Uterine lymphocyte distribution and interleukin expression during early pregnancy in cows

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Introduction

An interaction between the conceptus and the immune system is important during implantation (Hansen, 1997). Although the immune system is functional in the uterus and the bovine embryo expresses paternal major histo-compatibility complex (MHC) molecules of class I on day 7 of pregnancy (Templeton et al., 1987; Low et al., 1990), the conceptus nevertheless escapes the deleterious effect of maternal rejection. The exact mechanisms involved in the maternal tolerance of the foreign fetal antigens during early pregnancy are not fully understood but are likely to involve both local production of immunomodulatory signals and changes in the type of MHC antigens expressed on the trophoblast (Wegmann et al., 1993; Ellis, 1994). It has also been suggested that the stimulation of lymphocytes by the conceptus may in fact promote conceptus growth by increasing concentrations of cytokines available at the conceptus–maternal interface (Wegmann, 1988).

Immune responses are mainly divided into cell-mediated and delayed-type hormonal responses. The cell-mediated immune response (Th type 1) accounts for acute cytotoxic activity, whereas the hormonal response (Th type 2) involves the induction of lymphocyte proliferation and antibody production before the rejection of foreign bodies takes place. It is believed that the immune system is biased towards the Th type 2 response during pregnancy (Lin et al., 1993; Wegmann et al., 1993; Raghupathy, 1997). Type 2 responses are associated with preferential production of the cytokines IL-4, IL-5 and IL-10, whereas in type 1 responses, the pro-inflammatory cytokines interleukin 2 (IL-2), interferon γ (IFN-γ) and tumour necrosis factor α (TNF-α) predominate (Wegmann et al., 1993; Mosmann and Sad, 1996). Recurrent spontaneous embryo loss has been related to a high degree of maternal immunity during pregnancy (Hill et al., 1992; Yamada et al., 1994; Hill et al., 1995). In mice, the CBA × DBA/2 mating cross has a high rate of spontaneous fetal resorption and their placentae are deficient in IL-4 and IL-10 compared with the more fertile CBA × BALB/c cross (Chaouat et al., 1995). Furthermore, the rate of resorption in the CBA × DBA/2 mice can be reduced by treatment with either recombinant (r)IL-10 or with r ovine IFN-γ (Chaouat et al., 1995). Further evidence for the involvement of cytokines...
in fetal loss comes from studies showing increased concentrations of IL-2 in placental tissues associated with spontaneous abortions in both mice (Tangri and Raghupathy, 1993) and women (Hill et al., 1995; Marzi et al., 1996).

In dairy cows, about 25% of pregnancies fail in the first 3 weeks of gestation, representing a multimillion pound loss to the industry (Peters, 1996). In cattle, attachment of the fetal chorion to the maternal caruncles to form an epitheliocorial placenta does not begin until day 19 of pregnancy (Wathes and Wooding, 1980). This must be preceded by the signal for the maternal recognition of pregnancy to prevent luteolysis (Flint et al., 1994a; Wathes and Lamming, 1995). In cows, this signal consists of the production of IFN-\(\tau\) by the trophoblast from day 10 to day 25 of pregnancy (Roberts et al., 1992; Flint et al., 1994b). As well as inhibiting oxytocin receptor formation and hence luteolysis (Wathes and Lamming, 1995), IFN-\(\tau\) regulates lymphocyte proliferation in vitro (Newton et al., 1989; Skopets et al., 1992) and has antiviral activity (Pontzer et al., 1988).

Studies in ruminants have determined that pregnancy has a profound effect on the uterine population of lymphocytes and macrophages (French and Northey, 1983; Skopets et al., 1992). The numbers of both types of cell are significantly reduced between early and mid-pregnancy in cattle (Vander Wielen and King, 1984) and sheep (Staples et al., 1983; Lee et al., 1988; Lee et al., 1992). By mid-pregnancy, virtually no lymphocytes or macrophages are found in the caruncular endometrium although they are still present in the intercaruncular endometrium (Gogolin-Ewens et al., 1989; Low et al., 1990). The caruncles are the sites in which fetal chorion comes into close contact with the maternal endometrium to form placentomes, so these findings indicate that the maternal immune tolerance is both local and specific to the areas adjacent to fetal tissues. Indeed, unknown factors secreted from the ovine placenta also inhibit lymphocyte activity at various stages of pregnancy (Low et al., 1991).

