

## Effects of *Escherichia coli* and *Porphyromonas gingivalis* Lipopolysaccharide on Pregnancy Outcome in the Golden Hamster

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**This report describes the effects of two gram-negative bacterial endotoxin (lipopolysaccharide [LPS]) preparations on hamster pregnancy outcome variables. Single intravenous challenges with *Escherichia coli* and *Porphyromonas gingivalis* LPS on day 8 of pregnancy produced dose-dependent effects on fetal weight malformation and fetal resorption with *E. coli* LPS having potent embryolethal effects. Premating maternal exposure to *P. gingivalis* produced embryolethal effects similar to those of *E. coli*. These data suggest that maternal exposure to *P. gingivalis* LPS prior to and during pregnancy can induce deleterious effects on the developing fetus.**

To date the effects of endotoxins of oral origin on the fetal-placental unit have not been examined. This is of potential significance since the molecular structure and biological activities of endotoxins isolated from oral organisms are different from those of enteric endotoxins. Experiments were conducted to compare the effects of enteric versus oral endotoxins on fetal development in the pregnant golden hamster model. In addition, studies were performed to characterize both the dose-response effects of lipopolysaccharide (LPS) on pregnant dams and the effects of immunization prior to LPS challenge on the pregnancy outcome.

Early experiments (1, 2, 5, 6) showed that enteric endotoxins are capable of inducing placental necrosis, spontaneous abortions, fetal organ damage, fetal death, and malformations in various animal species. Endotoxin was found to be embryolethal and to produce several malformations, spontaneous abortions, and low fetal weight. Administering *Escherichia coli* LPS as a single intravenous bolus on day 8 of gestation, Lanning (4) demonstrated a decrease in fetal weight and an increase in the percentage of malformed fetuses with increasing LPS concentrations.

It is our hypothesis that the burden of oral endotoxin which is associated with periodontal infection in humans may present a risk for abnormal pregnancy outcomes. Although there are considerable data indicating that systemic exposure to oral LPS occurs, especially in the presence of periodontal infection, the critical question remains as to whether oral endotoxins can elicit adverse pregnancy outcomes. In this report, we demonstrate that *Porphyromonas gingivalis* LPS can induce fetal growth retardation, fetal death, resorptions, and malformations and that the dose-response characteristics differ significantly from those of *E. coli* LPS. Furthermore, repeated immunization with *P. gingivalis* does not afford protection but, rather, appears to potentiate the abortifacient and embryotoxic effects of LPS challenge during pregnancy.

In these studies we have used *P. gingivalis* A7436, a clinical

isolate from a refractory periodontitis patient extensively characterized by V. R. Dowell, Anaerobic Microbiology Laboratory, Centers for Disease Control, Atlanta, Ga. *P. gingivalis* grown on blood agar plates in an anaerobic chamber for 1 to 2 days until the culture reached an  $A_{660}$  of 1.2, which corresponded to approximately  $10^9$  CFU/ml. *P. gingivalis* LPS was extracted by a modification of the Westphal-Jann method as described by Kasper et al. (3) and resolved by CsCl density gradient centrifugation and *Limulus* amoebocyte lysate identification of fractions. *E. coli* LPS (serotype O127:B48) was purchased from Sigma (St. Louis, Mo.) and used as the enteric LPS.

Golden hamsters (Charles River Laboratories, Wilmington, Mass.) were maintained in a breeding colony, mated, challenged, and sacrificed by following the teratology-toxicology pregnancy model described initially by Ornay (5). For intravenous challenge, the female was anesthetized to allow injection with various dosages of LPS or buffer (final volume, 0.1 ml) into the lingual vein. Pregnant dams were weighed and monitored daily for signs of malaise, fever, and abortion. On day 15, pregnant dams were euthanized by CO<sub>2</sub> asphyxiation and weighed, and the uterus was dissected for a thorough examination of implantation sites, individual embryos, and lethality determination. The mean fetal weight was determined for each pregnant dam, pooling results for six or more dams to form a group mean  $\pm$  standard error at each LPS dosage tested. Fetal death was expressed as a percentage of total implantation sites per litter which had either a resorption site or a nonvital fetus, again pooling results for multiple dams at each LPS concentration. Malformations were determined by gross inspection (examining for skeletal anomalies), expressed as percentage of total implantation sites per litter. A significant association between pregnancy outcome (for example, fetal weight) and LPS concentration was determined by repeated-measures analysis of variance (ANOVA) at  $P < 0.05$ . When significant dose-response effects were observed by analysis of variance, pairwise comparisons between LPS responses at different dosages were tested for significance by using the nonpaired  $t$  test.

