (data not shown). Pretreating macrophages with misoprostol greatly suppressed the inflammatory generation of TNF-α in response to C. sordellii (Fig. 4E). This result was robust and not limited to rat cells, as similar degrees of suppression were seen using mouse peritoneal macrophages, mouse BMDMs, and human peripheral blood monocyte-derived

Misoprostol inhibits macrophage inflammatory mediator generation

Both misoprostol and PGE2 suppress proinflammatory cytokine production by macrophages stimulated with the Gram-negative bacterial outer membrane component LPS (21, 22). As expected, misoprostol and PGE2 inhibited macrophage TNF-α production in response to LPS, whereas the EP1/EP3 agonist sulprostone had no effect (Fig. 4. A and B). Less is known, however, about the regulation of cytokine production in response to pathogen-associated molecular patterns of Gram-positive bacteria, such as PGN, or to intact Gram-positive organisms like C. sordellii. Misoprostol and PGE2 each inhibited the production of TNF-α by rat peritoneal macrophages challenged with PGN (Fig. 4C). That these effects were driven by cAMP-dependent signaling is suggested by the lack of ability of sulprostone to regulate TNF-α production (Fig. 4C). To confirm this result in a reproductive tract macrophage, rat UMs (expressing the macrophage cell surface marker CD68) were isolated. When challenged with PGN, both misoprostol and PGE2, but not sulprostone, extinguished TNF-α production by UMs (Fig. 4D). Misoprostol and PGE2 similarly blocked production of the chemokine MIP-1α by rat UMs (data not shown).

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**FIGURE 3.** Misoprostol increases cAMP in macrophages via EP2 and EP4 activation. A, Rat peritoneal macrophages were treated with PGE2 or misoprostol (MISO) at the concentrations indicated (15 min) in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 250 µM). Intracellular cAMP levels were determined by EIA. Mean values were determined in triplicate from one of three independent experiments with similar results. B, cAMP levels were determined as in A with PGE2, the EP2 agonist butaprost free acid, or the EP4 agonist Ono-AE1-329. Mean values were determined in triplicate from one of three independent experiments with similar results. ***, p < 0.01 vs untreated cells.

**FIGURE 4.** Misoprostol impairs macrophage TNF-α production in response to LPS, PGN, or C. sordellii. A, Rat peritoneal macrophages were treated with PGE2 or misoprostol (MISO) at the concentrations indicated (60 min) before challenge with LPS (100 µg/ml) for 18 h. B, Rat peritoneal macrophages were treated with PGE2, misoprostol, or sulprostone (SP) at 10 µM concentration (60 min) before challenge with LPS (100 µg/ml) for 18 h. C, Rat peritoneal macrophages were pretreated for 1 h with PGE2, misoprostol, or sulprostone (SP) at the indicated concentrations and then exposed to PGN (10 µg/ml) for an additional 18 h. D, Rat UMs were treated as in C and supernatant TNF-α levels were determined. E, Rat peritoneal macrophages (rat PMs), mouse peritoneal macrophages (mouse PMs), mouse BMDMs, and human monocyte-derived macrophages (human MDMs) were pretreated for 1 h with misoprostol 1 µM and then exposed to heat-killed C. sordellii (at a multiplicity of infection of 100:1) for an additional 18 h. TNF-α levels were measured in cell-free supernatants and data are expressed as a percentage of inhibition of C. sordellii-treated controls in the absence of misoprostol. TNF-α levels in all experiments in cell-free supernatants were assessed by EIA. Mean data ± SEM from one of at least three independent experiments performed in triplicate. ***, p < 0.01 and ***, p < 0.001 vs control.
Misoprostol suppresses the phagocytosis of unopsonized C. sordellii

PGE₂ inhibits macrophage phagocytosis of unopsonized Gram-negative and Gram-positive bacteria (23, 24) as well as IgG-opsonized pathogens (6). Little is known about the effects of misoprostol on phagocytosis. To test the ability of misoprostol to inhibit phagocytosis of unopsonized C. sordellii, a fluorometric assay of phagocytosis using FITC-labeled C. sordellii was developed. The ingestion of FITC-labeled C. sordellii by rat peritoneal macrophages was time- and inoculum-dependent (data not shown). A 5-min preincubation with misoprostol significantly inhibited phagocytosis of FITC-labeled C. sordellii at a maximal inhibition of 63.1 ± 5.6% at 1 µM (Fig. 5A). Misoprostol and PGE₂ similarly impaired the ingestion of unopsonized FITC-labeled C. sordellii by human blood neutrophils (44.7% and 36.2% inhibition with 1 µM misoprostol or PGE₂, respectively; p < 0.001 for either compound vs vehicle-treated cells; data not shown). Like PGE₂, misoprostol suppressed FcγR-mediated phagocytosis by rat peritoneal macrophages (Fig. 5B), a finding of potential clinical relevance given the relatively high concentrations of IgG in cervicovaginal secretions. Thus, misoprostol impairs both inflammatory mediator generation and phagocytosis in C. sordellii-challenged macrophages, which might contribute to the effects of this drug in vivo. Mifepristone (1–10 µM) did not inhibit the phagocytosis of C. sordellii in these studies (data not shown).