These studies all relate to the period after placentation. To date, there has been no investigation of the uterine lymphocyte population during the critical pre-implantation period in cattle when high pregnancy losses occur. Therefore, the aim of this study was to compare the immune cell distribution in the uterus between pregnant and non-pregnant cows on day 16 using immunohistochemistry. The results were related to both the embryonic production of IFN-\(\tau\) and the concentrations of IL-1\(\alpha\), IL-2, IL-6 and IL-10 mRNAs in the uterine tissue.

Materials and Methods

Tissue collection

Holstein–Freisian cows were synchronized with two injections of Estrumate (PGF analogue, Coopers Animal Health, Crewe) at 12 days apart and subsequently either given a double insemination 72 h and 96 h after the second injection or left as un inseminated controls. The animals were killed on day 16 and the reproductive tract was removed and transported to the laboratory within 30 min. The uterus was bisected and each half was flushed with 40 ml sterile saline (0.9% (w/v) NaCl) for collection of embryos. The flushed uterine fluid was stored frozen at –20°C for measurement of antiviral activity. Complete cross-sections of uterine horns from the side containing the embryo or corpus luteum were frozen in isopentane immersed in liquid nitrogen and stored at –70°C for immunohistochemistry. Additional samples of uterine horns were frozen in liquid nitrogen and stored at –70°C for mRNA preparation.

The length of each flushed blastocyst was measured and the embryos were classified into either small (<0.5 cm, no elongation), medium (0.5–1.0 cm, some elongation) or large (>1.0 cm, clear elongation) size categories. On the basis of the flush results, the cows were classified into three groups: (i) cyclic controls (n = 8); (ii) pregnant (n = 15); and (iii) inseminated but not pregnant (n = 5). Not all animals were used for all the measurements reported.

Measurement of antiviral activity

Interferon activity in uterine flushes was measured by the Madin Darby bovine kidney (MDBK) cell Semiliki Forest virus cytoplasmic inhibition assay as described by Abayasekara et al. (1995). Samples with IFN-\(\tau\) concentrations initially below the detection limit of 5 antiviral activity units ml\(^{-1}\) were concentrated 10-fold by centrifugal filtration using MicrocapsTM (Flowgen Instruments Ltd, Lichfield) with a molecular cut off of 3000. A starting volume of 3 ml of flushed fluid was reconstituted in 300 \(\mu\)l. Samples above the detection limit in the initial assay were also concentrated 10-fold to validate this approach, resulting in a mean \(\pm\) SEM increase in IFN activity of 913 ± 58%.

Immunohistochemistry for localization of uterine lymphocytes

Immunohistochemistry was carried out on cross-sections of uterus as described by Wathes and Hamon (1993) with a few modifications. The primary antibodies used were monoclonal mouse anti-bovine CD4\(^+\) (T helper lymphocyte specific, CC8; Naessens et al., 1993), CD14\(^+\) (macrophage specific, CCG33; Berthon and Hopkins, 1996) and CD21\(^+\) (B lymphocyte specific, CC21; Sopp, 1996) kindly donated by Dr Chris Howard (Institute of Animal Health, Compton). Serial sections of a bovine lymph node were used as a positive control tissue. Briefly, cross-sections (5 \(\mu\)m thick) were cut on a cryostat and thaw-mounted onto poly-l-lysine coated (0.1 mg ml\(^{-1}\)) slides. The slides were fixed in acetone (100%) followed by hydrogen peroxide in methanol (1% (v/v); BDH, Poole) at 4°C for 30 s each. The slides were washed twice in 1 \(\times\) PBS for 5 min. All subsequent incubations were carried out in a humidified box at room temperature unless otherwise specified. After washing, the sections were incubated with normal rabbit serum (NRS, 2.5%; Sigma, Poole) for 20 min. The NRS was then removed by blotting before incubation with 100 \(\mu\)l primary antibody (collected from the tissue culture supernatant and diluted 1:5 in 1 \(\times\) PBS ) at 4°C for 1 h. Control sections were treated with non-specific mouse anti-IgG (1 \(\mu\)g ml\(^{-1}\); Sigma). An
additional CD8+ antibody (specific for cytotoxic T cells) was tested but did not produce positive staining in control tissues. Results from this antibody were not included in the study.