In certain experiments, animals were preimmunized with either whole formalinized *P. gingivalis* bacteria (strain A7436) or isolated LPS. Prior to mating, animals were immunized

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intravenously with daily doses of formalinized *P. gingivalis* starting at  $3 \times 10^7$  cells increasing incrementally 20% daily for 5 days. Similarly, other animals were administered incremental intravenous doses of *P. gingivalis* LPS for 5 days starting at 10  $\mu\text{g}/100$  g of body weight (gbw) and increasing 20% daily. Ten days later the animals were boosted by repeating the last dose and mated 2 weeks later. A postmating challenge of *P. gingivalis* LPS at various doses was administered at day 8 of pregnancy.

By comparing single doses of either *E. coli* or *P. gingivalis* LPS in the pregnant hamster, we have observed both qualitative and quantitative differences in pregnancy outcome responses. The effects of *E. coli* and *P. gingivalis* LPS on fetal weight are shown in Fig. 1. The dashed horizontal line indicates the mean fetal weight of control (non-LPS-treated) dams. This value of  $1.55 \pm 0.024$  g (mean  $\pm$  standard error) was obtained by pooling results for 18 mothers and 255 fetuses. There were no spontaneous abortions, no resorptions, no visible congenital anomalies, and an average of 14.2 fetuses per dam in control animals. The experimental data for mean fetal weight at each LPS dosage are shown, with standard errors shown by error bars. *E. coli* LPS (Fig. 1A) at dosages of 0.03 and 0.3  $\mu\text{g}$  of LPS per 100 gbw induced statistically significant increases in fetal weight ( $P < 0.01$ , nonpaired *t* test). However, as the LPS dosage increased there were statistically significant decreases in mean fetal weight with dosage of 10  $\mu\text{g}/100$  gbw, resulting in an 18.1% decrease ( $P < 0.01$ ). The decrease in fetal weight in response to *E. coli* LPS occurred over a relatively narrow dose-response range, because as the LPS dose increases, the relative number of lethal fetal resorptions also increases (Fig. 2A). The total number of implantation sites per female cannot change, since this event occurs prior to LPS challenge. Thus, as the *E. coli* LPS dose increases, the number and the size of the viable fetuses decrease. The effects of *P. gingivalis* LPS on fetal weight are shown in Fig. 1B. The dose-response curve shows a biphasic effect on fetal weight over the dose range of 0.003 to 10  $\mu\text{g}$  of LPS per 100 gbw, which was almost identical to that obtained from *E. coli* LPS. There are more data for intermediate *P. gingivalis* LPS concentrations emphasizing the ability of doses of 30 to 300 ng to induce significant increases in fetal weight. Larger challenges (3 to 10  $\mu\text{g}$ ) with *P. gingivalis* LPS doses cause a significant decrease in mean fetal weight. However, in contrast to *E. coli* LPS, doses of *P. gingivalis* LPS of 30  $\mu\text{g}$  and above do not influence fetal weight. This dose-response curve extends over 5 logs with an apparent window of embryotoxic activity which influences fetal weight over a more narrow range of approximately 2.5 logs. The data showing the effect of LPS on fetal resorptions (death) are shown in Fig. 2. There is little similarity between *E. coli* and *P. gingivalis* LPS dose responses. At 10  $\mu\text{g}$  of *E. coli* LPS per 100 gbw, 66.4% of all fetuses are nonvital and are resorbed (Fig. 2A) and the remaining viable fetuses are dramatically smaller (Fig. 1). At 30  $\mu\text{g}$  of *E. coli* LPS, there are no viable fetuses, as 100% of the implantation sites have resorbed. One-fourth of these animals also had signs of vaginal discharge. At doses of 100  $\mu\text{g}$ , all of the animals showed vaginal discharge and 100% resorption upon sacrifice. In this dose range of 30 to 100  $\mu\text{g}/100$  gbw, piloerection and lethargy were observed as signs of fever and malaise; however, no effects were seen on maternal weight at any of the dosages tested. The maximum effect of *P. gingivalis* LPS on fetal mortality (Fig. 2B) was observed at a dose of 300 ng of *P. gingivalis* LPS, at which 21.7% of the fetuses were resorbed, a statistically significant elevation as compared with results for control animals. As levels of *P. gingivalis* LPS challenge increased, there was not an increase in the percent resorptions