Misoprostol increases cAMP in human UECs

UECs provide an indispensable physical barrier between the uterine lumen and underlying cells and also recognize and respond to pathogens by elaborating cytokines and chemokines as well as small antimicrobial peptides (e.g., β-defensins) to directly control bacterial interlopers. Human endometrial epithelial cells are reported to express EP2 and EP4 receptors in situ (25). To establish the ability of these receptors in UECs to respond to misoprostol, intracellular cAMP production was assessed in two human UEC lines: ECC-1 and RL 95-2 cells. In both cell lines misoprostol (and PGE₂) increased intracellular cAMP (Fig. 6A). Interestingly, only EP4 receptors appeared functionally coupled to adenylate cyclase in UECs as Ono-AE1-329 increased cAMP to the same extent as PGE₂, whereas butaprost free acid had no effect (Fig. 6B). This response is consistent with the reported presence of EP4 and lack of EP2 in RL 95-2 cells (26), though we found both mRNA and protein expression of EP2 and EP4 in the ECC-1 cells (data not shown). The lack of functional coupling of the EP2 receptor to adenylate cyclase in ECC-1 cells requires further study.

Human β-defensins are directly toxic to C. sordellii in vitro

Like other epithelial cell populations, UECs generate a variety of antimicrobial peptides that prevent colonization and infection by directly killing microbes. The β-defensins are among the best characterized of the antimicrobial peptides. Two of the best-characterized human defensins are hβd-1 and hβd-2. The constitutively expressed hβd-1 and the cytokine-inducible hβd-2 are both produced by human UECs (27). Both ECC-1 and RL 95-2 cells express hβd-1 basally, whereas only RL 95-2 cells produced detectable hβd-2 mRNA or protein upon activation with proinflammatory cytokines such as TNF-α or IL-1β. There are no published data regarding the activities of hβd against clostridial pathogens. As shown (Fig. 7A), both human defensins exhibited concentration-dependent killing of C. sordellii. Maximal bacterial activity was identified at ~10 µg/ml and hβd-2 was slightly more potent than hβd-1 against C. sordellii. Bactericidal responses against C. sordellii were similar to those observed against the Gram-negative pathogen E. coli (data not shown). The bactericidal effect of β-defensins was rapid and time-dependent, as exemplified for hβd-2 (Fig. 7B).

Misoprostol increases UEC β-defensin expression

It is not known whether PGE₂ or its analogs regulate β-defensin expression, although it was recently reported that the pharmacological suppression of PG synthesis by infected human gingival epithelial cells resulted in increased hβd-2 production (28). Notably (Fig. 7C), hβd-1 mRNA levels fell ~80% in response to misoprostol (10 µM; 24 h of exposure) in both ECC-1 and RL 95-2 cells. Similarly, the TNF-α induced transcription of the hβd-2 gene in RL 95-2 cells was significantly inhibited by 10 µM misoprostol (Fig. 7D). Similar results were observed when IL-1β was used to stimulate hβd-2 in RL 95-2 cells (data not shown). Using a commercially available EIA for hβd-2, the mRNA results were confirmed at the protein level (Fig. 7E). These effects of misoprostol were shared by PGE₂ (data not shown). Exposure of UECs to C. sordellii or mifepristone did not consistently alter hβd-1 or hβd-2 expression (data not shown).
administration of this synthetic PGE2 analog. In this study, we
Seven of these cases were associated with the off-label intravaginal
tritis associated with misoprostol use have been reported (3).
largely uninvestigated. To date, eight cases of clostridial endome-
complicating medical abortion has thus far gone unexplained and
provide evidence to support a previously unexplored hypothesis
that high local concentrations of misoprostol suppress innate im-
response include macrophages and epithelial cells (8). Macrophages
and reduced amounts of Ig in uterine secretions (30). A more re-
cents mouse study reported that pharmacologically administered
PGE2 facilitated the establishment of chlamydial infections of the
murine female reproductive tract (31). Although little is known
regarding the potential of misoprostol to suppress reproductive
tract innate immunity, it is relevant that a recent comprehensive
review of the literature (n = 46,421 medical abortions) reported an
overall rate of diverse infectious complications of 0.21% when
misoprostol was given orally that increased to 1.33% (∼6.3-fold)
with intravaginal administration (32).