After incubation, the slides were washed twice in 1 × PBS for 5 min and incubated with 100 μl per slide of: (i) rabbit anti-mouse antiserum (1:100, Sigma) for 30 min; (ii) peroxidase anti-peroxidase (1:50, Sigma) for 30 min; and (iii) activated diaminobenzidinetetrahydrochloride (DAB; 0.5 mg ml–1; Sigma) for 10 min in the dark. After incubation, the excess DAB was removed by washing in distilled water. The slides were then counterstained with haematoxylin and eosin and subjected to a serial dehydration in 75%, 100% ethanol, and xylene (100%) each for 2 min. The slides were subsequently mounted for light microscope inspection.

Two approximately circular cross-sections of intact uterus were processed from each cow with each antibody. The stained sections were divided into four quarters from the centre and one quarter was used for counting. This quarter was further sub-divided into three regions: (i) the dense stromal cells immediately underlying the luminal epithelium (sub-epithelial stoma); (ii) the remaining endometrium including glands but excluding caruncles; and (iii) the myometrium. The cells within the luminal epithelium were not counted as they were few in number. The number of positively stained cells in each region was counted manually by an operator unaware of the pregnancy status of each animal. The area covered by each region was measured using an image analysis system (Seescan plc, Cambridge). Each area of measurement covered at least 0.13 mm2. The final results were expressed as the mean ± SEM number of stained cells per mm2 calculated from the two replicate sections from each animal.

**RNA isolation and RT–PCR**

Total RNA from intact bovine uterus was isolated according to the method of Chomczynski and Sacchi (1987). The purity and quality of the RNA extracted was determined by the A260:A280 ratio in spectrophotometry and in agarose-formaldehyde gel electrophoresis, respectively. Bovine (bIL) interleukin sequences including bIL-1α (accession M37210), bIL-2 (M12791), bIL-6 (X57317) and ovine interleukin (oIL) sequences including oIL-10 (U11421) and β-actin sequence (U39357) (the house keeping gene) were obtained from Gene Bank. The priming oligonucleotides used for RT–PCR were subsequently designed using the Primer program Wisconsin Package Version 9.0 (Genetics Computer Group (GCG) Madison, WI). The specificity of the identified primers was finally confirmed by the BLAST Program (Altschul et al., 1990). The primer sequences chosen for each individual interleukin are shown (Table 1).

RT–PCR was performed according to a coupled one-step procedure (Aatsinki et al., 1994). One microgram of total RNA per 50 μl reaction mixture was reverse transcribed at 42°C for 1 h, denatured at 97°C for 3 min and amplified for 35 (IL-1α, IL-10 and β-actin) or 40 (IL-2 and IL-6) cycles. During each cycle, the reaction mixture was subjected to denaturing at 96°C for 30 s, primer annealing at 56°C (IL-6, IL-10 and β-actin) or 52°C (for IL-1α and IL-2) for 30 s and DNA extending at 72°C. The enzymes used for the reaction were: avian myeloblastosis virus (AMV) reverse transcriptase (4 U; Promega, Madison, WI), RNasIn (20 U; Promega) and Taq DNA polymerase (1.25 U; Sigma). The PCR products (20 μl) were subjected to electrophoresis on 1–2% agarose gels and photographed under UV light. RT–PCR control reactions included lymphoid total RNA (positive control) and reaction controls without RNA or reverse transcriptase and bovine DNA as a template to exclude false positive reactions. Data were only included from mRNA samples that generated a positive β-actin response.

The specificity of bIL-1α and bIL-2 RT-PCR products from uterine RNA were confirmed by Southern blotting. Internal oligonucleotides (bIL-1α, 20 mer and bIL-2, 45 mer) labelled with [γ32P]-ATP using a DNA 5’ end-labelling system (Promega) were used as probes. The oligonucleotide sequences used were as follows: bIL-1α: GTG AGG ACC AGA TGA ATA AG; bIL-2: CTC TTG CAC TCG TTG CAA and bIL-6) for 30 s and DNA sequence (U39357) (the house keeping gene) was obtained from Gene Bank. The priming oligonucleotides used for RT–PCR were subsequently designed using the