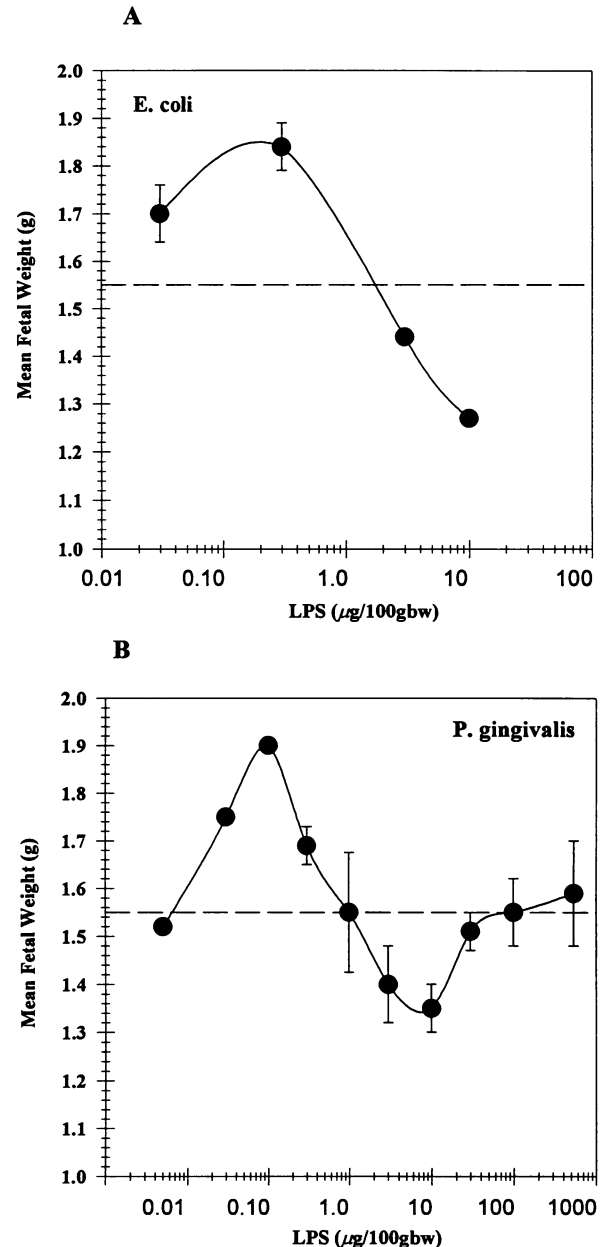


FIG. 1. The effects on mean fetal weight of *E. coli* (panel A) and *P. gingivalis* (panel B) LPS administered on day 8 of gestation in pregnant hamsters. The dashed horizontal line represents the mean value for control animals (no LPS).

as seen with *E. coli* LPS. In contrast, there was a return to baseline in fetal weight (Fig. 1B) and percent resorptions (Fig. 2B), suggesting that single challenges of *P. gingivalis* LPS at higher dosages failed to elicit an embryolethal effect.

This suggestion was also reflected in Fig. 3B, which indicates that *P. gingivalis* LPS had minimal activity as an inducer of congenital malformations. It was also significant that the highest levels of *P. gingivalis* LPS caused slight fever in some animals but failed to induce vaginal discharge, maternal shock, or maternal death (data not shown). In contrast, *E. coli* LPS (Fig. 3A) induced visible congenital malformations over the range of 3 to 10  $\mu\text{g}/100$  gbw. At 10  $\mu\text{g}$  of *E. coli* LPS, 33.4% of

