

Lipopolysaccharide-induced fetal resorption in mice is associated with the intrauterine production of tumour necrosis factor- α

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Summary. Certain strains of mice display an increased frequency of fetal resorption, but little is known about the effector mechanisms involved. We have examined the events associated with lipopolysaccharide (LPS)-induced fetal resorption in mice. Administration of 25 μg LPS on Day 12 of gestation resulted in the appearance of tumour necrosis factor- α (TNF- α) in the amniotic fluid and fetal resorption. Levels of LPS-induced TNF- α were reduced by 90% after pretreatment with the TNF- α -suppressing drug pentoxifylline (PXF). Treatment of pregnant mice during early gestation with 0.1 μg LPS resulted in fetoplacental resorption which was maximal when the LPS was given on Day 8. Resorption induced by 0.1 μg LPS on Day 8 of gestation was significantly reduced by pretreatment with PXF. Infiltration of asialo-GM1-positive cells was observed in the decidual-ectoplacental cone area of embryonic units from LPS-treated mice. In addition, treatment with anti-AGM1 antiserum prevented the LPS-induced resorption. Our results suggest that TNF- α and asialo-GM1-positive cells are involved in LPS-induced fetal resorption.

Keywords: abortion; lipopolysaccharide; TNF- α ; natural killer cells; pentoxifylline; mouse

Introduction

Spontaneous fetal resorption in CBA/J \times DBA/2 mice has been shown to be associated with early decidual infiltration of natural killer (NK)-like cells (Gendron & Baines, 1988). Previous results have shown that treatment of pregnant CBA/J \times DBA/2 mice with polyinosinic cytidylic acid (poly I:C), which is known to activate NK cells through macrophage interferon production, increases the resorption frequency (deFougerolles & Baines, 1987). These findings suggest that activation of non-specific immune mechanisms in the fetoplacental unit can influence fetal survival.

It has been previously shown that bacterial endotoxins induce fetal resorption in mice and rats (Zahl & Bjerknes, 1943; Rieder & Thomas, 1960; McKay & Wong, 1963). However, endotoxins do not appear to interrupt the normal endocrine functions of pregnancy and do not cross the placental barrier (Chedid *et al.*, 1962; Parant & Chedid, 1964; Gasic *et al.*, 1975).

One of the characteristic effects of bacterial endotoxins such as LPS is the triggering of tumor necrosis factor- α (TNF- α) release from primed macrophages (Beutler *et al.*, 1985a). If macrophages are primed first with gamma interferon they can be stimulated to produce TNF- α after exposure to very small amounts of LPS (Gifford & Lohmann-Matthes, 1987). Furthermore, LPS has been shown to stimulate interferon-gamma production by human peripheral blood mononuclear cells and mouse splenocytes (Le *et al.*, 1986; Blanchard *et al.*, 1986). Thus LPS can both trigger TNF- α release by interferon-primed macrophages and induce interferon production, resulting in an additive effect on the production of TNF- α . Although macrophages are considered a

major source of TNF- α , production of TNF- α by large granular lymphocytes (NK phenotype cells) has also been demonstrated (Peters *et al.*, 1986). Exposure to excessive quantities of TNF- α can elicit numerous systemic toxic and lethal effects (Tracey *et al.*, 1986; Kiener *et al.*, 1988).

In this study, we have examined LPS-induced resorption of the mouse conceptus.

Materials and Methods

Animals. Mice were maintained in an animal care facility with 12 h light/dark cycles (lights on 07:00–19:00 h) and were given free access to food and water. Female CBA/J mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Female CFW/SW and male DBA/2 mice were purchased from Charles River (St. Constant, Quebec, Canada). Pregnancies were obtained by housing 4 female mice with 1 male mouse per cage. Day of vaginal plug detection was designated as Day 1 of gestation. Animals were killed by cervical dislocation.