| Table 1. The primer sequences chosen for each individual interleukin |
|---------------------------------|-----------------|-----------------|-----------------|
| Interleukin      | Primer   | Sequence            | Product (bp) |
| bIL-1α           | Forward   | GAC CAC CTC TCT CTC AAT C | 146 |
|                  | Reverse   | CCA TCA CCA CAT TCT CC | |
| bIL-2            | Forward   | TTG CAC TAA CTC TCG ACT C | 108 |
|                  | Reverse   | CCA AAA GCA ACT GTA AAT CC | |
| bIL-6            | Forward   | TAT GAA CTC CCG CTT CAC | 236 |
|                  | Reverse   | CAC ACT CAT CAT TCT CCT TCA C | |
| oIL-10           | Forward   | AAA ACA AGA GCA AGG CCG | 573 |
|                  | Reverse   | CAG GGA CAA GAG AAA TTA GAG G | |
| β-actin          | Forward   | GAA GAT CTG CCA CCA CAC | 200 |
|                  | Reverse   | AGA GGC GTA CAG GGA CAG C | |
2 × SSC and 0.1% (w/v) SDS at room temperature. The films were exposed to X-ray film at –70°C for 3–14 days.

Statistical analysis

Data were analysed using the SPSS for Windows release 6.1 statistical program. The relationship between embryo size and the antiviral activity in the corresponding flushed fluid was determined using correlation analysis. The significance of the variation in the number of CD4+, CD14+ and CD21+ cells, measured as the stained cell count per mm², was determined using a two-way ANOVA (general model) with the pregnancy status of the cows and regions of uterus as factors. Significance was defined as $P < 0.05$. The expression of interleukins and β-actin in the bovine uterus or lymphoid tissues was identified on the agarose gels as either present (expressed) or absent (not expressed). No attempt was made to measure the concentration of the RT–PCR product.

Results

Relationship between embryo size and antiviral activity of the uterine flushing

Embryos were found in uterine flushings from 15 cows. The concentration of the uterine flushes measured in antiviral units ml⁻¹ (AVU), indicative of the concentration of IFN-τ associated with the different sizes of embryo were as follows: small embryos (< 0.5 cm) 4/5 undetectable (< 5 AVU) plus 18 AVU, $n = 1$; medium embryos (0.5–1.0 cm) 125 ± 77 AVU (mean ± SEM), range 5–417 AVU ($n = 5$); and large embryos (> 1.0 cm) 215 ± 100 AVU, range 8–593 AVU ($n = 5$). The antiviral activity was significantly correlated with blastocyst length ($r^2 = 0.33$, $P = 0.038$).

Population and distribution of immune cells in the bovine uterus

The lymph node used as the positive control tissue showed intense staining in the germinal centre of the node (in which activated lymphocytes accumulate) for CD4+ (T lymphocytes) and CD21+ (B lymphocytes); CD14+ (macrophages) were located mainly in the capsule of the lymph node (not shown).

In the uterus (Fig. 1), the highest concentration of CD4+ cells was found in the sub-epithelial stroma; much lower populations were observed in the remaining endometrium and myometrium (Table 2). There was a tendency towards higher concentrations of stained cells in the sub-epithelial stroma of the pregnant cows compared with the control and inseminated non-pregnant groups but the difference was not significant. CD4+ cells were rarely found within the luminal epithelium and were therefore not quantified in this location. The CD4+ cells in the endometrium and myometrium usually appeared in a cluster rather than randomly distributed within the area measured. CD21+ cells also tended to be highest in the sub-epithelial stroma, but the concentration among regions was not significantly different. There was also no significant variation among groups of cows (Table 2). The population of CD14+ macrophages was similar across the three groups in the sub-epithelial stroma. No significant variation in CD14+ cells was found in the endometrium and myometrium between pregnant and control groups (Table 2). CD14+ cells were not counted in the endometrium and myometrium of the inseminated non-pregnant group due to difficulty in identifying the specific regions on the slides from this group.

Neither the IFN-τ concentration nor the size of the embryo was related to the density of CD4+ T cells, CD14+ macrophages or CD21+ B cells in the day 16 pregnant uterus.

