Neutrophil migration into the bovine uterine lumen following intrauterine inoculation with killed *Haemophilus somnus*

B. M. Butt¹, P. L. Senger^{2*} and P. R. Widders^{1†}

¹Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA 99164-7040, USA; and ²Department of Animal Sciences, Washington State University, Pullman, WA 99164-6332, USA

Summary. Polymorphonuclear neutrophils (PMN) in bovine uterine flushings following intrauterine deposition of killed bacteria were measured and the effect of immune status on the influx of PMN into the uterine lumen during oestrus was determined. Holstein heifers were immunized with a 270-kDa outer-membrane protein (omp-270) from *Haemophilus somnus*. During oestrus, immunized heifers ($n = 21$) received an intrauterine inoculum of either a heat-killed suspension of a homologous strain of *H. somnus* containing omp-270 ($n = 7$), a heterologous strain of *H. somnus* lacking omp-270 ($n = 7$), or phosphate-buffered saline ($n = 7$). Five additional heifers were inseminated with extended bovine semen. Uterine contents were collected in saline lavage immediately before inoculation (t_0) and at 6, 24, 48, 72, 96, and 120 h after inoculation. The semen-inoculated heifers were lavaged only at t_{120} . All groups experienced PMN infiltration which peaked 6 h after inoculation and tended to decline thereafter. Differences were not observed between treatment groups, indicating that neither bacterial inoculation nor immune status was as important in eliciting PMN effusion as the flushing procedure itself.

Keywords: uterus; immunology; neutrophils; cow

Introduction

Bovine intrauterine infections contribute significantly to reproductive failure in cattle (Sagartz & Hardenbrook, 1971; Hartigan *et al.*, 1972; Studer & Morrow, 1978; Paisley *et al.*, 1986). Intra-uterine infections in cattle have been associated with abortion (Roberts, 1986; Widders *et al.*, 1986), early embryonic death (Kaneene *et al.*, 1986a, b) and infertility (Griffin *et al.*, 1974; Studer & Morrow, 1978). Understanding uterine defence mechanisms is essential for developing effective treatment and preventative strategies and for optimizing reproductive performance.

It is accepted that polymorphonuclear neutrophils (PMNs) are the predominant phagocytic cell type in the bovine uterus during the follicular phase of the cycle. These cells can function as non-specific phagocytes or in the presence of opsonizing antibody can contribute to specific immunity. However, PMNs can reduce fertility through their effects on sperm function and viability (Targowski *et al.*, 1985), embryo survival (Smith *et al.*, 1971; Bo *et al.*, 1975; Anderson & Alexander, 1979) and implantation (Waites & Bell, 1982).

Uterine PMN effusion is known to occur during the follicular phase of the normal bovine oestrous cycle (Skjerven, 1956; Hartigan *et al.*, 1972). At times other than oestrus, the presence of

*Reprint requests to.

†Present address: Veterinary Research Insititute, 475–485 Mickleham Rd, Attwood, Victoria 3049, Australia.

PMNs in the uterine lumen is considered pathological (Skjerven, 1956; Studer & Morrow, 1978). Ovarian hormones are thought to play a major role in mobilization and activation of PMN in the uterine lumen (Black *et al.*, 1960; Broome *et al.*, 1960; Hawk *et al.*, 1960a, b, c), but the kinetics of PMN migration has not been defined in normally cycling cows, or in heifers given intrauterine inoculations of semen or bacteria.

Immune status has been reported to be important in PMN mobilization to the guinea-pig uterus. Targowski (1984) has demonstrated that there was an increase in PMN mobilization into the uterus in systemically immunized guinea-pigs following a homologous intrauterine challenge when compared with controls.

The aim of this investigation was to define the kinetics of PMN mobilization into the bovine uterus at oestrus in response to inoculation of heat-killed *H. somnus* and to determine the effect of immune status on PMN recruitment to the uterus.

Materials and Methods

Preparation of a 270-kDa outer-membrane protein for immunization. Selection of the protein (omp-270) in this study was based on its antigenicity, ease of preparation and the availability of isolates of *H. somnus* that expressed or lacked omp-270. Suspensions of *H. somnus* 2336 were grown in brain and heart infusion broth (Difco Laboratories, Detroit, MI, USA) supplemented with 0.1% Tris and 1 µg thiamine monophosphate/ml to log phase, and centrifuged at 9148 g for 15 min. Pellets were discarded and the supernatant was filtered through 0.45-µm filters. This filtrate was ultracentrifuged at 245 000 g for 2 h to pellet membrane vesicles. This fraction contained omp-270 as well as other surface proteins (Yarnall *et al.*, 1988). Supernatants were discarded and membrane vesicles resuspended in the residual volume. Pooled samples were fractionated by gel filtration (Sephadex CL4B: Pharmacia LKB Biotechnology, Inc. Piscataway, NJ, USA). Fractions were analysed for the presence of omp-270 by sodium dodecyl sulphate polyacrylamide gel electrophoresis. Fractions containing only omp-270 were pooled and dialysed exhaustively against distilled water. The pooled, dialysed fractions were lyophilized.

