Effects of Gram-positive bacterial pathogens in ewes: peptidoglycan as a potential mediator of interruption of early pregnancy

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Bacterial cell walls contain peptidoglycan (PTG), which, among other actions, induces fever. The present experiment evaluated the effects of PTG treatment on early pregnancy and blood plasma concentrations of reproductive hormones. Ewes were injected i.v. with saline or 15, 30 or 60 $\mu\text{g kg}^{-1}$ sonicated PTG (*Streptococcus pyogenes*) on day 5 after mating. Each dose of PTG induced fever. Pregnancy rate at day 25 was not related to incidence of fever but tended to differ among treatments (control, 100%; low, 100%; medium, 67%; high, 60%; $P < 0.08$). Combined pregnancy rate in ewes from control and low dose groups (100%) was greater than that in ewes from medium and high dose groups (64%, $P < 0.01$). Ewes with high 13,14-dihydro-15-keto-prostaglandin $\text{F}_{2\alpha}$ (PGFM) concentrations had lower pregnancy rates (6 of 10) than those with low concentrations of PGFM (11 of 11; $P < 0.05$). Mean cortisol concentrations were

higher in treated ($2.8 \pm 0.28 \mu\text{g dl}^{-1}$) than in control ($1.1 \pm 0.03 \mu\text{g dl}^{-1}$) ewes ($P < 0.01$); the pattern of secretion was biphasic and increased in all treated ewes ($P < 0.01$). Neither means nor profiles of oestradiol differed with treatment. Mean concentrations and the pattern of concentrations of progesterone were reduced in all treated ewes, as indicated by the time by treatment and linear interaction with treatment (1.2 ± 0.1 versus $1.6 \pm 0.1 \text{ ng ml}^{-1}$, $P < 0.01$). Patterns of LH pulses did not differ from 0 to 4 h or 24 to 28 h after treatment; mean plasma LH concentration was lower in ewes treated with 0, 15 or 30 $\mu\text{g PTG kg}^{-1}$ than with 60 $\mu\text{g PTG kg}^{-1}$ ($P < 0.01$). Pregnancy status was not related to plasma concentrations or patterns of LH, oestradiol, progesterone or cortisol. Inflammatory mediators, such as $\text{PGF}_{2\alpha}$, may act directly on the embryo or uterus in ewes treated with PTG.

Introduction

Cell growth and lysis of bacteria after bacterial infection result in the release of antigenic cell wall components, leading to increases in inflammatory mediators, which can result in an inflammatory response. For example, Law (1923) observed a general immune response, including fever, in cows with bacterial infection of the udder (mastitis). Cullor (1993) indicated that endotoxin, a component of the cell wall of Gram-negative bacteria, induces luteolysis and reduces conception rate and early embryonic survival through the release of inflammatory mediators. Barker *et al.* (1998) found that clinical mastitis caused by either Gram-negative or Gram-positive pathogens reduces reproductive performance during early lactation. Each type of pathogen may act through similar mechanisms to increase inflammatory mediators, such as cortisol, interleukin 1 and $\text{PGF}_{2\alpha}$, leading to reproductive failure.

Cell walls of both Gram-negative and Gram-positive bacteria contain a mucopeptide, peptidoglycan (PTG; Rotta, 1975). Injections of PTG lead to a pyretic response in rabbits comparable to that elicited by endotoxin (Rotta, 1975). The induced fever is eliminated by treatment with antiserum to PTG. Peptidoglycan is implicated in the induction of acute changes in vascular permeability (Woolverton *et al.*, 1989), similar to those found during endotoxin treatment (Banks, 1999) and experimental mastitis (Matilla and Frost, 1989). Both endotoxin and PTG stimulate the immune system (Rotta, 1975; Lichtman *et al.*, 1994) by crossing the intestinal mucosa and entering the portal veins, subsequently stimulating the release of tumour necrosis factor α ($\text{TNF-}\alpha$) from Kupffer cells. However, the intracellular signalling pathways for endotoxin and PTG differ (Lichtman *et al.*, 1994).

Increased secretion of $\text{PGF}_{2\alpha}$ in response to PTG would be expected to decrease pregnancy rate by inducing luteal regression or compromising luteal function. Buford *et al.* (1996) found that even with replacement therapy with progestagens, luteal regression during days 4–9 after oestrus increased early embryonic

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death in cattle. Cows supplemented with progestagen and treated with $\text{PGF}_{2\alpha}$ on days 5–8 after mating have lower pregnancy rates (23%) compared with controls (72%) or cows treated on days 10–13 (50%) or 15–18 (60%) (Seals *et al.*, 1998). Similarly, Costine *et al.* (2001) showed that progestagen-supplemented ewes are sensitive to the embryotoxic effects of $\text{PGF}_{2\alpha}$ on days 4–7 after mating. Therefore, the early pregnant ewe should be a useful model for evaluation of the effects of PTG on reproductive performance.