Expression of interleukin (IL)-1α, IL-2, IL-6 and IL-10 in the bovine uterus

Bovine lymphoid tissue (positive control) showed expression of mRNAs for bIL-1α, IL-2, IL-6 and IL-10 as detected by RT–PCR (Fig. 2). For IL-1α mRNA, a single product of 146 bp was expected. Multiple RT–PCR products were obtained from both lymphoid and uterine tissues, but only products of about 150 and 110 bp were specific, as confirmed by hybridization with an internal oligonucleotide probe. IL-1α mRNA was present in all the control uteri on day 16 of the oestrous cycle (4/4 cows sampled) (Table 3). IL-1α mRNA was also present in 3 of 4 pregnant cows and 2 of 3 cows in the inseminated non-pregnant group on day 16. IL-2 mRNA was present as a single product of 108 bp as expected. IL-2 mRNA was not detected in any of the samples of pregnant bovine uterus (0/4), but it was expressed in all but one of the non-pregnant cows (6/7 control + inseminated non-pregnant groups). This finding was confirmed using Southern blotting with an internal oligonucleotide probe for IL-2. No products were detected from the RT–PCR reactions with extracts of pregnant uterus. In the lymph tissue, IL-6 was expressed as the expected product of 236 bp and IL-10 as the expected product of 573 bp. Expression of IL-6 and IL-10 mRNAs was not detected in uterine samples from any of the groups.

Discussion

Successful implantation in cows requires an adequate signal for the maternal recognition of pregnancy (Thatcher et al., 1995; Wathes and Lamming, 1995) and the suppression of a hostile maternal immune response (Hansen, 1995, 1997). Failure of either of these processes could result in early embryo loss. IFN-τ is the major signal produced by the trophectoderm during the preimplantation period. Inadequate IFN-τ secretion may be related to pregnancy failure due to the inability of the conceptus to prevent luteolysis (Mann et al., 1999). Furthermore, in vitro studies have shown that IFN-τ suppresses the activity and proliferation of lymphocytes caused by trophectoderm antigens (Davidson et al., 1994) and may, therefore, play a role in the onset of maternal immune tolerance. The results of the present study confirm that the concentration of IFN-τ, as assessed by antiviral activity, is
Fig. 1. (a) A lower power haematoxylin and eosin stained uterine cross-section to illustrate the histology of the bovine uterus. (b–d) Immunohistochemical staining of CD4+ (T lymphocytes), CD21+ (B lymphocytes) and CD14+ (macrophages) in the bovine uterus. A bovine lymph node was used as the positive control and a negative control, using mouse IgG instead of primary antibody, was also used to validate the staining process (not shown). (b) CD4+ T lymphocytes were found mainly in the cells of the sub-epithelial stroma. (c) CD21+ B lymphocytes in the myometrium, where they were often located around the blood vessels. (d) CD14+ macrophages were also present in the sub-epithelial stroma. The sections illustrated are from the pregnant group (b,d) and the cyclic control group (c), although no differences in distribution were detected between pregnant and non-pregnant cows (see Table 2). BV: blood vessels; Endo: endometrium; G: glandular epithelium; LE: luminal epithelium; Myo: myometrium; SES: sub-epithelial stroma. Scale bars represent (a) 750 μm, (b) 75 μm, (c) 250 μm, (d) 75 μm.
proportional to the size of the embryo. Although the embryos were at the same stage of pregnancy, the largest embryo produced more than 10-fold more IFN-\(\gamma\) than the smallest embryo. It is possible that cows with smaller embryos would be less likely to maintain their pregnancies, although this could not be determined from the present study, which terminated at day 16.

However, in the present study, neither the concentration of IFN-\(\gamma\) nor the size of the embryo was correlated with the density of CD4\(^+\) T cells, CD14\(^+\) macrophages or CD21\(^+\) B cells in the day 16 pregnant uterus. T (CD4\(^+\)) and B (CD21\(^+\)) lymphocytes were rarely found within either the cyclic or pregnant luminal epithelium or in the uterine lumen. Intracellular CD8\(^+\) lymphocytes have been identified in sheep (Hansen, 1995), although in the present study it was not possible to stain for this type of cell with the antibodies available. Thus, possible changes in the epithelial lymphocyte population might not have been detected. However, Vander Wielen and King (1984) identified lymphocytes in the bovine luminal epithelium and subepithelial stroma and found no significant variation in the number of lymphocytes in these regions between cyclic and pregnant animals on day 20. Lee et al. (1988, 1992) also found a relatively high population of lymphocytes in the subepithelial stroma of the ovine uterus throughout the oestrous cycle and showed that the number of intra-epithelial lymphocytes was only reduced between day 18 and day 46 of pregnancy. This lack of change before implantation is in agreement with the results of the present study. These observations indicate that the immunosuppressive mechanism affecting lymphocyte proliferation during pregnancy was not effective until after the first 3 weeks of gestation. This finding implies that IFN-\(\gamma\) does not have a direct effect on lymphocyte redistribution, as IFN-\(\gamma\) production ceases after implantation whereas maternal immune tolerance persists for the rest of the pregnancy.