Bacteria. Heat-killed suspensions of *H. somnus* strains 2336 and 1P were prepared for inoculation. The 2336 strain was isolated from the lungs of a cow which had died of pneumonia. This strain contains an omp-270 used to immunize all heifers in this experiment (Widders *et al.*, 1989). The 1P strain, which does not contain omp-270, was isolated from the prepuce of a clinically normal bull (Widders *et al.*, 1989). Bacteria were grown to log phase and centrifuged as described above. Pellets were resuspended in phosphate-buffered saline (PBS) to 5×10^7 colony-forming units/ml, determined spectrophotometrically. Bacteria were killed by heating at 60°C for 10 min and the sterility of the suspensions was confirmed by culture.

Animals and immunization. Thirty Holstein heifers, ~12 months old, were obtained from a commercial dairy in central Washington State, WA, USA. No animal contained demonstrable serum antibody titres to the omp-270 antigens prior to the experiment. All heifers were immunized (i.m.) with 10 µg omp-270 in Freund's incomplete adjuvant on Days 1 and 35 of the experiment.

Detection of oestrus. All animals were injected (i.m.) with 25 mg prostaglandin-2α (The Upjohn Company, Kalamazoo, MI, USA) on Days 50 and 60. Beginning 48 h after the second prostaglandin injection (Day 62) all animals were observed every 6 h for signs of behavioural oestrus. Only those which stood to be mounted were included in the experiment. A total of 26 heifers qualified.

Treatment groups. Four treatment groups were used to examine uterine effusion. Animals within each group received intrauterine inoculations of either *H. somnus* 2336 (containing omp-270), *H. somnus* 1P (lacking omp-270), PBS, or extended bovine semen.

Sampling technique and treatment administration. Eight hours after the first observation of standing heat, uterine lavage was performed using the method of Anderson *et al.* (1985). Epidural anaesthesia was performed to reduce rectal contractions and facilitate passage of the catheter through the cervix. The perineum was thoroughly cleaned with chlorhexidine (Virocan, Boehringer Ingelheim, St Joseph, MO, USA). A 14-gauge Rausch catheter was passed into the right uterine horn, the cuff inflated and 30 ml of sterile 0.01M PBS, pH 7.4, was infused into the right horn; 5–25 ml infusate was recovered by aspiration using a 30-ml syringe. After flushing and while the catheter was still in place, 0.5 ml of one of the following treatments was infused into the right uterine horn: 5×10^7 heat-killed *H. somnus* of either the homologous or heterologous strains; or PBS. Another 1.0 ml PBS was infused immediately after this treatment, to ensure that all bacteria entered the uterus. Additional flushings using 30 ml PBS were obtained at 6, 24, 48, 72, 96 and 120 h after the initial inoculation.

PMN quantitation. The total number of nucleated cells was counted in uterine flushings by haemocytometry. Additional aliquots were cytocentrifuged (Cytospin, Shandon Southern, Inc., Astmoor, Runcorn, Cheshire, UK)

and stained with Diff-Quik (Baxter Healthcare Corp., McGaw Park, IL, USA) and differential cell counts performed by a single observer on 100 cells. A 1 ml aliquot from each sample was evaluated for the presence of haemoglobin (Cyanmethaemoglobin technique using a Colter haemoglobinometer, Colter Electronics, Inc., Hialeah, FL, USA) to identify animals undergoing iatrogenic uterine trauma or metoestrus bleeding. Those samples positive for haemoglobin and subsequent samples from the same animals were removed from the study.

Because the volume of fluid recovered from the uterus varied (5–25 ml/flush) the concentration of PMN was standardized to total uterine protein (TP) to normalize for these volume differences. The concentration of TP will also differ between cows and with the magnitude of PMN infiltration. However, with lavage fluids this calculation permits the most accurate analysis of the kinetics of cellular infiltration currently available.