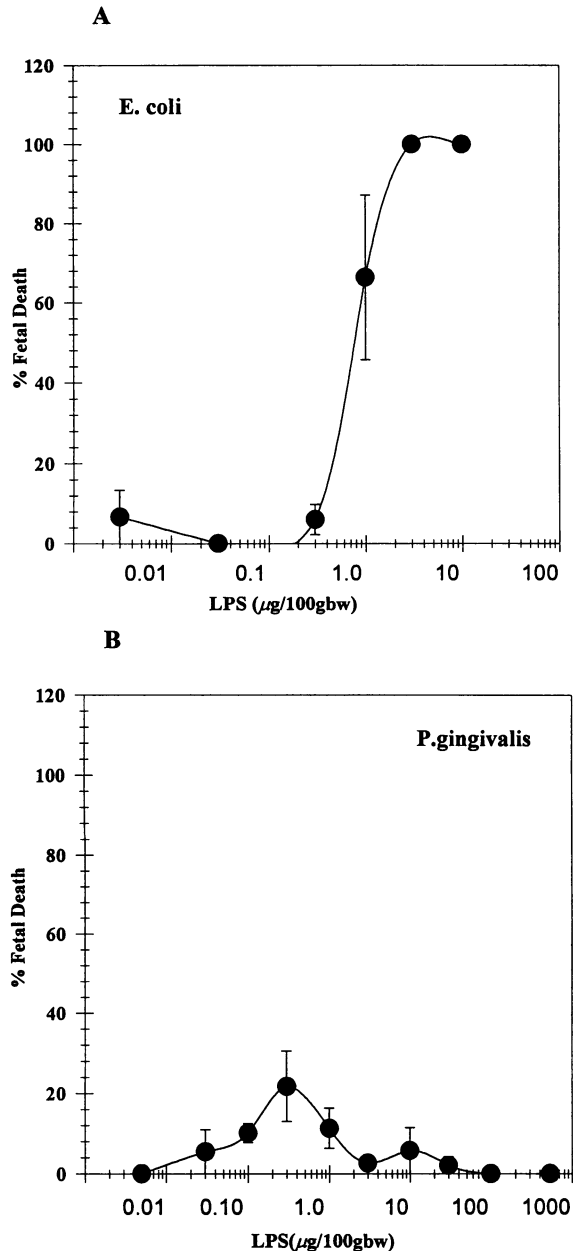


FIG. 2. The percent fetal death in response to *E. coli* (panel A) and *P. gingivalis* (panel B) LPS. In this pregnant hamster model, embryolethality is accompanied by resorption of the fetus.

all viable fetuses had visible skeletal malformations. Although not characterized systematically, malformations included neural crest defects and cleft palate and limb anomalies.

Although a single challenge with *P. gingivalis* LPS failed to demonstrate the same embryotoxicity as *E. coli* LPS, the effects of repeated treatment with either whole *P. gingivalis* or *P. gingivalis* LPS prior to mating on the pregnancy response elicited by a *P. gingivalis* LPS challenge during pregnancy are dramatic. Table 1 shows the effects of preparturient exposure with either *P. gingivalis* LPS or whole cells on pregnancy outcome. A single bolus of 1  $\mu\text{g}$  of *P. gingivalis* LPS during pregnancy did not result in a significant effect on fetal weight as compared to controls. A single challenge of 3  $\mu\text{g}$  of *P. gingivalis* LPS per 100