IL-2 is a pro-inflammatory cytokine. A novel finding of the present study was that IL-2 mRNA was not detectable in the pregnant bovine uterus whereas it was present in the non-pregnant cows on day 16. It has been demonstrated that PGE\(_2\) inhibits the expression of IL-2 in bovine lymphocytes (Emond et al., 1998) and that the basal synthesis of PGE\(_2\) in the endometrium is stimulated by IFN-\(\gamma\) (Danet-Desonyers et al., 1994). Similarly, Bergeron et al. (1996) reported that day 12 blastocoele fluid from rabbits inhibited IL-2 gene expression in stimulated lymphocytes. This finding provides a

### Table 2. Distribution of CD4\(^+\), CD14\(^+\) and CD21\(^+\) cells in the bovine uterus on day 16 of the oestrous cycle or early pregnancy

<table>
<thead>
<tr>
<th>Area</th>
<th>Sample(^a)</th>
<th>Number of cows</th>
<th>CD4(^+) (T lymphocytes)</th>
<th>CD21(^+) (B lymphocytes)</th>
<th>CD14(^+) (Macrophages)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sub-epithelial stroma</td>
<td>Preg 7-9</td>
<td>239 ± 51.0</td>
<td>151 ± 49.3</td>
<td>527 ± 86.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cont 6-8</td>
<td>214 ± 26.7</td>
<td>210 ± 50.7</td>
<td>487 ± 129.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>INP 4-5</td>
<td>156 ± 85.9</td>
<td>131 ± 45.0</td>
<td>383 ± 106.4</td>
<td></td>
</tr>
<tr>
<td>Endometrium</td>
<td>Preg 5</td>
<td>44 ± 11.3</td>
<td>44 ± 15.5</td>
<td>435 ± 105.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cont 5</td>
<td>53.5 ± 21.1</td>
<td>87 ± 16.9</td>
<td>320 ± 130.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>INP 5</td>
<td>59 ± 21.1</td>
<td>63 ± 11.2</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Myometrium</td>
<td>Preg 5</td>
<td>30 ± 7.0</td>
<td>59 ± 7.0</td>
<td>78 ± 12.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cont 5</td>
<td>14 ± 8.0</td>
<td>107 ± 21.0</td>
<td>187 ± 97.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>INP 5</td>
<td>30 ± 12.7</td>
<td>87 ± 8.4</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

The number of stained cells per mm\(^2\) was measured in two replicate sections per cow. Values are mean ± SEM. No significant variation was found in the number of cells within a region among any of the groups.

\(^a\)Animals were classified as either inseminated controls (Cont), pregnant (Preg) or inseminated but not pregnant (INP).

ND: not done.

Fig. 2. RT–PCR products of bovine interleukin (bIL)-1\(\alpha\), IL-2, IL-6 and IL-10 mRNA. Products of bIL-1\(\alpha\), IL-2 and IL-6 were subjected to electrophoresis in 2% agarose gels (whereas bIL-10 was in a 1% agarose gel) and photographed under ultraviolet (UV) light. The size of the molecular weight markers is given on the left in base pairs (bp). Arrows show the expected size of the RT–PCR products. The identity of the bIL-1\(\alpha\) and IL-2 products were confirmed by Southern blotting using internal oligonucleotides. L: lymphoid (positive control); u: uterus.

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Table 3. mRNA expression of interleukin (IL)-1α, IL-2, IL-6 and IL-10 in the bovine uterus on day 16 of the oestrous cycle or early pregnancy

<table>
<thead>
<tr>
<th>Sample</th>
<th>β-actin</th>
<th>IL-1α</th>
<th>IL-2</th>
<th>IL-6</th>
<th>IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preg</td>
<td>4/4</td>
<td>3/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>Cont</td>
<td>4/4</td>
<td>4/4</td>
<td>3/4</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>INP</td>
<td>3/3</td>
<td>2/3</td>
<td>3/3</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>Lymph node</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Interleukin expression was classified as either present or absent (not expressed) on the basis of a correctly sized band. No attempt was made to measure the concentration of the RT–PCR product. β-Actin was used as a positive control for RT–PCR and a bovine lymph node was used as a positive control for the detection of the relevant interleukins.

