THE CHARACTERISATION OF NATURAL ANTIMICROBIAL PEPTIDES IN THE FEMALE REPRODUCTIVE TRACT

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MD
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2003
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Abstract

Changes in lifestyle and contraceptive practices have favoured the spread of sexually transmitted infections. The female reproductive tract is thus increasingly exposed to potential pathogens. Mucosal epithelial surfaces provide a first line of defence against the entry of these pathogens. The innate immune response is fundamental to the initial recognition of pathogens and influences the subsequent cascade of effector mechanisms. The natural antimicrobial peptides are a recently described innate immune defence mechanism, which in common with other mucosal surfaces, have been identified within the female reproductive tract.

The aims of this thesis are (1) To characterise the natural antimicrobials, human β defensin 1 and 2 (HBD1 and HBD2), secretory leukocyte protease inhibitor (SLPI) and granulysin, within the human endometrium through the menstrual cycle and in early pregnancy in vivo. (2) To assess the effect of exogenous sex steroids and chlamydial infection on this expression. (3) To investigate the expression and regulation of the natural antimicrobial peptides and pattern recognition receptors in vitro. (4) To examine some of the early immune responses to Chlamydia trachomatis infection in vitro.

All the natural antimicrobials showed cyclical variation. HBD1 was maximal in the mid secretory phase, granulysin in the late secretory phase, HBD2 in the menstrual phase and SLPI in the late secretory and menstrual phases. SLPI, HBD1 and granulysin were present in first trimester decidua, while levels of HBD2 were undetectable. Exogenous sex steroid, administered as the combined oral contraceptive pill and the levonorgestrel releasing intrauterine system (LNG-IUS) significantly decreased mRNA expression of HBD1 and 2 and granulysin. There was no significant effect of chlamydial infection on natural antimicrobial mRNA expression in first trimester decidual samples. Natural antimicrobials may play a role in the acquisition of infection and this is an important consideration in future contraceptive development and in the outcome of pregnancy.
Mimics of infection such as lipopolysaccharide (LPS - a mimic of Gram negative infection) and polyinosinic-polycytidylic acid (a mimic of viral infection) altered the mRNA expression of the natural antimicrobials in primary endometrial epithelial cells in vitro. mRNA expression of HBD1 was increased while SLPI mRNA expression was decreased. These mimics of infection are recognised by pathogen recognition receptors, which include the Toll-like receptor (TLR) family. Variable modulation of TLR 4 and the co-receptor CD14 were observed following treatment with LPS. Infection of the cervical epithelial cell line (HeLa) with Chlamydia trachomatis in vitro resulted in the up-regulation of SLPI, TLR9 and inflammatory cytokines such as IL-8 and IL-1. These data suggest that epithelial cells and the resulting cascade of innate immune responses play a central role in the events following pathogen invasion of the female reproductive tract.

This work furthers our understanding of the modulation of the natural antimicrobials, which act as an initial innate immunological barrier to infection. As a result of this work, further research might aim to manipulate these substances, or the effector mechanisms that lead to their expression. It may thus be possible to prevent or treat sexually transmitted infections and subsequently impact on the consequences of these infections such as infertility and adverse pregnancy outcomes.
Declaration

Except where due acknowledgement is made by reference the studies undertaken in this thesis were the unaided work of the author. No part of this work has been previously accepted for, or is currently being submitted in candidature for another degree.

Dr Diana Claire Fleming
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I would like to thank my supervisors, Professors Rodney Kelly and Hilary Critchley for their unfailing support and encouragement. Their guidance, teaching and discussions have been invaluable.

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I wish to acknowledge Ann Johnstone, Joan Creiger, Catherine Murray and Lynn Horribine for assistance with patient recruitment and thank you to the gynaecology theatre and ward staff who have supported our recruitment process. Thanks to Dr A.R.W. Williams for his expert histological assessment of endometrial biopsies and the Reproductive Medicine Laboratories, Edinburgh, for performing oestradiol and progesterone radioimmunoassays. Thanks to Mara Rocchi for help with the FACS data. I would like to acknowledge the graphical assistance of Ted Pinner and Eleanor Meikle. I am indebted to the support provided by the Medical Research Council, who funded my research enabling me to submit a MD thesis.

Finally, I would like to thank my family and friends for their support throughout and Michael, my husband, for his encouragement.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<td>ABC</td>
<td>avidin biotin peroxidase detection system</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotrophin</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein-1</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine- 3',5'- monophosphate</td>
</tr>
<tr>
<td>CD</td>
<td>cluster differentiation</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CIITA</td>
<td>MHC class II transactivator</td>
</tr>
<tr>
<td>COCP</td>
<td>combined oral contraceptive pill</td>
</tr>
<tr>
<td>COX-1/2</td>
<td>cyclo-oxygenase-1/2</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP response element</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3'-diaminobenzidine</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double stranded RNA</td>
</tr>
<tr>
<td>EB</td>
<td>elementary body</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>EP</td>
<td>E prostanoid receptor (prostaglandin E receptor)</td>
</tr>
<tr>
<td>ER</td>
<td>oestrogen receptor</td>
</tr>
<tr>
<td>FAM</td>
<td>6-carboxyflourescein</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle stimulating hormone</td>
</tr>
<tr>
<td>G/M-CSF</td>
<td>granulocyte/macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>HBD</td>
<td>human beta defensin</td>
</tr>
<tr>
<td>HBSS</td>
<td>hanks balanced salt solution</td>
</tr>
<tr>
<td>HD5</td>
<td>human defensin 5</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>HNP</td>
<td>human neutrophil defensin</td>
</tr>
<tr>
<td>HSP</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>intracellular adhesion molecule-1</td>
</tr>
<tr>
<td>IDO</td>
<td>indoleamine 2,3-dioxygenase</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IkB</td>
<td>inhibitor of NFkB</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
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</tr>
<tr>
<td>IL-1ra</td>
<td>interleukin-1 receptor antagonist</td>
</tr>
<tr>
<td>IL-1R1/11</td>
<td>interleukin-1 receptor type 1/11</td>
</tr>
<tr>
<td>IMDM</td>
<td>iscove's modified dulbecco's medium</td>
</tr>
<tr>
<td>IP-10</td>
<td>IFNgamma inducible protein-10</td>
</tr>
<tr>
<td>IRAK</td>
<td>IL-1 receptor associated kinase</td>
</tr>
<tr>
<td>LBP</td>
<td>lipopolysacchride binding protein</td>
</tr>
<tr>
<td>LH</td>
<td>luteinizing hormone</td>
</tr>
<tr>
<td>LMP</td>
<td>last menstrual period</td>
</tr>
<tr>
<td>LNG-IUS</td>
<td>levonorgestrel intrauterine system</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LTA</td>
<td>lipoteichoic acid</td>
</tr>
<tr>
<td>MAL</td>
<td>MyD88-adaptor-like protein</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>MOMP</td>
<td>major outer membrane protein</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>N-9</td>
<td>nonoxynol-9</td>
</tr>
<tr>
<td>NBF</td>
<td>neutral buffered formalin</td>
</tr>
<tr>
<td>NCAM</td>
<td>neural cell adhesion molecule</td>
</tr>
<tr>
<td>NF IL-6</td>
<td>nuclear factor interleukin-6</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor kappa B</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>P4</td>
<td>progesterone</td>
</tr>
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<td>PAMP</td>
<td>pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PG</td>
<td>prostaglandin</td>
</tr>
<tr>
<td>PGDH</td>
<td>prostaglandin dehydrogenase</td>
</tr>
<tr>
<td>PGES</td>
<td>prostaglandin E synthase</td>
</tr>
<tr>
<td>PGFS</td>
<td>prostaglandin F synthase</td>
</tr>
<tr>
<td>PID</td>
<td>pelvic inflammatory disease</td>
</tr>
<tr>
<td>PLSD</td>
<td>protected least significant difference</td>
</tr>
<tr>
<td>Poly I:C</td>
<td>polyinosinic-polycytidylic acid</td>
</tr>
<tr>
<td>PR</td>
<td>progesterone receptor</td>
</tr>
<tr>
<td>PRR</td>
<td>pathogen recognition receptor</td>
</tr>
<tr>
<td>Q-RT-PCR</td>
<td>quantitative-reverse transcription-polymerase chain reaction</td>
</tr>
<tr>
<td>RANTES</td>
<td>regulated upon activation, normal T cell expressed</td>
</tr>
<tr>
<td>RB</td>
<td>reticulate body</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RPMI</td>
<td>roswell park memorial institute medium</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal RNA</td>
</tr>
<tr>
<td>RU486</td>
<td>mifepristone</td>
</tr>
<tr>
<td>s.e.m</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SLPI</td>
<td>secretory leukocyte protease inhibitor</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>STI</td>
<td>sexually transmitted infection</td>
</tr>
<tr>
<td>TAMRA</td>
<td>6-carboxytetramethylrhodamine</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>Th1/2</td>
<td>T helper 1/2</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/IL-1 receptor homology</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TPA</td>
<td>tissue plasminogen activator</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF receptor associated factor</td>
</tr>
<tr>
<td>uNK</td>
<td>uterine NK</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
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</table>
Chapter 1