The present study tested the null hypothesis that embryonic survival and concentrations of inflammatory mediators and reproductive hormones are not affected by the treatment of ewes with PTG. Specifically, the objective of this study was to evaluate the effect of PTG on (i) pregnancy rate and (ii) concentrations of inflammatory mediators (cortisol and $\text{PGF}_{2\alpha}$) and reproductive hormones (LH, oestradiol and progesterone) in ewes.

Materials and Methods

Animals

Twenty-two ewes of mixed breeding, four years of age or older, were fed haylage and corn silage twice a day and were given free access to fresh water. Oestrus (day 0) was synchronized with two i.m. injections of 5 mg $\text{PGF}_{2\alpha}$ (Lutalyse; Pharmacia and Upjohn Inc., Kalamazoo, MI), 3 h apart (Hawk, 1975). Ewes were observed for oestrus twice a day from synchronization to day 18 using a vasectomized ram, and were mated to two rams of known fertility. Pregnancy diagnosis was carried out on day 25 in ewes that had not returned to oestrus, by transrectal ultrasonography as described by Schrick and Inskeep (1993), using an Aloka 500 console with a 7.5 MHz linear array transducer designed for examination of the human prostate gland (Corometrics Medical Systems, Wallingford, CT). Use of animals in this study was approved by the West Virginia University Institutional Animal Care and Use Committee as protocol #9801-06.

Dose determination and treatments

Solubilized PTG, isolated from the Gram-positive pathogen *Streptococcus pyogenes*, was obtained from Lee Laboratories (Grayson, GA). A single dose of PTG results in a pronounced inflammatory response in rats (Lee Laboratories, personal communication). Therefore, ewes received a single injection (i.v.) of PTG. All ewes were treated on day 5 after mating. Luteolysis is induced in cows with $1.5 \mu\text{g}$ endotoxin kg^{-1} body weight (BW) (Gilbert and Bosu, 1990). Treatment of goats with $1.8 \mu\text{g}$ endotoxin kg^{-1} BW induces luteolysis; however, treatment with $1.5 \mu\text{g}$ endotoxin kg^{-1} BW is ineffective (Fredricksson *et al.*, 1985). Rotta (1975)

determined that $150 \mu\text{g}$ PTG elicits a pyretic response in rabbits comparable to that induced by $5 \mu\text{g}$ endotoxin. Therefore, $60 \mu\text{g}$ PTG (equivalent to $2 \mu\text{g}$ endotoxin) would be expected to induce fever and luteolysis and $15 \mu\text{g}$ PTG (equivalent to $0.5 \mu\text{g}$ endotoxin) would not be expected to induce fever or luteolysis. Ewes were assigned at random to one of four treatments: control, saline only ($n = 5$); low, $15 \mu\text{g}$ PTG kg^{-1} BW ($n = 6$); medium, $30 \mu\text{g}$ PTG kg^{-1} BW ($n = 6$); or high dose, $60 \mu\text{g}$ PTG kg^{-1} BW ($n = 5$). Rectal body temperature was taken hourly from 0 to 4 h and at 8, 12 and 24 h after treatment.

Assays

Samples of jugular blood (10 ml) were collected into heparinized tubes every 15 min from 0 to 4 h and 24 to 28 h after treatment for LH determinations. Samples were collected every 4 h up to 48 h and daily to day 18 for determinations of the other hormones. Samples were centrifuged at $1000g$ for 20 min and plasma was stored at -20°C . Validated radioimmunoassays were used to determine concentrations of progesterone (Sheffel *et al.*, 1982), oestradiol (Rozell and Keisler, 1990), LH (Fogwell *et al.*, 1978), cortisol (human cortisol, radioimmunoassay kit; Diagnostic Products Corporation, Los Angeles, CA) and 13,14-dihydro-15-keto-prostaglandin $\text{F}_{2\alpha}$ (PGFM; Silvia and Taylor, 1989). For each hormone, all samples ($200 \mu\text{l}$ in duplicate) from an animal were included in one assay. Intra- and interassay coefficients of variation (CVs) for progesterone, oestradiol, LH and PGFM were 9.6 and 20.5, 5.3 and 18, 10.9 and 17.4, and 4.4 and 2.9%, respectively. Concentrations of cortisol were determined in a single assay, with an intra-assay CV of 11.5%. Sensitivities for the assays were 0.1 ng ml^{-1} , 0.5 pg ml^{-1} , 0.5 pg ml^{-1} , 10 pg ml^{-1} and $0.5 \mu\text{g dl}^{-1}$ for progesterone, oestradiol, LH, PGFM and cortisol, respectively.