The present work describes a novel animal model of C. sordelli
fection. Uterine infection with this pathogen was rapidly fatal
when rats were infected with 10^8–10^10 microorganisms. The rel-
atively high inoculum size required to cause death was similar to
the bacterial loads required for Clostridium perfringens to cause
necrotic soft tissue infections in a mouse model (33), suggesting
that the innate immune system of the rat uterus can control rela-
atively large burdens of C. sordelli in vivo. The i.u. inoculation of
rats with C. sordelli led to a rapidly fatal disease that was made
significantly worse by the concomitant administration of misopros-
but only when the drug was delivered into the uterus directly.
Gastric delivery of misoprostol did not alter the virulence of this
pathogen, reflecting the relative lack of human cases associated
with the oral administration of misoprostol. Interestingly, miso-
prostol enhanced the virulence of the toxin-producing pathogen C. sordelli, but did not turn a relatively nonpathogenic commensal
organism like L. crispatus into a lethal opportunistic bacterium.
This finding might help explain the apparently specific association
between medical abortion and clostridial endometritis, rather than
infections caused by more commonly encountered pathogens.
Whether misoprostol increases the virulence of other female re-
productive tract pathogens (or commensal bacteria) remains to be
determined.

**Human C. sordelli infection is marked by leukocytosis and
hemocoagulase (18), so the augmentation of these parameters
by misoprostol also suggests a more severe infection and capillary
leak. In addition to causing an increase in mortality, i.u. adminis-
tered misoprostol significantly increased both the circulating leu-
kocyte count and hematocrit level of infected rats. Moreover, i.u.
administered misoprostol impaired the clearance of C. sordelli
from the uterus (Fig. 2A), suggesting that cells of the innate im-
une system were pharmacological targets of this PGE2 analog.
An impairment of uterine macrophage function, in particular, was
suggested by the significant inhibition of local TNF-α generation
following C. sordelli infection in misoprostol-treated animals be-
cause macrophages are believed to be the major cellular producer
of this cytokine within the uterus (34).

Two key cellular participants in the uterine innate immune re-
sponse include macrophages and epithelial cells (8). Macrophages
make up ∼25% of the total leukocyte count of the decidua during
during early pregnancy, the remainder being predominantly lymphocytes
(35). Decidual macrophages are of paramount importance in host
defense against bacterial infection and also regulate immunity at
the fetal/maternal interface to induce tolerance of the fetus by the
mother (36). Few studies have specifically examined host-microb-
ial interactions between invading bacterial pathogens and female
reproductive tract macrophages. UECs not only provide an indis-
pensable physical barrier between the uterine lumen and underly-
ing cells, they also produce small antimicrobial peptides (e.g.,
β-defenseins) to directly control bacterial interlopers (37). We hy-
thesized that both macrophages and UECs would be pharmaco-
logical targets for misoprostol actions.

The suppression of macrophage innate immune functions by
PGE2 is mediated by the intracellular second messenger cAMP

**Discussion**

The pathogenesis of clinical cases of fatal C. sordelli endometritis
complicating medical abortion has thus far gone unexplained and
largely uninvestigated. To date, eight cases of clostridial endome-
tritis associated with misoprostol use have been reported (3).
Seven of these cases were associated with the off-label intravaginal
administration of this synthetic PGE2 analog. In this study, we
provide evidence to support a previously unexplored hypothesis
that high local concentrations of misoprostol suppress innate im-
une defenses against C. sordelli within the female reproductive tract.
Our in vitro and in vivo data provide the first experimental
insight into the immune responses necessary for clearance of C. sordelli in the reproductive tract.