Literature review
1.1 The human endometrium and the menstrual cycle.

The human endometrium is a unique tissue in that it undergoes repetitive monthly cyclical changes, with tissue proliferation, loss at menstruation and healing without scarring. The regular and predictable nature of the menstrual cycle results from regulated interactions between the hypothalamus, pituitary, ovaries and genital tract. The regulation of the menstrual cycle and the endometrial changes that consequently occur will be considered below.

1.1.1 Regulation of the human menstrual cycle

The menstrual cycle is regulated by hormones and peptides secreted from components of the hypothalamic-pituitary-ovarian-uterine axis. It is believed that the ovarian steroids are the main controlling factors (Chabbert Buffet et al. 1998), through a series of positive and negative feedback loops. The menstrual cycle is divided into two phases, a follicular or proliferative phase and a luteal or secretory phase. This can be further subdivided based upon anatomical and functional changes within the endometrium into the menstrual, proliferative, early secretory, mid secretory (with preparation for implantation) and late secretory phases. The median length of the normal menstrual cycle is 28 days, although this can vary from 25-35 days (Treleor et al. 1967). A 28 day cycle is assumed within this thesis.

Within the ovary, follicular growth is initiated in the last few days of the luteal phase; it terminates at the time of ovulation. As the corpus luteum regresses at the end of the luteal phase, plasma oestrogen and progesterone concentrations fall. This is associated with a decrease in the levels of inhibins A and B. Follicle stimulating hormone (FSH) is no longer inhibited and begins to rise, initiating the development of follicles. As the follicles develop there is a concomitant increase in oestrogen and inhibin resulting in a negative feedback and suppression of FSH. This inhibits the development of further follicles and a dominant follicle is recruited. A positive feedback occurs with increasing
oestrogen secretion from the dominant follicle leading to increases in the number of granulosa cells and FSH and luteinizing hormone (LH) receptors. Just before ovulation the oestrogen secretion by the follicle rises dramatically initiating a LH surge from the pituitary. LH initiates the luteinisation of the theca and granulosa cells and enhances progesterone secretion which stimulates the mid cycle surge of FSH. Plasma levels of oestrogen then fall and the LH levels increase dramatically. 10-12 hours after the peak of the LH surge, ovulation occurs. Post ovulation, LH levels decrease dramatically, either secondary to the low oestrogen levels or due to depletion of LH within the pituitary. The corpus luteum then enlarges and secretes high levels of progesterone, which allow the oestrogen primed endometrium to undergo changes in preparation for implantation. In the absence of implantation, the corpus luteum regresses, oestrogen and progesterone plasma levels decline and FSH levels begin to rise again, and the cycle repeats. Figure 1 summarises these changes.
Figure 1: Concentrations of pituitary gonadotrophins luteinizing hormone (LH), follicle stimulating hormone (FSH) (both U/L), oestradiol (pmol/L), progesterone (nmol/L), inhibin A and inhibin B (both pg/mL) in the circulation through the menstrual cycle. This is correlated to the ovarian cycle with selection of a follicle, maturation and ovulation followed by formation of a corpus luteum, which then regresses. Cyclical changes within the endometrium are also shown with the broad characterisation into the proliferative, secretory and menstrual phases. (Adapted from Wilson et al. 1998)
1.1.2 Endometrial histological changes

The endometrium can be divided morphologically into an upper two-thirds functionalis layer, and a lower one-third basalis layer. The basalis layer is a regenerative layer that does not undergo cyclical changes, while the functionalis layer undergoes cyclical changes as it prepares for implantation of the blastocyst. These changes have been described in classic publications by Noyes (Noyes et al. 1950)(Figure 2) and Markee (Markee 1940) and reviewed by others (Critchley and Healy 1998; Dallenbach-Hellweg 1981; Speroff et al. 1999; Wilson et al. 1998). These changes will be briefly described in the following sections 1.1.2.1 – 1.1.2.9.
**Figure 2:** Dating the endometrium. Morphological features observed through the menstrual cycle, which allow accurate dating of the endometrium. From Noyes et al (Noyes et al. 1950).

### Dating the Endometrium

**Approximate Relationship of Useful Morphological Factors**

<table>
<thead>
<tr>
<th>Menses</th>
<th>Early Proliferation</th>
<th>Mid Proliferation</th>
<th>Late Proliferation</th>
<th>Secretion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Gland Mitoses</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Proliferation sites. They occur during menstruation because repair and breakdown are progressing simultaneously at that time.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pseudostatification of Nuclei</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>This is characteristic of the proliferative phase but persists until active secretion begins. It is not resumed until the glands have involuted during menstruation.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Basal Vagulation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>This is the earliest morphological evidence of ovulation found in the endometrium. It begins approximately 36 to 48 hours following ovulation.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Secretion</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>This curve represents visible secretion in the gland lumen; active secretion falls off more abruptly. In the later stages the secretion becomes inspissated.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Stromal Edema</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>This factor varies with the individual, particularly the rise during proliferation which accompanies secretion is more constant.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pseudodecidual Reaction</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>This is evident first around the arterioles and progresses until just before menstruation. A superficial compact layer is formed.</td>
</tr>
<tr>
<td></td>
<td>Stromal Mitoses</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>These are most abundant during the proliferative phase, absent during active secretion but reappear during the stage of decidual formation.</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Leucocytic Infiltration</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Throughout the cycle there are always a few lymphocytes. Polymorphonuclear infiltration begins about two days before the onset of flow.</td>
<td></td>
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</tr>
</tbody>
</table>

![Diagram of dating the endometrium](chart.png)
1.1.2.1 Proliferative phase (Days 4-13)
Reconstruction and growth of the endometrium is seen. Glands are narrow and tubular, mitoses and pseudostratification are present. The underlying stromal compartment, consisting of pluripotent mesenchymal cells, is initially dense and cellular becoming oedematous and then forming a loose syncytial-like structure. Spiral blood vessels are initially unbranched and uncoiled.