Treatments. For the induction of resorption, pregnant mice received tail vein injections (i.v.) of 0.1 μ g *E. coli* (0:55: B5) lipopolysaccharide (LPS) dissolved in PBS (Sigma No. L-2880, St Louis, MO, USA) on various days of gestation. Treatment with LPS was used alone or in combination with a prior single (i.v.) 0.2 ml injection of sterile anti-asialo-GM1 antibody (aAGM1) (Wako, TX, USA), at a dilution of 1/20 (previously shown to decrease the spontaneous fetal resorption frequency in mice; deFougerolles & Baines, 1987). Control groups received (i.v.) 0.2 ml injections of PBS. To suppress the production of TNF- α (Strieter *et al.*, 1988), daily injections of pentoxifylline (PXF) were administered to pregnant mice intraperitoneally (i.p.) at a dose of 1.15 mg per mouse per day. PXF was dissolved in PBS vehicle and filter sterilized. PXF was kindly provided by Hoechst Inc. (Sommerville, NJ, USA). Control groups received equal volumes of PBS (i.p.).

Resorption and tissue processing. Fetoplacental resorption was assessed by viewing the contents of the pregnant uterus. Resorbing uterine contents were characterized by haemorrhage and tissue maceration. CBA/J \times DBA/2 pregnant uteri were removed on Day 10 after LPS treatment on Day 9, embedded and frozen in OCT compound (Miles Scientific, Elkhart, IN, USA) for immunohistochemical analysis (see below). Resorption was assessed 1–2 days after treatment with LPS or PBS. CFW/SW \times DBA/2 pregnant mice were used on Day 12 of gestation in the analysis of amniotic fluid for TNF- α . In these pregnancies, amniotic fluid was harvested 2 h after treatment with 25 μ g LPS or PBS by aspirating the amniotic fluid from the amniotic sac using a 25-gauge needle and syringe. Amniotic fluid was then centrifuged to remove debris and red blood cells and stored at -80°C until assayed. The 2-h time point was chosen to attain peak TNF- α levels (Beutler *et al.*, 1985b).

Immunohistochemistry. Cryostat sections of uteri from LPS-treated CBA/J \times DBA/2 pregnancies were analysed for the infiltration of NK-like cells utilizing an indirect immunoperoxidase technique with anti-asialo-GM1 as primary antiserum as previously described (Gendron & Baines, 1988). Pregnant mice were intravenously treated with 0.1 μ g LPS or PBS vehicle on Day 9 of gestation and killed 24 h later. Stained sections were scored for the density of asialo-GM1 (AGM1)-positive cells by counting 5–10 high-power fields (0.185 mm²) pooled from the mesometrial decidual and ectoplacental cone areas of each fetoplacental unit. All fetoplacental units were counted and used to calculate the mean number of AGM1-positive cells per high-power field. Metrial gland areas were not included in the cell counts.

TNF- α assay. For the analysis of TNF- α in amniotic fluid, samples were titrated on actinomycin D-treated L929 cells as previously described (Fisch & Gifford, 1983). TNF- α was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye reduction assay using recombinant murine TNF- α (4×10^4 units/ μ g) (Genzyme, Boston, MA, USA) for creating the standard curves (Mosman, 1983). Dye reduction was quantified on an EAR 400 AT plate reader SLT-Labinstruments (Austria). To confirm that the cytotoxin was indeed TNF- α , amniotic fluid samples were also tested at 1:2 dilutions in the presence of a 1:20 dilution of rabbit-anti-TNF- α antiserum (Genzyme).

Data analysis. Analysis of variance was used to assess frequency data from LPS-induced resorption as a function of gestational day. The modulation of LPS-induced resorption frequency by aAGM1 treatment, amniotic fluid TNF- α levels and PXF modulation of resorption frequency were analysed by Student's *t* test. Data were considered significant at a probability level of $P < 0.05$.