Previous work by other investigators suggests that exogenously
applied PGE2 analogs can reduce innate immune defenses within
the reproductive tract. The stable analog 16,16-dimethyl-PGE2 (an
agonist of the EP2, EP3, and EP4 receptors) shares with misopros-
tol its uterotonic facilitation of medical abortion (29). Slama et al.
(30) administered the 16,16-dimethyl-PGE2 directly into the ma-
ternal cervix of dairy cows for 1 wk after calf delivery. This treat-
ment led to increased purulent uterine secretions, an increase in the
frequency and severity of bacterial contamination of the uterus,

![Figure 7](http://www.jimmunol.org/)

**Figure 7.** Misoprostol impairs hβd production by UECs. A. C. sor-
delli (10^6 CFU) were incubated in the presence or absence of hβd-1 or
hβd-2 for 3 h under anaerobic conditions. Data shown are mean ± SEM of
three independent experiments, expressed as a percentage of the growth of
bacteria not exposed to defensins. B, C. sordelli were incubated as in A, in
the presence or absence of 20 μg/ml hβd-2 for varying times under ana-
erobic conditions. Data shown are mean ± SEM of one representative
experiment of two performed in triplicate. Results are expressed as a per-
centage of the growth of bacteria not exposed to defensins. C. Real-time
PCR was used to determine the levels of mRNA for hβd-1 (relative to
GAPDH) in ECC-1 and RL 95-2 cells exposed for 18 h to misoprostol (MISO, 10 μM) or vehicle. Data are mean of three determinations.
D, Real-time PCR was used to determine the levels of mRNA for hβd-2
(relative to GAPDH) in TNF-α-treated (10 ng/ml) RL 95-2 cells exposed
overnight to misoprostol (10 μM) or vehicle. Data are mean of three de-
terminations. E, The hβd-2 protein levels in the cell supernatants from D
were determined by EIA (n = 3). *, p < 0.05 vs untreated cells (C) or
TNF-α exposed cells (D and E).
Misoprostol is known to increase cAMP in macrophages (21), a finding we confirmed. Our results suggest that both EP2 and EP4 mediate this effect on rat macrophages. Misoprostol potently suppressed the ability of macrophages to generate TNF-α in response to stimulation with either PGN or C. sordellii, supporting the in vivo results. Although misoprostol was previously reported to suppress macrophage cytokine production in response to the Gram-negative cell membrane component LPS (21, 22), this time is, to our knowledge, the first misoprostol to have been shown to suppress cellular responses to Gram-positive bacterial pathogens.

Misoprostol dose-dependently inhibited the ability of macrophages (and human blood neutrophils) to phagocytose unopsonized C. sordellii. This effect was also likely mediated by cAMP, as we also found that cAMP analogs similarly impaired C. sordellii ingestion (data not shown). Our data support a recent study demonstrating that the administration of a single dose of misoprostol to healthy human volunteers suppressed the ability of circulating leukocytes to phagocytose E. coli (38). It is unclear what receptor-mediated phagocytic pathways are used by macrophages during the uptake of unopsonized C. sordellii, and is an area requiring further study. The phagocytosis of the related pathogen C. perfringens involves class A scavenger receptors and a minor contribution from mannose receptors (39). Mifepristone, conversely, did not suppress macrophage phagocytosis of C. sordellii (data not shown), consistent with recent reports showing a lack of regulation of macrophage phagocytosis by this agent (40, 41).

We speculate that macrophages are important cellular participants in the defense against clostridial uterine infection and further hypothesize that the suppression of macrophage functions by misoprostol contributes to the enhanced proliferation and virulence of C. sordellii. This is important to note, as macrophages were recently found to be critical to immune defense against clostridial infection in vivo, macrophages were recently found to be critical to immune defense against C. perfringens in the setting of skin and soft infections (33). Furthermore, C. perfringens uses two toxins (a phospholipase C and a cholesterol-dependent cytolysin) to escape macrophage phagolysosomes, thereby avoiding intracellular death within these cells (33). Interestingly, C. sordellii similarly expresses both a phospholipase C and a cholesterol-dependent cytolysin (18, 42), though whether it can escape macrophage phagolysosomes requires further investigation.

In addition to providing a tight barrier protecting the underlying endometrium and myometrium from invading microbes, the UEC secretes antimicrobial peptides to deter bacterial colonization of the uterus. Although it was previously reported that a chicken homolog of hβd-1 was bactericidal against C. perfringens (43), the finding that hβd is bactericidal against clostridial pathogens is novel. We speculate that hβd helps prevent clostridial colonization of the uterus, though this requires further study. There are few data regarding cross-talk between defensins and PGs; however, it was found that cyclooxygenase-derived PGs inhibited hβd-2 production by human gingival epithelial cells infected with Actinobacillus actinomycetemcomitans (28). In accord with that report, we found that misoprostol suppressed both constitutive hβd-1 expression and cytokine-induced hβd-2 expression by UECs. The regulation of hβd by misoprostol correlated with the drug’s ability to activate adenylyl cyclase via the EP4 receptor. The molecular basis of cAMP-directed suppression of hβd gene expression requires further investigation. Such data also provide a foundation for studying the role of endogenous PGE2 in the regulation of epithelial cell antimicrobial peptide generation.