1.1.2.2 Early secretory phase (Days 14-18)
After ovulation, epithelial proliferation ceases due to decreased levels of oestrogen and increasing levels of progesterone. Glands continue to expand, becoming tortuous and coiled. Within the glandular cells subnuclear intracytoplasmic glycogen vacuoles appear, and active secretion of glycoproteins and peptides into the endometrial cavity follows.

1.1.2.3 Mid secretory or implantation phase (Days 19-23)
Initially secretory glands are prominent with little intervening stroma. The stroma then becomes very oedematous. Spiral vessels undergo intense coiling. Leukocytes begin to infiltrate the endometrium.

1.1.2.4 Late secretory phase (Days 24-28)
The functionalis layer can be divided into the superficial stratum compactum, with large stromal decidua-like cells, and the middle portion, the stratum spongiosum. This middle layer has loose oedematous stroma with numerous tightly coiled spiral vessels. Leukocytes greatly increase within the endometrium around days 25-28.

1.1.2.5 Menstruation (Days 1-4)
In the absence of pregnancy, oestrogen and progesterone levels decline. The endometrium becomes serrated in appearance and decreases in height as glandular secretion becomes exhausted. Blood flow in the spiral vessels decreases. The arterioles undergo constriction and vasodilatation leading to endometrial ischaemia and stasis. Apoptosis occurs with menstruation and subsequent healing.
1.1.2.6 Glands of the endocervix
The mucus secreting glands of the endocervix also undergo cyclical changes under the influence of ovarian hormones. As oestrogen increases in the latter half of the follicular phase the quantity of mucus increases up to 30 fold. At the time of ovulation the mucus changes in quality becoming watery and elastic. Progesterone in the luteal phase then reverses these changes.

1.1.2.7 Morphological changes in the endometrium following use of the combined oral contraceptive pill (COCP)
In the first few months after a combined steroid contraceptive is used, cyclical endometrial changes may occur, but after prolonged usage the regenerative capacity of the endometrium becomes reduced as a result of progesterone predominance and the endometrium becomes atrophic (Buckley and Fox 1989). Introduction of progesterone on day 5 of the menstrual cycle shortens the proliferative phase, resulting in poorly developed glands, sparse and lacking tortuosity, and stromal cells remaining spindled in appearance. As oestrogenic priming is inadequate and the resultant progesterone receptors are few in number, the secretory changes in the endometrium are weak and poorly developed. Glands remain uncoiled and there is only a trace of secretions within the lumina. Glands and stroma at different stages of maturity are observed and spiral arterioles fail to develop. In the latter part of the cycle there is regression of the secretory change and the endometrium becomes inactive. With prolonged pill taking, the endometrium becomes inactive and atrophied with sparse, tiny glands. The stromal cells decrease in number and collagenous fibres predominate.

1.1.2.8 Morphological changes in the endometrium following use of the levonorgestrel intrauterine device (LNG-IUS)
The LNG-IUS releases a synthetic progestogen, levonorgestrel, directly into the uterine cavity, which then acts in a paracrine manner. Cyclical activity of the endometrium is
lost within one month following LNG-IUS insertion. With extended use morphological changes are seen throughout the endometrium (Silverberg et al. 1986). Extensive decidualization of the stroma is observed. The proliferative activity of the endometrium is inhibited and the secretory activity of the epithelial glands cease, with time epithelial glands atrophy. There is also an associated down-regulation of oestrogen and progesterone receptors in all compartments of the endometrium after insertion of the LNG-IUS, with a gradual return 6-12 months post insertion. An initial infiltrate of CD56+ uterine natural killer cells and CD68+ macrophages has been described (Critchley et al. 1998a; Jones and Critchley 2000) with a subsequent decrease in macrophage numbers after 1 month of use. Vascular changes are also observed, with thickening of the uterine arteries, suppression of spiral arterioles and the presence of large distended vessels.

1.1.2.9 Decidualisation
Decidual cells are derived from the stromal cells of the endometrium, under the stimulation of progesterone. They appear in the mid to late luteal phase (approximately day 23) initially around the spiral arterioles, beneath the surface epithelium. The decidual cell is characterised by the accumulation of glycogen and lipid droplets and the expression of substances such as prolactin, insulin-like growth factors and insulin-like growth factor binding proteins. The glands in the upper two-thirds of the decidua become atrophied while those in the lower one-third remain secretory. If implantation occurs decidual cells continue to differentiate and/or proliferate eventually lining the entire endometrial cavity.

1.1.3 Regulation of local mediators in the endometrium

There are a number of potential regulatory mediators within the endometrium. Three of these will be considered here. Progesterone and oestrogen are two fundamental hormones involved in the cyclical changes observed within the endometrium, while Nuclear Factor-κB (NFκB) is a transcription factor involved in inflammatory and
immune responses (Baldwin 1996; Siebenlist et al. 1994), and may play a role in regulation of events within the endometrium.

1.1.3.1 Oestrogen and progesterone receptor expression in the endometrium

Both oestrogen and progesterone receptors are present within the endometrium, and their spatio-temporal distribution has been reviewed elsewhere (Critchley and Healy 1998; Lessey et al. 1988; Snijders et al. 1996). As their role is crucial to the control of events within the endometrium these changes will be summarised here. There are two predominant forms of the human progesterone receptor (PR), PR-A and PR-B, there are also two oestrogen receptors (ER), ERα and ERβ. The receptors are primarily located in the cell nucleus. ER expression increases through the proliferative phase, peaking in the early secretory phase and then declining. The glandular decline is delayed when compared to the stroma. ERα expression is observed in glandular and stromal tissues, while ERβ is expressed by the glandular epithelium, stromal tissues and endometrial endothelium (Critchley et al. 2001; Matsuzaki et al. 1999). ERβ declines in glandular cell nuclei, but not stroma, within the functionalis layer during the late secretory phase. A parallel but delayed increase in PR in the stroma and glandular compartments occurs in the proliferative phase. This is followed by decline in the glandular component in the secretory phase. Some PR expression persists in the stroma in the secretory phase, particularly in the cells adjacent to the blood vessels. While both PR-A and PR-B are expressed in the proliferative phase, mainly PR-A is expressed in the stroma in the secretory phase (Mote et al. 1999; Wang et al. 1998).