Statistical analyses

Pulse patterns of LH were examined by PC-Pulsar (Gitzen and Ramirez, 1986). Concentrations of hormones were examined by ANOVA and pregnancy rates were examined by point biserial correlation (SAS Institute Inc., 1989). For PGFM, variances differed among treatments and time periods, thus a weighted ANOVA was used. Each observation was weighted by the inverse of the variance among observations within a given treatment and time period. Days were grouped into four periods, each consisting of four or five observation days, for the purpose of calculating variances.

Association of treatment with incidence of fever was analysed by chi-squared test (SAS Institute Inc., 1989). The assumptions of normality and homogeneity of variances were not met, and the data did not follow a linear model, because the concentrations of PGFM of

Fig. 1. (a) Body temperature (fever response) in ewes after treatment with peptidoglycan (PTG). Fever (body temperature increase $> 0.5^{\circ}\text{C}$) occurred in treated (13 of 17) but not in control ewes (0 of 5; $P < 0.01$; SEM = 0.23). (b) Concentrations of 13,14-dihydro-15-keto PGF_{2 α} (PGFM) in ewes did not differ as a result of treatment with PTG ($P < 0.01$; SEM = 0.77). (c) Concentrations of cortisol differed between control ewes and ewes treated with PTG ($P < 0.01$; SEM = 0.77). (d) Concentrations of progesterone were reduced in all ewes treated with PTG ($P < 0.01$; SEM = 0.37). (a–d) Groups were as follows: control (○), saline only; low (■), $15\text{ }\mu\text{g PTG kg}^{-1}$ body weight (BW); medium (●), $30\text{ }\mu\text{g PTG kg}^{-1}$ BW; high (▲), $60\text{ }\mu\text{g PTG kg}^{-1}$ BW.

many ewes were not detectable ($< 10\text{ pg ml}^{-1}$). Thus, Fisher's exact test (Finney *et al.*, 1963) was used to determine the effects of PGFM classification (low and high 50% groups based on concentrations of PGFM) on pregnancy status.

Results

Haematocrits were similar in all ewes throughout the sampling period. Fever (body temperature increase $> 0.5^{\circ}\text{C}$) occurred in treated (13 of 17) but not in control ewes (0 of 5; $P < 0.01$). Body temperature peaked at 4 h and returned to pre-treatment values by 8 h after PTG treatment (Fig. 1a). However, pregnancy rate was not related to incidence of fever (ewes with fever: 10 of 13 pregnant; ewes with no fever: 8 of 9 pregnant). Pregnancy rate tended to differ among treatments but the effect was not significant (control, 100%; low, 100%; medium, 67%; high, 60%; $P < 0.08$). Combined pregnancy rate in ewes from control and low dose groups (100%) was

greater than that in ewes from medium and high dose groups (64%, $P < 0.01$).

Mean concentrations and profiles of PGFM did not differ with treatment ($P > 0.10$; Fig. 1b). Pregnancy rate was lower in ewes with high concentrations of PGFM (6 of 10) than in ewes with low concentrations of PGFM (11 of 11; $P < 0.05$). Concentrations or patterns of the other hormones were not associated with pregnancy status. The pattern of cortisol secretion was biphasic and increased in all ewes treated with PTG ($P < 0.01$; Fig. 1c). Mean concentrations of cortisol were higher in treated than in control ewes (2.8 ± 0.28 versus $1.1 \pm 0.03\text{ }\mu\text{g dl}^{-1}$; $P < 0.01$). Mean concentrations of LH were lower ($P < 0.01$) in ewes treated with 0, 15 or $30\text{ }\mu\text{g PTG kg}^{-1}$ (0.9 ± 0.1 , 1.2 ± 0.1 and $0.9 \pm 0.1\text{ ng ml}^{-1}$, respectively) than with $60\text{ }\mu\text{g PTG kg}^{-1}$ ($1.9 \pm 0.1\text{ ng ml}^{-1}$). However, characteristics of LH pulses did not differ. Mean concentrations (1.2 ± 0.1 versus $1.6 \pm 0.1\text{ ng ml}^{-1}$; $P < 0.01$) and the pattern of concentrations of progesterone

were reduced in all treated ewes, as indicated by the time (h) by treatment and linear interaction with treatment (Fig. 1d). Neither mean concentrations nor patterns of oestradiol differed as a result of treatment. Concentrations of oestradiol for all ewes averaged $2.5 \pm 0.1 \text{ pg ml}^{-1}$.