The anti-progesterone/anti-glucocorticoid agent mifepristone has also been suggested as a putative factor in C. sordellii infection after abortion (4). In addition to causing cervical dilatation and loss of the protective cervical mucous plug, mifepristone evokes decidual ischemia and tissue necrosis, providing access and a ripe milieu for anaerobic bacteria. There are conflicting data regarding the manner in which mifepristone modulates inflammation (44, 45). Although debated, it is unlikely that human macrophages express progesterone receptors, and the influence of this receptor on innate immunity is unclear. In our animal model of infection, mifepristone (1 and 10 mg/kg i.g. injection) did not worsen rat mortality either in the presence or absence of misoprostol (data not shown).

Furthermore, mifepristone showed conflicting results in our in vitro assessment of TNF-α production in response to C. sordellii challenge. The production of TNF-α by rat peritoneal macrophages was significantly enhanced by pretreatment with mifepristone over a range of concentrations (0.1–10 μM; data not shown), which was consistent with a study showing increased basal TNF-α production by the murine macrophage-like P388D1 cell line in response to this agent (46). However, in our hands this priming by mifepristone was entirely suppressed by misoprostol (1 μM; data not shown). It is noteworthy that we did not observe any effects of mifepristone on TNF-α expression by mouse peritoneal macrophages, mouse BMDMs, or human monocyte-derived macrophages, suggesting that the effects of mifepristone may be cell or species specific (results not shown). Lastly, mifepristone did not share misoprostol’s ability to counter-regulate hβd expression by UECs (data not shown).

Our new data, summarized in Fig. 8, shed new light on the interrelations among C. sordellii, female reproductive tract innate immune cells, and misoprostol. C. sordellii endometritis is likely an ascending infection with bacteria first colonizing the vagina/cervix then gaining access to the uterus. On the basis of our findings, it can be speculated that when applied locally in sufficiently high concentrations, misoprostol promotes colonization and infection with this organism via a number of mechanisms. These include reduced UEC synthesis of antimicrobial peptides such as β-defensins, impaired macrophage phagocytosis, and the reduced generation of macrophage-derived inflammatory mediators. Other possible mechanisms whereby intravaginal misoprostol might increase the risk of bacterial endometritis (that were not investigated in this study) include the impairment of macrophage and neutrophil bacterial killing and the enhancement of uterine contamination following the dilation of the cervix promoted by misoprostol. Finally, the possibilities that misoprostol either facilitates the dissemination of clostridial toxins via vasodilation, or sensitizes host cells to the effects of these toxins cannot be excluded.
Our work has several limitations. We modeled C. sordellii endometritis in nonpregnant rats, whereas human infections of the uterus have followed both abortion and childbirth. It is possible that different results would have been obtained if similar in vivo studies were conducted in rats infected after medical abortion or term delivery. This possibility, suggested by the distinct immunological and endocrine changes associated with pregnancy, requires further study. These investigations were also limited by the use of vegetative C. sordellii organisms instead of spores. Clostridia may exist in vivo as spores, causing clinically overt infection on transition to the vegetative form. The present experiments attempt to model human infection following the emergence of C. sordellii from their (dormant) sporulated form. Future studies will be needed to explore how misoprostol modulates interactions between the innate immune system and clonaltridal spores.

In summary, we have developed the first animal model of a C. sordellii infection (of any organ system) and document for the first time an adverse impact of misoprostol on host defenses against this infection both in vivo and in vitro. These studies support the therapeutic use of misoprostol via nonvaginal routes of administration, which might reduce the risk of immunosuppression within the female reproductive tract. Beyond the immediate relevance of our data to the understanding of clostridial endometritis after medical abortion, these findings suggest that PGE_{\alpha}, in which production is exaggerated within the reproductive tract during pregnancy, might be an important (and pharmaco logically modifiable) causal determinant in the pathogenesis of more common infectious complications of pregnancy (such as chorioamnionitis). In addition, these results identify a general caution that must be considered in other circumstances in which PGs are administered locally for therapeutic purposes.

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Disclosures

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