Data concerning ER and PR expression in the decidua of pregnancy are inconsistent. Perrot-Applanat et al have reported that ER and PR are absent from the epithelium in pregnancy (Perrot-Applanat et al. 1994). However others (Wang et al. 1992; Wu et al. 1993) report that during early pregnancy PR are expressed in stromal cells and in the endothelium. ER expression in early pregnancy is minimal in decidualised cells of the decidua parietalis, but stronger in the decidua capsularis. Staining of ER and PR has also
been observed in the glandular cells and arteriolar cells of the decidua parietalis (Wu et al. 1993).

In the absence of hormone the receptors are inactive, complexed to other proteins. On binding hormone the receptor dissociates from the complex and can bind to specific hormone responsive elements in DNA, altering gene expression (Carson-Jurica et al. 1990)

1.1.3.2 Transcription factor - Nuclear Factor-κB (NFκB)

NFκB is a transcription factor, one of the Rel transcription factor family. It is classically a heterodimer consisting of a p50 and p65 protein. NFκB will usually act with other transcription factors such as activator protein 1 (AP-1) to regulate gene transcription (McKay and Cidlowski 1999). NFκB is held inactive in the cytoplasm by endogenous inhibitors, IκB (which include IκBα, IκBβ and IκBγ) (Baeuerle and Baltimore 1988). These inhibitors mask the NFκB nuclear localisation sequence. Upon stimulation of a cell IκB becomes phosphorylated (Beg and Baldwin 1993; Beg et al. 1993), ubiquinated (Scherer et al. 1995) and then dissociates from the NFκB. NFκB can then translocate to the nucleus to regulate gene expression. A number of genes have NFκB binding sites in their promoter regions, such as IL-1, IL-6, IL-8 (Mukaida et al. 1991) and COX-2 (Schmedtje et al. 1997). IκBα is also up regulated by NFκB resulting in a negative feedback loop (Sun et al. 1993). NFκB activity can also be inhibited by progesterone, which stimulates the synthesis of IκB or binds to the nuclear receptor competing for NFκB binding sites on the target gene (Kalkhoven et al. 1996).
1.2 Inflammatory mediators within the endometrium

The menstrual cycle and implantation have both been associated with inflammatory-like events (Finn 1986; Kelly et al. 2001; Salamonsen et al. 1999). Mediators of these responses include cytokines, chemokines and other factors such as the prostaglandins. Representative mediators in each of these groups will be discussed in more detail below.

1.2.1 Cytokines

Cytokines are soluble polypeptides synthesised and secreted by activated immune cells, as well as by the stromal, epithelial and endothelial cells within the human endometrium (Table 1). They play an important part within the endometrium, in the pregnant and non-pregnant state (Kelly et al. 2001; Tabibzadeh 1991). In addition cytokines can interact with pituitary and hypothalamic hormones and may thereby influence neuroendocrine events. In vivo a cell is likely to be exposed to a number of cytokines, acting synergistically or antagonistically therefore differentiating individual functions can be difficult. Cytokines can be considered to be pro-inflammatory (e.g. interleukin (IL)-1, interferon gamma (IFNγ)) or anti-inflammatory (e.g. Interleukin (IL)-10). Three pro-inflammatory cytokines will be considered further; IL-1, IFNγ and tumour necrosis factor alpha (TNFα).
Table 1: Examples of Cytokines and Growth factors found within human endometrium, and their cellular source

<table>
<thead>
<tr>
<th>Stromal cells</th>
<th>Epithelial cells</th>
<th>Endothelial cells</th>
<th>Macrophages</th>
<th>T cells</th>
<th>uNK cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>IL-1α</td>
<td>IL-1</td>
<td>IL-1</td>
<td>IFNγ</td>
<td>TGFβ</td>
</tr>
<tr>
<td>IL-1β</td>
<td>IL-1β</td>
<td>TNFα</td>
<td>M-CSF</td>
<td></td>
<td>TNFα</td>
</tr>
<tr>
<td>IL-6</td>
<td>EGF</td>
<td>EGF</td>
<td>G-CSF</td>
<td>IFNγ</td>
<td>GM-CSF</td>
</tr>
<tr>
<td>EGF</td>
<td>TGFα</td>
<td></td>
<td>GM-CSF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGFA</td>
<td>TNFα</td>
<td></td>
<td>TNFα</td>
<td></td>
<td>G-CSF</td>
</tr>
<tr>
<td>TNFα</td>
<td>IL-6</td>
<td></td>
<td>IFNα</td>
<td></td>
<td>M-CSF</td>
</tr>
</tbody>
</table>

Adapted from (Bronson et al. 1996; Tabibzadeh and Sun 1992)

1.2.1.1.1 Interleukin-1

IL-1 is a multifunctional cytokine. Two forms of IL-1, IL-1α and IL-1β and two IL-1 receptors (type 1 (IL-1RI) and 2 (IL-1RII)) have been described, as well as an IL-1 receptor antagonist (IL-1ra), which binds to the type 1 receptor.

IL-1α and IL-1β are 17kDa proteins consisting of 159 amino acids and 153 amino acids respectively. At the protein level they show only 27% homology, but are nearly identical in 3 dimensional structure (www.copewithcytokines.com). Their biological effects are generally indistinguishable. They both bind to the IL-1 type 1 and 2 receptors. IL-1RI transduces a signal while IL-1RII binds IL-1 but does not transduce a signal (see below). IL-1 is produced by a number of cell types, including monocytes, endothelial cells, fibroblasts, smooth muscle cells and epithelial cells (Dinarello 1991).

Within the endometrium IL-1 has been detected in the endothelium, stroma and epithelium, with an increase in expression in the secretory phase of the cycle (Simon et al. 1993a; Tabibzadeh and Sun 1992), while IL-1ra expression is decreased in the secretory phase (Simon et al. 1995). The IL-1 type 1 receptor is expressed by
endometrial epithelial cells and expression is maximal in the secretory phase (Simon et al. 1993a; Simon et al. 1993b).

IL-1 secretion can be induced by many factors including TNFα, IFN-γ and endotoxins. Inhibitors of secretion include PGE₂, glucocorticoids, IL-1ra and IL-6. IL-1 can act directly or indirectly via the induction of PGE₂ (Tabibzadeh et al. 1990), IL-6 (Laird et al. 1994) and IL-8. It has numerous biological effects, activating T helper cells and NK cells, stimulation of immunoglobulin production, proliferation of fibroblasts, up-regulation of adhesion molecules and some chemotactic activity for neutrophils to list but a few functions. IL-1 also synergises with other cytokines such as TNFα. Within the endometrium it has been suggested that IL-1 may play a role in the defence against infection as well as in menstruation and implantation.

1.2.1.1.2 IL-1 receptor
The extracellular domains of the IL-1RI and IL-1RII proteins are members of the immunoglobulin superfamily, and are each comprised of three immunoglobulin G-like domains. IL-1 Type 1 receptor is the primary signal transducer (Sims et al. 1993). An IL-1 accessory protein has been described and once IL-1 has bound with low affinity to the type 1 receptor, the accessory protein docks and high affinity binding occurs. The type 1 receptor has a single transmembrane segment and a cytoplasmic domain. The type 2 receptor lacks a signal transducing cytoplasmic domain. The cytoplasmic domain has 45% amino acid homology with the cytosolic domain of the Drosophila Toll gene (Gay and Keith 1991) (see section 1.4.1).