Results

Production of TNF- α during LPS-induced fetal resorption and inhibition by PXF

LPS has been shown to trigger the release of TNF- α from primed macrophages (Beutler *et al.*, 1985a). Since we could not detect TNF- α in the serum or in supernatants of decidual cell suspensions from LPS-treated pregnant mice, we analysed amniotic fluid for TNF- α at the earliest time

point (Day 12) at which a sufficient volume of this fluid could be collected. Animals received 25 µg LPS to induce maximal release of TNF-α into the amniotic fluid. Treatment of pregnant mice with 25 µg LPS on Day 12 of gestation resulted in the appearance of significant amounts of TNF-α in the amniotic fluid 2 h after treatment (Table 1). The cytotoxic activity in the amniotic fluids from LPS-treated pregnant mice was shown to be due to TNF-α as neutralization with anti-TNF-α antiserum completely abrogated the bioactivity. TNF-α concentrations in control PBS-treated pregnant mice were below the detection limit of the assay. The dose of LPS used in the analysis of amniotic fluid TNF-α levels (25 µg on Day 12) resulted in 100% resorption in all animals by 24 h after treatment in separate experiments. There was no detectable increase (above background levels) in TNF-α production in the serum of pregnant mice 2 h after treatment with 25 µg LPS on Day 8 or Day 12 (data not shown).

Pentoxifylline is a methylxanthine which has been shown to suppress TNF-α mRNA expression and TNF-α production (Strieter *et al.*, 1988). Pretreatment of pregnant mice with PXF on Days 9–12 followed by treatment with 25 µg LPS on Day 12 resulted in a significant decrease in amniotic fluid TNF-α levels compared with LPS treatment alone (Table 1).

Table 1. TNF-α in amniotic fluid of CFW/SW × DBA/J pregnant mice treated with lipopolysaccharide (25 µg) and inhibition by pentoxifylline

Treatment/days	Amniotic TNF-α (U/ml)	No. of mice
LPS Day 12	37.4 ± 6.7	6
PBS Day 12	<2.0	5
LPS Day 12	30.6 ± 5.9	3
LPS Day 12 (+ a-TNF-α Ab)	<2.0	3
LPS Day 12	34.4 ± 5.0	5
PXF Days 9, 10, 11, 12 + LPS Day 12	4.4 ± 1.0*	6

Values are mean ± s.e.

**P* < 0.05.

LPS-induced fetoplacental resorption varies with day of gestation

Since little is known about the effects of LPS on early pregnancy, we studied the response to LPS as a function of gestational day. A differential effect of LPS on fetoplacental resorption in normal pregnancy depended on the day of gestation on which the LPS was administered. As shown in Fig. 1, sensitivity to 0.1 µg LPS was maximal on Day 8 of gestation (100% resorption) and then declined between Days 9 and 11. The frequencies of LPS-induced resorption on Days 9 and 10 were not significantly different. Resorption frequencies after control injections of PBS on Days 7 through 11 were 2–3%. Resorptions occurring in response to treatments with 0.1 µg LPS on Day 8 were characterized by extensive haemorrhage and complete maceration of the uterine contents within 24 h after LPS administration, but were not accompanied by any other noticeable effects in the treated gravid female mice. Resorptions occurring in response to treatments with 0.1 µg LPS on Days 7 and 9–11 were not grossly detectable until 48 h after LPS administration and appeared to be characterized by a slower time course. Abortion could be induced in later pregnancy, but required higher doses (10–25 µg) of LPS. Higher doses in late gestation (Days 15–20) were associated with systemic effects such as severe diarrhoea, pilo-erection and hunched posture.

