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Placental Viral Infection Sensitizes to Endotoxin-Induced Pre-Term Labor: A Double Hit Hypothesis

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Abstract

Problem—Among pregnant women, acquired viral infections with a concurrent bacterial infection is a detrimental factor associated to poor prognosis. We evaluate the effect of a viral infection that does not lead to pre-term labor on the response to low doses of lipopolysaccharide (LPS). Our objectives were (i) to characterize the effect of a viral infection concurrent with exposure to microbial products on pregnancy outcome and (ii) to characterize the placental and fetal immune responses to the viral sensitization to LPS.

Method—C57B/6 wild-type mice were injected with murine gammaherpesvirus 68 (MHV68) at E8.5. Either PBS or LPS was injected i.p. at E15.5. Pregnancy outcome and cytokine / chemokine profile from implantation sites were analyzed by multiplex.

Results—LPS treatment of MHV-68-infected animals induced pre-term delivery and fetal death in 100% of the mice. Pre-term labor was characterized by a upregulation of pro-inflammatory cytokines and chemokines in both placenta and decidua. Similar profiles were observed from MHV-68-infected human primary trophoblast and trophoblast cell lines in response to LPS.

Conclusion—We describe for the first time that a sub-clinical viral infection in pregnant mice might sensitize to a bacterial infection leading to pre-term delivery. We propose the ‘Double Hit Hypothesis’ where the presence of a viral infection enhances the effect of bacterial products during pregnancy leading not only to pre-term labor but likely larger adverse outcomes.

Keywords

Double hit hypothesis; lipopolysaccharide; placenta; toll-like receptors; trophoblast; viral infection

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Introduction

Acute viral infections and the inflammatory process they elicit can pose a substantial challenge to pregnancy and to fetal well-being.[1–3] Viral pandemics are a good example of the increased susceptibility of pregnant women to viral infections, increasing the risk for pre-term labor and fetal death.4,5 However, most viral infections during pregnancy are subclinical,6 do not cause strong a inflammatory response, and are not thought to induce pre-term labor.7 For example, the majority of women of child-bearing years in the USA carry Epstein-Barr Virus (EBV),[8] a member of the gamma-herpesvirus subfamily, but only a small fraction have adverse pregnancy outcome (APO).9

Most bacterial infections are associated with a severe local or systemic inflammatory response, which may lead to acute respiratory distress syndrome (ARDS), maternal complications, APO and death.10,11 A variety of bacterial products interact with cellular pathogen-associated molecular pattern (PAMP) receptors including the Toll-like receptors (TLR) to drive immune responses.12 A major initiator of septic shock is lipopolysaccharide (LPS), a component of the cell wall of the Gram-negative bacterial membrane. Intraperitoneal (i.p.) injection of a high dose of LPS into pregnant mice has been associated with APO; however, low doses have been shown to have little or no effect on pregnancy outcome.13–15

Viral infections are known to pre-dispose to increased numbers of bacterial infections.16 However, there are very few studies exploring comorbidity during pregnancy.[17] In this study, we used murine gammaherpesvirus 68 (Murid herpesvirus 4; MHV-68), a gammaherpesvirus of rodents that shares significant genomic colinearity with two human pathogens, EBV and Kaposi's sarcoma-associated herpesvirus (KSHV),[18] to evaluate the role of a subclinical viral infection combined with exposure to low dose LPS, known to have mild effects on pregnancy outcome.19,20 We demonstrate that infection with MHV-68 sensitizes pregnant mice to LPS, resulting in pre-term delivery and significantly increased inflammatory chemokine production. Overall, this model provides solid evidence for investigating pregnant women for a wider spectrum of viruses. Moreover, it suggests the need to evaluate the presence of viral infection that might pre-dispose to bacterial infection on patients with adverse pregnancy outcome.

Methods

Virus Culture

Murine herpes virus 68 expressing green fluorescent protein (MHV-68 GFP, kindly provided by Ren Sun) was passaged in NIH 3T3 cells. After complete lysis, cell culture supernatant was harvested and filtered (0.45 μm pore) under sterile conditions. Viral stock concentrations were determined limiting dilution titration on NIH 3T3 cells. NIH 3T3 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 100 U of penicillin per ml, 100 mg of streptomycin per ml and 2 mM L-glutamine.

Cytokine Analysis

Cytokine and chemokine content from decidua and the placenta were determined by multiplex (Luminex). After centrifugation, homogenized tissues (1 mL lysis buffer (beadlyte) with PIC / gram protein) were diluted in assay buffer. Plates were read using Luminex 200 and analyzed using BioRad v5.0 according to the manufacturers protocol.