Following binding to the receptor, several biochemical events occur. There are multiple and sequential phosphorylations or dephosphorylations of kinases, resulting in nuclear translocation of transcription factors and activation of proteins involved in translation of RNA. This includes the activation of the adapter protein MyD88, the IL-1 Receptor Associated Kinases (IRAK) and TNF Receptor-Associated Factor-6 (TRAF-6). Most of the effects of IL-1 occur following nuclear translocation of nuclear NFkB (see section
1.1.3.2) and activating protein-1 (AP-1) (Daun and Fenton 2000). NFκB and, or, AP-1 binding sites are present in the promoter regions of many IL-1 inducible genes, such as PGE₂, IL-6 and IL-8. This amplification system allows for very few receptors to be expressed on primary cells, resulting in an efficient signalling mechanism.

1.2.1.2 Tumour Necrosis Factor α

TNFα is a 17kDa protein consisting of 157 amino acids. It is secreted by a number of different cells including macrophages, monocytes, neutrophils, T lymphocytes, NK cells and fibroblasts, smooth muscle cells and granulosa cells (Zhang and Tracey 1998). The TNF promoter region has NFκB (Goldfeld et al. 1993), AP-1 and cAMP response element (CRE) (Leitman et al. 1992) as well as other regulatory sites. The receptors (type 1 and 2) are part of the TNF receptor superfamily and are found on all somatic cells except erythrocytes.

TNFα expression has been demonstrated within the human endometrium, predominantly in the glandular epithelium (Hunt et al. 1992). Different groups have examined the cyclical nature of expression, but results have been inconsistent. TNFα mRNA appears to be low in the proliferative phase and increases in secretory tissue (Hunt et al. 1992; Philippeaux and Piguet 1993). In the late secretory phase, expression in glandular epithelium declines, but stromal cells of the functionalis region show strong expression of TNFα mRNA (Hunt et al. 1992).

TNFα secretion is induced by a variety of factors, including IFN-γ, bacterial and viral products and tumour cells. There are a wide range of TNFα inhibitors, including PGE₂, IL-4, IL-10, glucocorticoids and spermine. The biological actions of TNFα are extensive, both pro-inflammatory (Old 1985) and cytotoxic. Effects also include cytolysis, cytostasis, monocyte and neutrophil chemotaxis (Ming et al. 1987), angiogenic effects (Frater-Schroder et al. 1987) and endocrine effects (Zhang and
Its role within the endometrium is not well defined and may like IL-1 include roles in immune defence, menstruation and implantation.

1.2.1.3 Interferon γ
IFNγ is a dimeric protein of 146 amino acids. Receptors are found on all human cells except mature erythrocytes. A soluble receptor has also been described. IFNγ is secreted predominantly by T cells (Celis et al. 1986) and NK cells, which have been activated by antigens, mitogens or alloantigens. Biological activities include antiviral and anti-parasitic effects as well as the inhibition of proliferation of normal and transformed cells. IFNγ has immunomodulatory effects such as augmenting MHC expression (Tabibzadeh and Satyaswaroop 1988) and affecting T cell growth and functional differentiation (Pestka et al. 1987).

Within the endometrium T cells, uNK cells and neutrophils are the source of IFNγ (Tabibzadeh 1994; Yeaman et al. 1998), particularly from the lymphoid follicles in the basalis region of the endometrium. Its prominent role is in the modulation of the immune response. Yeamen et al (Yeaman et al. 1998) found no correlation between stage of the menstrual cycle and IFNγ expression.

1.2.2 Chemokines
Chemokines are chemoattractant cytokines that promote the recruitment of leukocytes into tissues via specific receptors on the attracted cells. They have four conserved cysteine residues and two disulphide bridges. The chemokine superfamily has been divided into four subgroups depending on the amino acid configuration with regards to the first two cysteine residues; CC, C, CXC and CXXXC, with the CC and CXC groups being the best studied (Zlotnik and Yoshie 2000) (see table 2). CXC chemokines, such as IL-8 and IFNγ-inducible protein (IP)-10, tend to be chemoattractant for neutrophils. CC chemokines, such as RANTES and monocyte chemotactic protein (MCP) 1-4, tend to be chemoattractant for monocytes, lymphocytes basophils, eosinophils, but not for
neutrophils. Lymphotactin is the only C chemokine, and fractalkine is a CXXXC chemokine. Chemokines act via 7-transmembrane spanning G-protein coupled receptors (Murphy 1996).

Table 2: Examples of Chemokines in human endometrium

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>Sub group</th>
<th>Chemoattractant for:</th>
<th>Cellular location</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RANTES (regulated upon activation, normal T cell expressed and secreted)</td>
<td>CC</td>
<td>Eosinophils, basophils, monocytes, activated T cells, NK cells</td>
<td>stroma</td>
<td>(Hornung et al. 1997)</td>
</tr>
<tr>
<td>MCP-1</td>
<td>CC</td>
<td>monocytes, activated T cells, NK cells, basophils</td>
<td>Epithelium, stroma, perivascular cells</td>
<td>(Jones et al. 1997)</td>
</tr>
<tr>
<td>IL-8</td>
<td>CXC</td>
<td>Neutrophils, T cells, basophils</td>
<td>Epithelium, perivascular cells</td>
<td>(Jones et al. 1997; Milne et al. 1999)</td>
</tr>
</tbody>
</table>

Adapted from (Salamonson and Lathbury 2000)

1.2.2.1 IL-8

IL-8 is produced by a number of cell types including monocytes (Yoshimura et al. 1987), NK cells, neutrophils (Fujishima et al. 1993), fibroblasts, endothelial cells (Gimbrone et al. 1989), epithelial cells and some tumour cells. Other CXC chemokines have a far more limited cellular source. IL-8 can be induced by cytokines (e.g. IL-1 and TNFα (Fujishima et al. 1993)), bacterial and viral products (Wuyts et al. 1998). There are four CXC receptors. The effects of IL-8 are predominantly through the CXCR1 receptor, which binds IL-8 with high affinity (Samanta et al. 1989).

IL-8 has neutrophil (Schroder et al. 1987) and T cell chemotactic (Larsen et al. 1989) properties. Within the endometrium it has angiogenic and proliferative properties, inducing the proliferation of endometrial stromal cells (Arici et al. 1998). Other actions
include augmenting the bacteriocidal activity of neutrophils (Baggiolini et al. 1989) and chemotaxis of endothelial cells (Koch et al. 1992) and smooth muscle cells (Yue et al. 1994).

Within the endometrium IL-8 has been localised to the perivascular cells and surface epithelium and glands (Critchley et al. 1994; Jones et al. 1997), increasing in the late secretory phase. In vivo levels are seen to increase 48 hours after progesterone withdrawal (Critchley et al. 1999) while in vitro IL-1 and TNFα increase stromal cell production.

1.2.2.2 Monocyte chemotactic protein -1
MCP-1 is released by a wide range of cell types including monocytes (Yoshimura et al. 1989), fibroblasts (Yoshimura and Leonard 1990) and endothelial cells (Sica et al. 1990). It is induced by factors including IL-1, TNFα (Sica et al. 1990) and LPS and is inhibited by progesterone (Kelly et al. 1997). A number of CC receptors have been described, and the effects of MCP-1 are mediated primarily through CCR2 (Bacon et al. 1998).