Discussion

Peptidoglycan-induced pyretic response was achieved in the present study, as 76% of ewes treated with PTG responded with a fever. The response was acute; body temperature peaked at 4 h and returned to baseline by 8 h. Although the profile in the present study was monophasic, endotoxin or PTG produces a biphasic pyretic response in rabbits (Rotta, 1975). Changes in mean concentrations of cortisol are biphasic and similar to those found in men after induced hyperthermia (Collins *et al.*, 1969). Cortisol has been shown to decrease release of pyrogenic substances from leucocytes (Gander *et al.*, 1968; Dillard and Bodell, 1970). It is possible that concentrations of cortisol increased in response to fever and controlled the pyretic response. Incidence of fever and concentrations of cortisol were increased in all treatment groups. Pregnancy loss in treated ewes was not a function of fever or concentration of cortisol *per se*, because ewes receiving the low dose of PTG maintained pregnancy even though they showed fever and had high concentrations of cortisol.

Loss of pregnancy could occur through decreased luteal function. Secretion of LH is decreased in studies with endotoxin treatment (Stoebel and Moberg, 1982; Lopez-Diaz and Bosu, 1992). Corticotropin-releasing factor (Lopez-Diaz and Bosu, 1992) and adrenocorticotrophic hormone (Besedovsky *et al.*, 1986) increase after a challenge of endotoxin and either by acting directly or through increases in cortisol, can decrease LH secretion (Besedovsky *et al.*, 1986; Lopez-Diaz and Bosu, 1992; Cullor, 1993). However, in the present study, PTG treatment did not affect the pattern of secretion of LH; in fact, mean LH was greater in ewes receiving the high dose of PTG. In addition, treatment with PTG did not affect mean concentrations or pattern of oestradiol. Thus, embryo mortality was not related to increased systemic concentrations of oestradiol, as observed previously during maternal recognition of pregnancy in cattle (Pritchard *et al.*, 1994), nor to reduced concentrations of LH.

PTG treatment results in decreased concentrations of progesterone at each dose. However, ewes in the low dose group maintained pregnancy despite decreased progesterone. PTG treatment may have resulted in inadequate formation of corpora lutea, and the subsequent loss of other factors may have resulted in loss of pregnancy.

It is possible that treatment with PTG resulted in the synthesis or release of additional inflammatory mediators

that could act directly on the embryo. Increases in $\text{PGF}_{2\alpha}$ occur after treatment with endotoxin (Giri *et al.*, 1984, 1991; Fredricksson *et al.*, 1985; Cort and Kindahl, 1990; Cullor, 1993). Costine *et al.* (2001) showed that progestagen-supplemented ewes are sensitive to the embryotoxic effects of $\text{PGF}_{2\alpha}$ on days 4–7 after mating. Although in the present study mean concentrations and profiles of PGFM did not differ due to treatment, pregnancy was maintained in only six of ten ewes with high plasma concentrations of PGFM, but in all ewes with lower PGFM. Increases in $\text{PGF}_{2\alpha}$ were insufficient to induce luteolysis. It is possible that $\text{PGF}_{2\alpha}$ has direct adverse effects on the embryo. In addition, increases in acute phase proteins such as tumour necrosis factor α (TNF- α) may affect embryonic survival directly after PTG treatment, as *in vitro* culture of preimplantation mouse embryos with TNF- α results in the absence of embryos reaching the blastocyst stage and decreased total cell number (Kurzawa *et al.*, 2001).

Redl *et al.* (1989) compared the effects of lipopolysaccharide and PTG on cardiovascular function in ewes. To date there is no reported work evaluating the effects of PTG on reproductive function in ewes. The goal of the present study was to examine whether Gram-positive pathogens, like the extensively studied Gram-negative pathogens, can have adverse effects on reproduction. The fever and cortisol data from the present study demonstrate that PTG treatment results in an immune response. Pregnancy loss was not due to fever, increased cortisol and decreased progesterone, as ewes in the low dose group maintained pregnancy despite these changes. Instead, inflammatory mediators such as $\text{PGF}_{2\alpha}$ and TNF- α , which could act directly on the embryo, might adversely affect embryonic survival after PTG treatment in ewes.

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