Animals were classified as either unstimulated controls (Cont), pregnant (Preg) or inseminated but not pregnant (INP).

*+, positive RT–PCR result.

Mechanism to explain the difference in IL-2 expression and indicates another potentially important role for IFN-γ during early pregnancy. Comparative information on uterine IL-2 expression is limited. Jokhi et al. (1997) were unable to detect IL-2 protein or mRNA in first trimester human endometrium and Fox et al. (1998) did not detect IL-2 mRNA in uterine intercaruncular epithelium in late pregnant ewes. In contrast, Grunig and Antczak (1995) detected IL-2 mRNA in horse endometrium but not trophoblast between day 30 and day 55 of gestation, but mares are unusual in that they mount a strong immune response to invading trophoblast.

IL-1α is also a pro-inflammatory cytokine. The IL-1 system has two ligands, IL-1α and IL-1β, which share 26% sequence homology and act through the same receptor. In the present study, IL-1α was present in most uteri from both cyclic and pregnant cows on day 16. This is consistent with previous reports: in the mouse uterus, both IL-1 mRNA and bioactivity increase between day 4 and day 8 of pseudopregnancy associated with the process of decidualization (Choudhuri and Wood, 1993) and IL-1α and IL-1β mRNA expression in isolated uterine epithelial cells increases during a 4 day culture period (Kover et al., 1995).

Similarly, IL-1α mRNA was present in sheep endometrial epithelium (Fox et al., 1998), and in women, IL-1β immunoreactivity in the endometrium increased in the late secretory phase (Tabibzadeh, 1991). IL-1β was present in uterine flushes from both pregnant and non-pregnant cows on days 14 and 17, whereas it was not detectable on days 25 and 30 (Davidson et al., 1995). IL-1 stimulates prostaglandin synthesis, particularly PGE, through a mechanism involving increased cyclo-oxygenase (Bany and Kennedy, 1995; Davidson et al., 1995). IL-1α also plays an important role in modifying the endometrial structure for implantation and during the human menstrual cycle (Singer et al., 1997; Huang et al., 1998). An IL-1 type I receptor antagonist administered from days 3–9 of pregnancy causes an almost complete block of implantation in mice (Simon et al., 1994). Together, these results indicate that uterine production of IL-1 plays an important role during implantation in a variety of species.

In the human uterus, IL-6 immuno- and bioactivity have been reported in both stromal and epithelial cells throughout the cycle (Tabibzadeh et al., 1989; Tabibzadeh, 1991; Jacobs et al., 1992). In the pseudopregnant mouse uterus, peaks of IL-6 bioactivity were detected between day 4 and day 8 (Choudhuri and Wood, 1993). Although IL-6 mRNA and protein were not detected in fresh uterus, they increased after culture of uterine epithelial and stromal cells (Jacobs et al., 1992; Kover et al., 1995). IL-6 mRNA has also been reported in elongating bovine, ovine and porcine conceptuses (Mathialagan et al., 1992), although the protein inhibits embryonic growth and attachment in vitro in mice (Jacobs et al., 1992). IL-6 mRNA was not detected in any uterine samples in the present study, indicating that it is not an important regulator of uterine activity at this stage of pregnancy in cows.

IL-10 is a Th type 2 anti-inflammatory cytokine which inhibits the production of cytokines that stimulate Th1 immune responses (Moore et al., 1993). IL-10 is produced by human placenta during gestation and the concentration decreases only at parturition (Paradowska et al., 1997; Simpson et al., 1998). IL-10 is also an important placental product in mice and anti-IL-10 can increase the fetal resorption rate in CBA × DBA/2 matings (Chouaet et al., 1995). IL-10 mRNA was not detected in the pregnant bovine uterus on day 16 in the present study. This finding was unexpected, but it is possible that IL-10 may be produced by either fetal or placental tissue later in pregnancy.

In conclusion, the lack of significant variation of CD4+ T cells, CD21+ B cells and CD14+ macrophages in the bovine uterus on day 16 between pregnant and non-pregnant cows indicates that differentiation of the maternal immune response from Th1 to Th2 may occur at a later stage of pregnancy and that IFN-γ does not have a role in this process. The pattern of uterine cytokine expression on day 16 suggests that IL-1 production in the late luteal phase, acting in concert with IFN-γ, stimulates stromal PGE2 synthesis and that this in turn inhibits uterine IL-2 expression. This effect may help to prevent a maternal immune response to implantation.

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