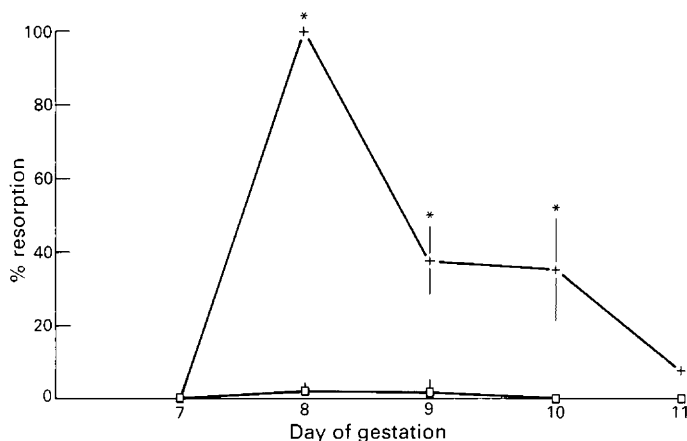


Fig. 1. Mean resorption frequency (\pm standard error) in CFW/SW (\times DBA/2) pregnant females as a function of administration of 0.1 μ g LPS (+) or PBS (\square) on different days of gestation: 4–9 pregnant animals were studied for each treatment group per day. * $P < 0.05$ vs PBS control.

LPS-induced fetoplacental resorption can be prevented by pretreatment with pentoxifylline

Since PXF decreased concentrations of TNF- α induced by LPS in amniotic fluid, we studied the effect of PXF on LPS-induced early resorption. As shown in Table 2, PXF treatment on Days 5 through 8 of gestation completely prevented the 100% resorption frequency observed for Day 8 LPS treatment alone. Pregnant animals in this group (killed on Day 18 of gestation) appeared to contain viable, healthy fetuses. There was no significant difference in the mean number of implantation sites in PXF-protected pregnancies compared with normal, untreated pregnancies. Pretreatment of pregnant mice with PXF on Days 7 and 8 of gestation decreased the LPS-induced resorption frequency considerably but was less effective than daily PXF pretreatment on Days 5–8 (Table 2).

Table 2. Protection against LPS-induced resorption by pentoxifylline (PXF) in CFW/SW \times DBA/2 pregnant mice

Gestational day of treatment		LPS (0.1 μ g)	Implantation sites	% Resorptions \dagger	No. of mice
PBS	PXF (1.15 mg/day)				
5, 6, 7, 8	—	8	—*	100 ^a	4
—	5, 6, 7, 8	8	10 \pm 0.4	0 ^b	4
—	7, 8	8	7.5 \pm 0.95	18 \pm 11 ^a	4
—	5, 6, 7, 8	—	8.6 \pm 0.94	6.5 \pm 3.5 ^b	7
—	—	—	7.7 \pm 1.15	2.2 \pm 1.47 ^a	9

Values are mean \pm s.e.m.

*Not assessed because of extensive maceration of the uterine contents.

\dagger Assessment on Day 10^(a) or Day 18^(b).

The relation between LPS sensitivity and asialo GM1-positive cells

Our previous studies had revealed that spontaneous resorptions in CBA/J \times DBA/2 pregnancies were associated with the infiltration of AGM1-positive cells into the decidual–ectoplacental cone

junction area of approximately 30% of the fetoplacental units from each pregnancy. We suggested that this 30% frequency distribution would change after LPS administration if the effects of LPS were related to AGM1-positive cells. We therefore measured the frequency of AGM1-positive cells in the decidual–ectoplacental cone region of CBA/J × DBA/2 pregnant mice treated with LPS. LPS was administered on Day 9 to avoid the complete uterine maceration observed for Day 8 treatment. Treatment with 0.1 µg LPS led to a significant increase in the density of AGM1-positive cells in the decidual–ectoplacental cone region of 92% of the fetoplacental units 24 h after treatment (13.0 ± 0.8 cells per high-power field (mean ± s.e.) for 39 fetoplacental units from 4 LPS-treated pregnant mice and 1.9 ± 0.3 cells per high-power field for 23 fetoplacental units from 3 PBS-treated pregnant mice). In the PBS-treated pregnant mice, 31% of the fetoplacental units contained AGM1-positive infiltrates, as predicted by our previous studies of spontaneous resorptions (Gendron & Baines, 1988). The infiltrating AGM1-positive cells displayed the morphology of large granular lymphocytes, similar to the resorption-associated NK-like cells described previously.