MCP-1 recruits and activates monocytes (Rollins 1991), and also T cells (Carr et al. 1994), basophils (Bischoff et al. 1992) and NK cells (Allavena et al. 1994) in sites of inflammation. It has also been described as an angiogenic agent, a mitogen for smooth muscle (Bacon et al. 1998) and a regulator of cytokine expression (Jiang et al. 1992). MCP-1 has been identified in the endometrium (Arici et al. 1995; Jones et al. 1997), predominantly in the perivascular cells. Expression increases on progesterone withdrawal (Jones et al. 1997) and MCP-1 may therefore be involved in leukocyte recruitment at this time.

1.2.3 Prostaglandins
Prostaglandins (PG) are a group of inflammatory mediators (PG A to J) first described in 1936 (Von Euler 1936). They are produced by de novo synthesis from arachidonic acid released from membrane bound phospholipids. Via the action of the cyclooxygenase
enzymes, COX-1 and COX-2, PGH₂ is derived. COX-1 is considered to be synthesised constitutively, while COX-2 expression is regulated (O’Banion et al. 1992). The COX-2 gene has a NFκB binding site, and can be up-regulated by factors such as LPS, IL-1 (Huang et al. 1998) and TNFα and inhibited by others such as progesterone (Abel and Baird 1980). Modification of PGH₂ by the enzymes PGE synthase (PGES) and PGF synthase (PGFS) results in the two PGs considered in this thesis, PGE₂ and PGF₂α respectively. Prostaglandin 15-dehydrogenase (PGDH) is then responsible for the catabolism of the PGs. PGDH has been shown to be progesterone dependent in reproductive tissues (Greenland et al. 2000).

There have been a number of PGE receptors identified, including EP1, 2, 3a, 3b, 3c, 3d and EP4. The EP1 receptor is calcium dependent, EP2 and EP4 receptors are both cyclic adenosine monophosphate (cAMP) dependent (Fedyk and Phipps 1996) and the EP3 receptor can act via calcium and cAMP.

Prostaglandins have a wide range of actions. These include immunomodulatory effects (Harris et al. 2002) such as the regulation of other cytokines, stimulation of IL-8 and IL-10 (Denison et al. 1999b) and inhibition of IL-12 from activated monocytes (Kraan et al. 1995) and antibody class switching of B cells (Phipps et al. 1991). Prostaglandins also have vasoactive properties and play a role in menstruation (Baird et al. 1996). Prostaglandins are found in high levels in semen and this may result in immunomodulatory effects that are advantageous to the spermatozoa, but may be detrimental in terms of acquisition of sexually transmitted infections (Kelly 1995; Kelly and Critchley 1997).

Concentrations of all prostaglandins are greater within the endometrium than in the myometrium. PGF₂α, which is found in the greatest concentrations, is highest in the mid-secretory phase in both endometrium and myometrium. Within the myometrium the concentration of PGE₂ does not vary through the menstrual cycle (Lumsden et al. 1986). However, the ability to produce and release PGE₂ appears to be maximal in the menstrual phase of the cycle (Rees et al. 1984).
1.3 Endometrial Immunity

1.3.1 Leukocytes within the endometrium
As a mucosal surface exposed to pathogens as well as foreign antigens in seminal plasma and trophoblast, the endometrium (which is normally sterile) has to be able to mount a regulated immune response. A wide range of immune cells has been identified in the endometrium within the stratum basalis, stratum functionalis and in an intraepithelial position. Their numbers vary over the menstrual cycle (Loke and King 1995) (Table 3 and figure 3). The cells identified include granulocytes (neutrophils and eosinophils), T and B-lymphocytes, macrophages and uterine natural killer (NK) cells.

<table>
<thead>
<tr>
<th>Cycle Stage</th>
<th>Percentage of stromal cells expressing CD45 (common leukocyte antigen) (Mean and range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferative</td>
<td>8.2</td>
</tr>
<tr>
<td></td>
<td>(6.3-12.2)</td>
</tr>
<tr>
<td>Early secretory</td>
<td>8.7</td>
</tr>
<tr>
<td></td>
<td>(6.1-12.5)</td>
</tr>
<tr>
<td>Late secretory</td>
<td>23.2</td>
</tr>
<tr>
<td></td>
<td>(15.4-29.4)</td>
</tr>
<tr>
<td>Early pregnancy</td>
<td>31.7</td>
</tr>
<tr>
<td></td>
<td>(26.2-36.2)</td>
</tr>
</tbody>
</table>

From (Bulmer et al. 1991b)

Table 3: Proportion of leukocytes in human endometrium
Figure 3: Leucocyte populations (number of leukocytes/10 high power fields) in human endometrium and first trimester decidua. Adapted from (Loke and King 1997).
(prolif = proliferative phase endometrium; secretory = secretory phase endometrium)
1.3.1.1 Neutrophils
Neutrophils are the most abundant leukocyte in the human immune system, but within the endometrium they are barely detectable through most of the cycle. Perimenstrually the numbers rise to make up 6-15% of the total cell number in the tissue at this time (Salamonsen and Woolley 1999). They are found scattered through the stratum functionalis.

1.3.1.2 Eosinophils
Like neutrophils, eosinophils are absent from the endometrium through most of the menstrual cycle until just prior to menstruation. Their numbers then increase and form 3-5% of the total cell number in the tissue at this time, mostly found as aggregates (Salamonsen and Lathbury 2000).

1.3.1.3 B-lymphocytes
B-lymphocytes are only detected in very low numbers throughout the menstrual cycle. They are found in aggregates in the stratum basalis, and do not vary in number through the menstrual cycle (Bulmer et al. 1991a). At other mucosal sites (gastrointestinal tract and respiratory tract), local production of secretory IgA antibodies provide a local immune defence system. A sub set of B-lymphocytes, IgA synthesising plasma cells, have been demonstrated in the vagina, ectocervix and endocervix, but not within the human endometrium. Plasma cells are not considered to be a normal component of endometrial stroma and their presence is used as evidence of endometritis (Buckley and Fox 1989). IgG, IgM and IgA have been localised in the stroma of secretory endometrium, but it is proposed that these derive from the plasma in association with stromal oedema (Bulmer et al. 1986).

1.3.1.4 T-lymphocytes
T-lymphocytes are found in the aggregates in the stratum basalis where their numbers do not vary (Bulmer et al. 1991a). They are also found scattered within the stratum functionalis and in intraepithelial sites (Loke and King 1995). Their numbers increase
slightly perimenstrually, but are only 1-2% of the total cell number in the tissue at this time. The majority of the T cells present are αβ T Cell Receptor +ve (TCR+) (85%), but a small population of γδ TCR+ cells are found within the endometrium (Chen et al. 1995). The role of the γδ TCR+ cells is unclear and include possible immunosuppressive (Suzuki et al. 1995) and/or cytolytic activity (Mincheva-Nilsson et al. 2000). It has been noted that there is an increase in the ratio of CD8+ cells to CD4+ cells in the endometrium, when compared to peripheral blood T cells.