Animals and Animals-Related Procedures

C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Adult mice between 8 and 12 weeks of age were used for experimentation. Timing of pregnancy was determined by visual inspection of the vaginal plug, which was defined as 0.5 days post-conception. Mice were infected intraperitonally with either 1×10^6 plaque-forming units of MHV-68 GFP (in 200 μL volume) of MHV-68 or DMEM media (vehicle) at day E8.5. Either PBS or LPS (Sigma E coli 0111:B4) was administered i.p. on day E15.5. The presence of pre-term labor was defined as the delivery of fetuses in the following 48 hr after the LPS injection. Animals were sacrificed using a CO_2 chamber. The organs were removed, fixed in 4% paraformaldehyde and / or stored at −80°C. All animals were maintained in the Yale University School of Medicine Animal Facility under specific pathogen-free conditions. All experiments were approved by the Yale Animal Resource Committee.

Statistical Analysis

Data are expressed as mean ± S.E. for the *in vitro* studies and median ± first or third quartiles for the *in vivo* studies. Statistical significance ($P < 0.05$) was determined using either two-tailed Mann–Whitney *U*-test for non-parametric data.

Results

MHV-68 Infection Sensitizes to LPS During Pregnancy

MHV-68 infection of pregnant mice does not cause pre-term labor and does not alter the total number of pups per gestation.21 On the other hand, bacterial infections are known to induce a potent inflammatory response and elicit pre-term delivery. Low doses of LPS administered to pregnant mice have been shown to have no detrimental or a mild effect on pregnancy outcome, while high doses trigger preterm labor / delivery.15,20 Our first objective was to determine whether a MHV-68 infection might affect the sensitivity to LPS. To investigate this, pregnant C57BL / 6 mice were infected with MHV-68 on day E8.5 followed by LPS injection on day E15.5. We selected a dose of LPS that had a mild effect on pregnancy outcome (20 ug / kg). Control mice received PBS on day E8.5 and LPS on day E15.5 or MHV-68 on day E8.5 and PBS on day E15.5 (Fig. 1a).

LPS treatment of MHV-68-infected animals induced pre-term delivery in 100% of the mice in less than 24 hr, while this occurred in only 29% of mice in the absence of viral infection (Fig. 1h). Moreover, the pre-term labor in all MHV-68-infected mice treated with LPS was associated with vaginal bleeding (Fig. 1d) when compared to controls receiving LPS or virus alone (Fig. 1b,c). In parallel experiments, mice were euthanized on E17.5, and gestational sac gross morphology was determined. Infection with MHV-68 alone (Fig. 1e), or treatment with LPS (Fig. 1f) in the absence of pre-term labor / delivery had no visible effect. In mice receiving both virus and LPS, however, there was marked necrosis and gestational sac anomalies (Fig. 1g).

To determine the potential mechanism by which MHV-68 + LPS treatment promotes pre-term labor, we evaluated the cytokine profile of placenta and decidua samples obtained from treated mice. In all cases (except for RANTES), viral infection alone did not significantly alter the cytokine profile in either the placenta or decidua when compared with the control group (Fig. 2a,b). Expectedly, treatment with LPS induced a low level inflammatory response. Surprisingly, in mice infected with MHV-68 followed by LPS treatment, we found synergistic effect; the pro-inflammatory cytokine profile was characterized by a significant increase in the levels of IL-6, G-CSF and MCP-1 compared to virus or LPS alone, in both placenta and decidua (Fig. 2a,b) The levels of IL-1β, RANTES and MIP-1b were increased only in decidua (Fig. 2b).

Next, we evaluated whether the trophoblast response to LPS is affected by viral infection. We used first trimester primary cultures of human trophoblast cells and trophoblast cell lines.[22,23] Cells were infected with MHV-68 for 24 h, and a high degree of infection (>95%) was confirmed by florescent microscopy (Data not shown). Cells were then treated with LPS (1 μg /mL) or PBS as control, for an additional 72 hr. Supernatants were then collected, and cytokines were assessed using multiplex Luminex. As shown in Fig. 2c, LPS alone, but not virus, induced IL-6 expression by first trimester trophoblast cells at concentration of 1 μg / mL; however, this effect was significantly enhanced in MHV-68-infected trophoblast treated with LPS. Furthermore, there was an increased expression of additional inflammatory cytokines such as GRO-α, G-CSF and IL-8 when both virus and LPS were present, when compared with either treatment alone (Fig. 2c).

To confirm that the increased cytokine expression was the result of trophoblast responses, we evaluated the same type of response but using first trimester trophoblast cells. Similar enhancement of cytokine responses was observed in trophoblast cell lines infected with MHV-68 and treated with LPS, when compared with either treatment alone (Fig. 2d).

Discussion

For the first time, we demonstrated that an asymptomatic maternal viral infection sensitizes pregnant animals to LPS, leading to pre-term labor. Bacterial infection has been identified as an important cause of pre-term labor.[24,25] In this study, we tested the 'double hit' hypothesis where a pre-existing viral infection can sensitize the placental response to bacteria.