Pretreatment of normal pregnant mice with anti-AGM1 was effective when given 24 h before LPS administration on Day 9, resulting in significant protection from LPS-induced resorption (Table 3). Pretreatment with PBS had no effect on the LPS-induced resorption frequency. Pretreatment with anti-AGM1 on Days 7 or 9 followed by LPS on Days 9 or 10, respectively, had little or no effect on the LPS-induced resorption frequency.

Table 3. Prevention of LPS-induced resorption by treatment with anti-AGM1 in CFW/SW × DBA/2 pregnant mice

Gestational day of treatment		LPS (0.1 µg)	% Resorption† (mean ± s.e.)	No. of mice
PBS	Anti-AGM1			
7	—	9	37.5 ± 11.0	13
—	7	9	28.2 ± 18.3	5
—	8	9	$2.5 \pm 2.3^*$	6
9	—	10	32.8 ± 13.2	9
—	9	10	28.3 ± 12.6	9

* $P < 0.05$ compared with PBS alone.

†Assessed 2 days after LPS treatment.

Discussion

Evidence presented in this report indicates that the normal mouse conceptus is exquisitely sensitive to LPS during early gestation. The sensitivity to LPS-induced resorption was maximal on Day 8 of gestation and declined thereafter. Resorption induced by LPS was associated with the appearance of TNF-α in the amniotic fluid. The effects of LPS in early pregnancy appeared to be site-specific as no other tissues showed any gross signs of pathology. Treatment of pregnant animals with the TNF-α-suppressing drug PXF decreased LPS-induced amniotic fluid TNF-α levels and decreased the LPS-induced resorption frequency. Histopathological examination of fetoplacental tissues demonstrated high densities of AGM1-positive cells infiltrating the decidual–ectoplacental cone area of fetoplacental units of LPS-treated pregnancies. Furthermore, the LPS-induced resorption was prevented by treatment with anti-AGM1 antiserum. Our results suggest that LPS-induced resorption may involve fetoplacental necrosis in response to local TNF-α production by primed macrophages or NK cells in the decidual–ectoplacental cone tissue.

Previous studies on the abortifacient effect of endotoxins on pregnancy in mice have concentrated on late gestation (Days 10–20) (Zahl & Bjerknes, 1943; Rieder & Thomas, 1960; Chedid *et al.*, 1962; Parant & Chedid, 1964; Gasic *et al.*, 1975). Our results indicate that a window during early gestation is the most sensitive period for LPS-induced resorption (Days 8–10). The dose of LPS used for the induction of resorption in early gestation was 10–90% less than doses used in previous studies (Rieder & Thomas, 1960; Parant & Chedid, 1964; Rioux-Darrieulat *et al.*, 1978). The presence of low concentrations of LPS associated with bacterial infection therefore may result in the induction of resorption at a critical time in gestation.

The mechanism of LPS-induced fetoplacental resorption may be due to the direct effect of TNF- α on the placental vasculature resulting in haemorrhage and necrosis. A similar mechanism for the action of TNF- α has been described in the haemorrhagic necrosis and regression of an established sarcoma (Carswell *et al.*, 1975; Havell *et al.*, 1988; North & Havell, 1988). Both LPS and TNF- α have been detected in the amniotic fluids of pregnant women with preterm labour (Casey *et al.*, 1989). Furthermore, placental necrosis has been demonstrated in rats treated with recombinant human TNF- α (Silen *et al.*, 1989). Minor infections of the genito-urinary tract during a critical period in early gestation may be sufficient to trigger the production of TNF- α and may therefore lead to resorption. TNF- α has been demonstrated to inhibit DNA synthesis and proliferation in trophoblast cells (Hunt, 1989). It is therefore also possible that TNF- α may act through the inhibition of trophoblast proliferation after LPS treatment, causing disruption of placental development.