The CD8+ cells are cytolytically active in the proliferative phase of the menstrual cycle, but inactive during the subsequent secretory phase (White et al. 1997). In pregnancy decidual T cells appear to have an activated phenotype (Mincheva-Nilsson et al. 1994). The likely role of these cells is as part of the immune defence armoury, and as a source of cytokine production. There has been some speculation about the existence of suppressor T-lymphocytes, but evidence is inconclusive at this stage (Kuhnert et al. 1998; Mahmoud et al. 2001).

1.3.1.5 Macrophages

Macrophages are present in the stratum basalis aggregates through the menstrual cycle, and are also present scattered throughout the stratum functionalis. There is a small increase in numbers in the functionalis from the proliferative through to the secretory phase (Bonatz et al. 1992), reaching a maximum of 6-15% of the total cell number in the tissue prior to menstruation.

Most of the endometrial macrophages express MHC class II and this expression does not seem to vary through the menstrual cycle (Bulmer et al. 1988a). This suggests that these cells are acting as antigen-presenting cells. As prostaglandin secretors, macrophages may play a role in intrauterine immunosuppression. They also secrete and can respond to a number of cytokines. Finally macrophages are well described as phagocytic cells and their role may be the removal of tissue debris perimenstrually.
1.3.1.6 Natural Killer cells

CD56 is the classic NK cell antigen, and is an isoform of the neural cell adhesion molecule – NCAM, while CD16 is the low affinity Fc-γ receptor. “Classical” CD 56'16' NK cells are rare throughout the menstrual cycle. In contrast “uterine” CD 56'16' NK (uNK) cells are found occasionally in intraepithelial sites and in the stratum functionalis where they form a major cellular component of the endometrial stroma. Although few uNK cells are present in the proliferative phase, they increase dramatically in the secretory phase, being 15-25% of the total number of cells in the stroma (Bulmer et al. 1991b; King et al. 1989; Salamonsen and Woolley 1999) at their maximum. The increase of cells is both as a result of migration into the endometrium followed by differentiation, as well as proliferation of cells already within the endometrium. Scattered single cells and aggregates adjacent to endometrial glands and around spiral arterioles are observed. In the absence of pregnancy, numbers of uNK cells decline. It is controversial as to whether or not this unique cell type undergoes apoptosis (Jones et al. 1998a; Jones et al. 1998b; King 2000; Loke and King 1995).

These cells were recognised in non-pregnant endometrium by Weill in 1921 (Weill 1921) and have been described morphologically as large lymphocytes with prominent cytoplasmic granules (King 2000; King et al. 1991). These cells have been referred to in the literature as large granular lymphocytes (LGL), endometrial stromal or granulated lymphocytes, Kornchennzellen or K cells, decidual granulated lymphocytes, or uterine NK (uNK) cells. They shall be referred to as uNK cells through this thesis.

The role of uNK cells has been widely speculated. As they express the NK cell marker CD56, and have a number of cytoplasmic granules, the uNK cells may be functioning as traditional cytotoxic, non MHC restricted NK cells. Cytotoxic activity has been demonstrated in vitro (Ferry et al. 1990). uNK cells have receptors for HLA class I molecules and as an extension to this it has been suggested that uNK cells may play a role in controlling the invasion of fetal trophoblast cells into the maternal uterine tissue.
(Loke and King 2000). uNK cells also secrete a wide range of cytokines and may play a role in decidualisation as well as implantation and early placental development (King 2000).

1.3.2 Leukocytes in Pregnancy
Leukocytes are a major component of decidua throughout pregnancy (Bulmer and Sunderland 1983). There are 3 major populations, T-lymphocytes, macrophages and uNK cells. Macrophage numbers stay relatively constant through pregnancy, accounting for approximately 10 - 20% of cells and are found throughout the decidua (Bulmer et al. 1988b). T-lymphocytes account for less than 20% of the leukocytes in first trimester decidua and are predominantly CD8+. Later in pregnancy T-lymphocytes account for a larger proportion of the leukocytes as uNK cells decrease in number (Bulmer and Sunderland 1984). uNK cells are predominant in the first trimester, scattered throughout the decidua as well as aggregating around the glands and arterioles (Bulmer et al. 1991a). These cells comprise more than 75% of the leukocytes in first trimester decidua (Bulmer et al. 1991b), they then decline in the second trimester and are uncommon at term.

1.3.3 Regulation of Leukocyte distribution
The mediators regulating leukocyte numbers and distribution have not been fully elucidated. Cells may increase in number due to local proliferation (Klentzeris et al. 1992), or due to influx from peripheral blood circulation in the tissue (Marzusch et al. 1993). Changes occurring through the menstrual cycle are likely to be hormonally mediated either directly or indirectly via other factors such as chemokines or cytokines. Immunohistochemical analysis of T lymphocytes, macrophages and uNK cells have failed to show either progesterone or classic oestrogen receptors (ERα) on these cells (King et al. 1996; Stewart et al. 1998). In contrast, expression of cytokine receptors on these cells has been demonstrated. Stromal cells express the progesterone receptor (Mote et al. 1999; Wang et al. 1998) and it has therefore been proposed that progesterone acts directly on endometrial stromal cells inducing production of secretory products which
then influence endometrial leukocytes. uNK cells require progesterone for their recruitment, as evidenced by the fact that they are not seen in ovariectomised women, unless they had been exposed to both oestrogen and progesterone (Loke and King 1995). Adhesion molecules are necessary for attachment to endothelium, extravasation and trafficking through tissues. These molecules include NCAM, ICAM-1, VCAM-1 and E-selectin, and have been detected in the endometrium (Tabibzadeh et al. 1994). Expression of adhesion molecules is modulated by a wide range of cytokines and these may play a role in recruitment of leukocytes.
1.4 Innate Immunity

The immune system is divided into two, the acquired immune system and the non-acquired innate immune system. Within the human endometrium the cellular components of these systems have been described above in sections 1.3.1 and 1.3.2. The innate system encompasses a range of first line responses to invading pathogens. It consists of mechanical barriers such as skin and mucosal membranes, secretions, such as complement, lysozyme and natural antimicrobials and has a cellular component such as phagocytic cells and natural killer cells. It does not require previous exposure to pathogens, or immunological memory. The innate immune response is relatively non-specific and rapidly kills invading microbes. This is in contrast to the adaptive immune response, which is specific in its response and maintains memory. As such, the adaptive immune response can take time (days) to develop and can produce inflammatory side effects. The innate immune response can activate the adaptive immune response. Two components of the innate immune response and their role in the endometrium will be considered further:

i) Toll-like receptors, which recognise pathogens

ii) Natural antimicrobials, a component of the secretory innate immune response

1.4.1 Pathogen recognition: Toll-like receptors

The early recognition of infection by the host is a fundamental component of the immune response, providing an early line of defence against pathogens (Janeway 1992a). It is now recognised that there is more specificity in the activation of the innate immune system than was initially believed. This has been attributed to germline-encoded pattern recognition receptors (PRRs) expressed on host cells. These PRRs recognise highly conserved motifs, defined as pathogen-associated molecular patterns (PAMPs) that are shared by large groups of microorganisms (Medzhitov and Janeway 1997). Examples of PAMPs include lipopolysaccharide (LPS) of Gram negative bacteria, lipoteichoic acid (LTA) of Gram positive bacteria, double-stranded RNA of several RNA viruses and unmethylated CpG DNA motifs (Medzhitov and Janeway
Ligation of particular PRRs results in intracellular signalling events that activate cells in defined ways.