All flushings were centrifuged at 1300 *g* for 10 min. Total protein content was measured colorimetrically with a commercial staining kit (Bio-rad protein assay: Bio-rad Laboratories, Richmond, CA, USA) using the following modification. A 5 µl sample was mixed with 300 µl dye in 96-well plates. After 5 min at room temperature optical density was measured at 950 nm.

Statistical analysis. The rank sum test was used to detect differences between treatment groups at any sampling time and to detect differences within any one treatment group with respect to time.

Results

Exfoliate epithelial cells, macrophages, lymphocytes, eosinophils and PMNs were observed in uterine flushings. The predominant cell type in t_0 flushings was the exfoliate epithelial cell (97%), which as a percentage of total cells declined in subsequent flushings. Macrophages were absent from t_0 flushings and reached their peak at t_{48} , comprising ~10% of the luminal cells. Lymphocytes and eosinophils were seen sporadically and at low concentrations.

Flushings obtained immediately before the challenge (t_0) contained 2.7% PMNs ($n = 21$) (Fig. 1a). An increase in percentage PMN ($P < 0.005$) occurred between t_0 and t_6 for all treatment groups. This represented the peak PMN effusion in the saline and homologous groups, PMN reaching 44, 51 and 55% in the saline, homologous *H. somnus* and heterologous *H. somnus* groups, respectively. Treatment influenced neither the degree nor the timing of PMN effusion ($P > 0.25$). Mean %PMN declined with succeeding sampling times in all groups, with the exception of a slight increase at t_{24} in the heterologous group. At t_{120} , %PMN did not differ from t_0 levels ($P > 0.05$). Uterine flushings from heifers inseminated with extended bovine semen at t_0 and lavaged only at t_{120} contained 3.0% PMN.

The PMN concentration (cells/ml) in uterine flushings was standardized to TP concentration (µg/ml). At t_0 , 11 PMN/µg TP was observed ($n = 19$) (Fig. 1b). A significant increase in PMN/TP occurred in all groups between t_0 and t_6 ($P < 0.05$), with values of 884, 1075 and 732 cells/µg in the homologous, heterologous and PBS groups, respectively. As with %PMN, PMN/TP remained above t_0 values in all groups from t_6 to t_{96} ($P < 0.05$). Uterine flushings obtained at t_{120} did not differ significantly from t_0 samples ($P > 0.3$). Uterine flushing obtained 5 days after artificial insemination contained mean PMN/TP values similar to those observed at t_0 (9.0 ± 8.4 vs. 18.0 ± 23.1).

Discussion

These results describe the temporal pattern of PMN infiltration into the bovine uterine lumen. At the first sampling point (t_0), 8 h after observation of standing heat, PMN concentrations were low relative to the total number of cells and to TP concentration in uterine flushings. Peak PMN effusion was observed 6 h after the initial uterine lavage. No detectable differences were noted in the magnitude or the time of onset of PMN infiltration between cows receiving saline lavage alone and those receiving an inoculum of killed bacteria.

Our results suggest that uterine lavage had little immediate effect because PMN effusion was negligible at t_0 . However, uterine lavage did influence subsequent flushings. All treatment groups experienced marked PMN effusion 6 h after the first lavage was obtained. Williamson *et al.*

Fig. 1. Results of flushings obtained from bovine uteri following immunization with *Haemophilus somnus*, homologous strain ▨, heterologous strain ■ or phosphate-buffered saline (control) □. (a) Percentage of cells obtained from uterine flushings which were polymorphonuclear neutrophils (PMN); (b) concentration of PMN as a proportion of the total uterine protein (TP) shown in the 120 h following immunization.

(1987) reported a significant uterine PMN effusion in mares 6 h after cervical or uterine stimulation. Therefore, delayed effusion in cows may be due, in part, to physical stimulation of the endometrium with fluid installation.

During oestrus there was minimal PMN content in the uterus. Time zero samples contained a very low proportion of PMN compared with other cell types present or with TP. While PMNs have been shown to phagocytize sperm *in vitro* (Mattner, 1969), our results indicate that sperm are initially exposed to a uterine environment with few PMNs. Five days after artificial insemination PMN levels were also low, indicating that embryos are not normally exposed to a leukocytic environment upon entering the uterus.

Unlike Targowski's (1984) findings in guinea-pigs, we were unable to demonstrate that immune status influenced PMN recruitment into the bovine uterus. Homologous inoculation with heat-killed *H. somnus* did not increase PMN mobilization into the uterus when compared with controls. No significant differences were detected between the homologous and heterologous groups, or between the homologous challenge group and the PBS control group. Immunization, if it had an effect, was less important than lavage itself.

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