Pretreatment of pregnant mice with PXF prevented resorption induced by LPS. PXF has been shown to inhibit TNF- α mRNA expression and TNF- α production by primed macrophages (Strieter *et al.*, 1988), and also decreases the activation of neutrophils by LPS, TNF- α and other cytokines (Sullivan *et al.*, 1988; Hammerschmidt *et al.*, 1988). Our previous results demonstrated that the decidual-ectoplacental cone areas of early spontaneously resorbing fetoplacental units show increased total cellular densities indicative of leukocytic infiltration (Gendron & Baines, 1989). The mechanism of PXF protection against LPS-induced resorption may involve inhibition of TNF- α production and suppression of neutrophil function in the fetoplacental unit.

The infiltration of NK-like lymphocytes appears to precede spontaneous resorption in CBA/J \times DBA/2 pregnant mice (Gendron & Baines, 1988). As shown here, LPS administration increases AGM1-positive cellular infiltrates in the same location of the fetoplacental unit as the AGM1-positive cellular infiltrates found in spontaneous resorption. We have previously demonstrated that decidual AGM1-positive cellular infiltrates increase sharply between Days 7 and 8 during spontaneous resorption (Gendron & Baines, 1988). Furthermore, spontaneous fetal resorptions are significantly reduced by anti-AGM1 treatment on Day 8 (deFougerolles & Baines, 1987). This suggests the existence of a short period during early gestation in which the presence of a population of AGM1-positive cells in the fetoplacental unit could affect fetal survival. Our data showing the maximal effectiveness of anti-AGM1 treatment on Day 8 (Fig. 1) correlate with this finding.

The exact phenotype and role of the resorption-associated AGM1-positive cells remains to be determined. AGM1-positive cells may participate in direct cell killing through lymphokine activation. Recent evidence demonstrates that the toxic effects of recombinant interleukin-2 in mice are mediated by AGM1-positive cells (Gately *et al.*, 1988). Fetal resorption can be induced by administration of interleukin-2 to pregnant mice (Tezabwala *et al.*, 1989) and this may be related to the activation of decidual AGM1-positive cells. Alternatively, AGM1-positive NK cells may act indirectly through the release of gamma interferon, which primes macrophages for TNF- α production (Gifford & Lohmann-Matthes, 1987). Since large granular lymphocytes (NK cells) are capable of TNF- α production (Peters *et al.*, 1986), it is possible that infiltrating AGM1-positive cells may themselves produce TNF- α during the early effector stages of resorption. Since activated macrophages are known to express surface AGM1 and are present in decidua (Mercurio *et al.*, 1984; Matthews *et al.*, 1985), it is possible that some of the cells being depleted by anti-AGM1 treatment

are of the monocyte/macrophage lineage. Macrophages that have been primed by interferon-gamma produce and release TNF in response to small doses of LPS that are insufficient to induce TNF production by unprimed macrophages (Gifford & Lohmann-Matthes, 1987). Decidual macrophages may be naturally primed during early pregnancy. This priming could perhaps occur through the production of interferon by early placental tissue (Fowler *et al.*, 1980). Priming of decidual macrophages may explain the exquisite sensitivity to LPS observed on Day 8 of gestation. Treatment with anti-AGM1 may therefore partly deplete a population of AGM1-positive activated macrophages that could be involved in the effector stages of fetal resorption. Since granulated metrial gland (GMG) cells have also been shown to be AGM1-positive (Redline & Lu, 1989), it is possible that this cell population may be involved in LPS-induced resorption. However, GMG cells are not able to kill either classical NK targets or targets sensitive to a TNF- α -related cytotoxin (Parr *et al.*, 1990; Liu *et al.*, 1987), suggesting that, if GMG cells are involved in the effector stages of LPS-induced resorption, the mechanism may be a novel one.

Our present findings demonstrate that LPS-induced resorption involves an AGM1-positive cellular effector component in addition to the production and release of TNF- α within the pregnant uterus. These two components may act synergistically in the effector stages of fetal resorption in response to bacterial endotoxin.

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