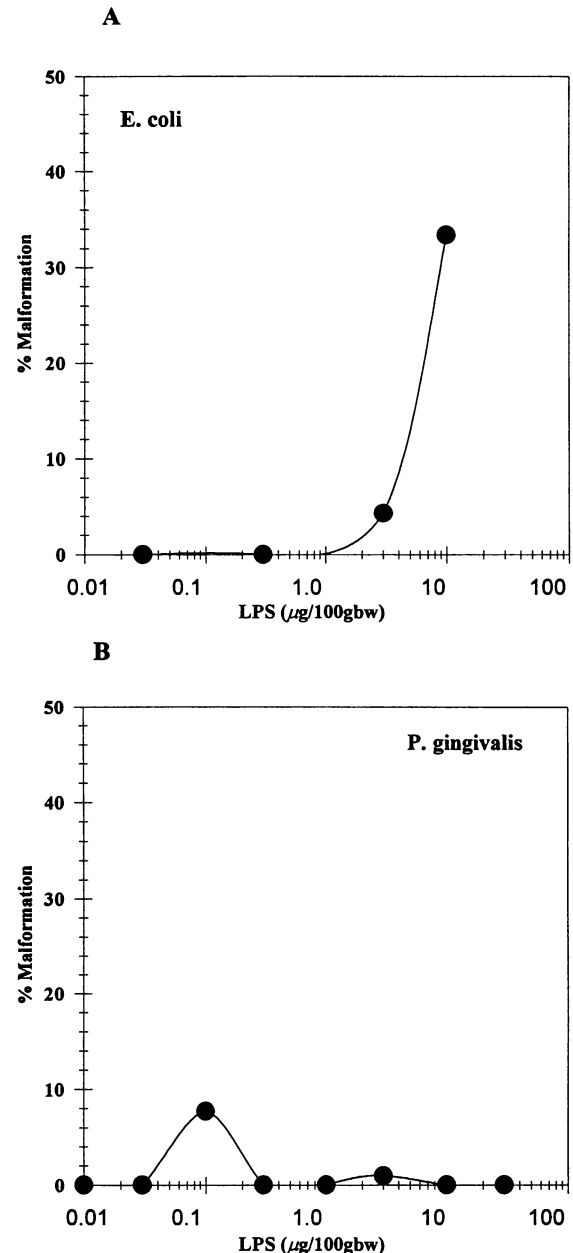


FIG. 3. The percent of congenitally malformed fetuses in response to *E. coli* (panel A) and *P. gingivalis* (panel B) LPS.

gbw during pregnancy resulted in a decrease in mean fetal weight from 1.55 g (control dams) to  $1.4 \pm 0.08$  g. This is equivalent to a 9.7% reduction in weight. In contrast, animals that were primed with LPS before mating, and challenged with the same 3- $\mu\text{g}$  *P. gingivalis* LPS dose during pregnancy, had a more pronounced decrease in fetal weight ( $P = 0.02$ ). Priming with *P. gingivalis* whole cells prior to mating also induced a greater decrease in fetal weight following 1- $\mu\text{g}$  or 3- $\mu\text{g}$  pregnancy challenges. For example, 3- $\mu\text{g}$  challenges in nonprimed animals resulted in a mean weight of  $0.78 \pm 0.08$ , or a 49.7% reduction in the weight of surviving fetuses, at  $P < 0.001$  relative to controls. Immunizations with whole cells resulted in a 44.3% reduction in fetal weight relative to nonimmunized

TABLE 1. Effects of intravenous treatment with either *P. gingivalis* whole cells or isolated LPS on adverse pregnancy outcomes induced by LPS

Premating treatment and pregnancy challenge (mg of LPS)	Fetal wt (g) (mean $\pm$ SE)	% Fetal death (resorptions)	% Congenital malformations
None			
1	1.55 $\pm$ 0.13	11.3	0
3	1.40 $\pm$ 0.08	2.6	1.0
LPS (3)	1.14 $\pm$ 0.08 <sup>a</sup>	31.3	8.3
Whole cell			
1	1.26 $\pm$ 0.05	26.7	0
3	0.78 $\pm$ 0.08 <sup>b</sup>	83.3	0

<sup>a</sup> P = 0.044 compared with 3  $\mu$ g pregnancy challenge and no premating challenge.

<sup>b</sup> P = 0.0003.

animals when challenged with 3  $\mu$ g of LPS per 100 gbw during pregnancy.

LPS exposure before conception also potentiated the embryo-lethal effects of *P. gingivalis* LPS, as shown in Table 1. In unexposed animals, few resorptions occurred with *P. gingivalis* LPS, reaching an apparent peak value of 21.7% at 0.3  $\mu$ g/100 gbw. In contrast, animals that were primed with either *P. gingivalis* LPS or whole cells were much more susceptible to resorptions, closely mimicking the potent embryo-lethal response observed for *E. coli* LPS. However, priming did not appear to induce a large increase in the teratogenic response, as reflected in the percent congenital malformations. Thus, multiple preexposure with either whole *P. gingivalis* cells or purified *P. gingivalis* LPS results in a marked potentiation of the effects on decreasing fetal weight and increasing fetal mortality.

This report demonstrates that the LPS from oral bacteria can cause adverse pregnancy outcomes. We have shown that increasing doses of LPS from both *E. coli* and *P. gingivalis* produce biphasic effects on fetal weight with significant decreases in fetal weight at higher doses. Single doses of *E. coli* LPS >10  $\mu$ g/100 gbw were potent inducers of pathological mechanisms leading to fetal morbidity and mortality. The fact that single doses of *P. gingivalis* LPS failed to produce comparable responses appears to be a result of primary versus secondary challenge. Immunizing dams prior to mating with the *P. gingivalis* LPS increased the pathogenicity of the day 8 pregnancy challenge to levels comparable with those for *E. coli*. These findings pose new questions regarding the mechanisms of potential adverse effects of chronic oral infections with gram-negative organisms during pregnancy.

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