Our data shows that low doses of LPS are not sufficient to trigger an inflammatory process that will lead to the termination of the pregnancy; however, viral infection may sensitize the mother to an enhanced immune response against a concurrent bacterial infection. We showed that MHV-68-infected pregnant mice had pre-term labor following injection of low dose of LPS at a higher rate than uninfected animals. These results suggest that a viral infection during pregnancy increases the risk of pre-term labor and other APO, including maternal death, in response to other microorganisms such as bacterial infection.

While clinical scenarios such as pandemics have shown that concomitant viral and bacterial infections can affect pregnancy outcomes,[5] one major question is whether infection with endogenous viruses or subclinical infections could enhance the susceptibility to equally subclinical bacterial infections during pregnancy leading to APO. Previous studies have shown that there is an increased sensitivity to LPS in mice infected with vesicular stomatitis virus, probably secondary to the up-regulation of TNF-α and IFN-γ[16], but this study did not examine this phenomenon in the context of pregnancy.

In this study, we evaluated the role of the placenta and more specifically the trophoblast in relation to viral and bacterial product responses. The placenta can function as an active immunologic barrier and is able to recognize and respond to microorganisms through the expression of TLRs.[26–28] Trophoblast responses, through these pathogen recognition receptors, influence the function and activity of the maternal immune system.[29,30] Through our *in vivo* model, we have demonstrated that sensitization to LPS occurs in pregnant mice with a previous placental viral infection that by itself did not lead to adverse pregnancy outcome. We propose that the ongoing local inflammatory response to the virus present in the placenta (first hit) primes the whole organ to mount a robust cytokine immune response against bacterial products, even at low doses (second hit). Importantly, our data suggests that a fraction of APOs are the result of prior infection with an apparently 'innocuous' virus, such as EBV, combined with a subclinical bacterial infection.

In conclusion, we propose that viral infections can modulate placental immune responses to bacteria leading to pre-term labor and APO. The data presented here support the concept that there is increased morbidity following viral and bacterial infection at what would normally be subclinical through an alteration in the placental inflammatory responses. Future studies are needed to further evaluate the mechanism of viral sensitization to bacterial infections during pregnancy.

Acknowledgments

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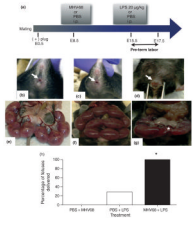

Fig. 1.

(a) Experimental timeline. C57BL/6 mice were infected with either 1×10^6 pfu MHV-68 or PBS at day E8.5 followed by a single dose at day E15.5 of either LPS 20 μg / kg or PBS. (b–d) Gross morphology of pregnant mice at day E17.5, which received the following treatments: MHV-68 (b), LPS (c), or MHV-68 + LPS (d). Note the presence of vaginal bleeding (white arrows) and dilation from MHV-68 + LPS injected pregnant mice (d). Gestational sacs from MHV-68 infected (e), LPS treated (f), or MHV-68 + LPS (g) injected mice at day E17.5. The asterisk shows the empty uterine wall with necrotic placentas left inside of the uterus after the delivery in the MHV-68 + LPS injected group (g). MHV-68 infection sensitizes to bacterial LPS (h). Fetuses delivered from mice treated with MHV-68 (line), LPS (white) and the combination of MHV-68 infection and LPS (black) were enumerated at day 17.5. $n = 6$ mice per group. $^*P < 0.05$ MHV68 + LPS vs PBS + LPS.

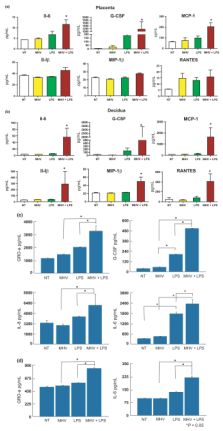

Fig. 2.

Cytokine/chemokine profile of placenta (a) and decidua (b) from each treatment group. Mice were treated according to the experimental timeline. Lysates from placenta and decidua were obtained and cytokines/chemokines were analyzed by multiplex. Note the significant increase in the levels of Il-6, G-CSF, MCP-1 in the cotreated group compared to MHV-68 alone, LPS, or controls in both placenta (a) and decidua (b). Il-1b, RANTES and MIP-1b were increased only in decidua (b). Bars show median ± S.E. (c–d) Cytokine/chemokine profile of LPS treated human trophoblast cells infected with MHV-68. The human trophoblast cell line HTR-8 (c) or primary cultures of isolated human first trimester trophoblast cells (d) were infected with MHV-68 for 24 hr followed by LPS treatment (1 ug/mL) for 72 hr. Supernatants were collected, and cytokines/chemokines were analyzed by multiplex. Bars show mean ± S.E.M. n = 6 mice per group. *$P < 0.05$.