The receptors involved in direct activation of signalling pathways after PAMP recognition have remained, until recently, elusive. It is now understood that the Toll-like receptor (TLR) family mediate the recognition of a number of PAMPs. Mammalian TLR proteins derive their name from the *Drosophila* Toll protein. This was originally shown to be critical for dorsal-ventral patterning in fly embryos (Stein et al. 1991). In addition it was observed that mutations in the Toll receptor resulted in increased susceptibility to fungal infection and that Toll controlled the expression of the antifungal peptide gene drosomycin (Lemaitre et al. 1996; Lemaitre et al. 1997). In 1997 Medzhitov et al (Medzhitov et al. 1997) reported the discovery of the human equivalent “hToll”, now known as TLR4. Subsequently a total of ten human TLRs have been identified (TLR1-10). Ligands have been characterized for 5 of the TLR (Table 4). Cells may use a number of different TLR to detect several features of a microbe simultaneously. In addition some of the receptors can dimerise (e.g. TLR2/1, TLR6/1, TLR2/6) allowing additional specificity (Ozinsky et al. 2000). This allows the nature of the microbe to be elucidated and an effective immune response, both innate and adaptive, to be activated.

TLR proteins are characterised by an extracellular leucine-rich repeat (LRR) domain, small extracellular cysteine-rich domains, and a cytoplasmic domain that is responsible for signal transduction. The number and arrangement of the cysteine-rich domains varies between different members of the Toll family. The mechanisms of microbial recognition are not well defined. It may be a direct recognition of the PAMP by the receptor, or recognition of a molecule that initially recognises the PAMP (Wright 1999). In flies Toll is activated by a secreted ligand, Spätzle. A mammalian homologue of Spätzle has not been identified. The cytoplasmic domain is homologous to the cytoplasmic domain of the IL-1 receptor (IL-1R) family. This area is therefore referred to as the Toll/IL-1R homology (TIR) domain. This TIR domain is responsible for signal transduction. The extracellular domain of the TLR bears no homology to the IL-1R. Downstream
signalling events after ligand binding to the TLR follow those seen after activation of the IL-1R (see section 1.2.1.1.2). The protein MyD88 is an adapter of signal transduction with a TIR domain, which is recruited following TLR ligation. Myd88-adaptor-like (MAL) is another such protein (Fitzgerald et al. 2001). Myd88 appears to be a signal adaptor used by several TLRs (including TLR4), while MAL is used predominately by TLR4 (O'Neill 2002). Recruitment of MyD88 or MAL result in a cascade of events culminating in the nuclear translocation of NF-κB (Cario et al. 2000; Kopp and Medzhitov 1999; Muzio et al. 1998) and activation of the AP-1 transcription factor family. This results in the activation of a number of genes, including pro-inflammatory cytokines and antimicrobial peptides (Becker et al. 2000) (Figure 4). Although a common inflammatory “danger signal” is activated following ligation of TLR, the exact genes and resultant proteins expressed differ according to the TLRs activated (Hirschfeld et al. 2001; Jones et al. 2001a) and the cell type involved (Thoma-Uszynski et al. 2000). It has been suggested that the signal transducers recruited such as MyD88 and MAL may tailor the response generated (O'Neill 2002).
## Table 4: Stimuli that activate signalling through TLRs

<table>
<thead>
<tr>
<th>Receptors</th>
<th>Ligands</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR2+(TLR6 or other TLR)</td>
<td>Bacterial lipoproteins (on most bacteria)</td>
<td>(Aderem and Ulevitch 2000; Lien et al. 1999)</td>
</tr>
<tr>
<td></td>
<td>Peptidoglycan</td>
<td>(Aderem and Ulevitch 2000)</td>
</tr>
<tr>
<td></td>
<td>LTA</td>
<td>(Aderem and Ulevitch 2000)</td>
</tr>
<tr>
<td></td>
<td>LPS – <em>Leptospira interrogans</em> Porphyromonas gingivalis</td>
<td>(Werts et al. 2001)</td>
</tr>
<tr>
<td></td>
<td><em>M. tuberculosis</em> – lipoarabinomannan phosphatidylinositol diamannoside</td>
<td>(Means et al. 1999a; Means et al. 1999b; Thoma-Uszynski et al. 2001)</td>
</tr>
<tr>
<td></td>
<td>Zymosan (a yeast cell wall preparation)</td>
<td>(Underhill et al. 1999)</td>
</tr>
<tr>
<td>TLR3</td>
<td>double-stranded RNA</td>
<td>(Alexopoulou et al. 2001)</td>
</tr>
<tr>
<td>TLR4</td>
<td>LPS</td>
<td>(Aderem and Ulevitch 2000)</td>
</tr>
<tr>
<td></td>
<td>HSP60 (human and chlamydial)</td>
<td>(Ohashi et al. 2000)</td>
</tr>
<tr>
<td></td>
<td>Taxol</td>
<td>(Kawasaki et al. 2000)</td>
</tr>
<tr>
<td></td>
<td>Fibronectin extra domain A (EDA)</td>
<td>(Okamura et al. 2001)</td>
</tr>
<tr>
<td></td>
<td>F protein – respiratory syncytial virus</td>
<td>(Kurt-Jones et al. 2000)</td>
</tr>
<tr>
<td></td>
<td><em>Escherichia coli</em> P fimbriae</td>
<td>(Frendeus et al. 2001; Hedlund et al. 2001)</td>
</tr>
<tr>
<td>TLR5</td>
<td>Flagellin</td>
<td>(Hayashi et al. 2001)</td>
</tr>
<tr>
<td>TLR9</td>
<td>unmethylated CpG DNA motifs</td>
<td>(Bauer et al. 2001; Hemmi et al. 2000; Takeshita et al. 2001)</td>
</tr>
</tbody>
</table>

Adapted from Underhill and Ozinsky 2002
Figure 4: Overview of signalling pathway for Toll-like Receptors.

Agonists such as LPS, LTA and CpG bind to the receptor, directly or in conjunction with co-receptors such as CD14 and MD-2. This results in sequential activation of signal transduction components including Myd88 or MAL, Map kinases and TRAF6, ultimately leading to IκBα phosphorylation and release of NFκB. NFκB translocates to the nucleus and promotes activation of regulated genes such as the proinflammatory cytokines IL-1 and TNFα.
Extracellular

CpG DNA

LPS

LTA

MD-2

Intracellular

CD14

MyD88/MAL

Map kinases

TRAF6

NFκB

Pro-inflammatory cytokines e.g. IL-1, TNFα

IkBα

IkBα-P

44
The cellular distribution of TLRs is wide (Medzhitov et al. 1997; Muzio et al. 2000) and different TLR are differentially expressed and regulated (Hornung et al. 2002; Zaremba and Godowsk 2002). In general, cells expressing TLRs include effectors of the immune system such as antigen presenting cells (APC) – e.g. macrophages (Akashi et al. 2000; Yang et al. 1999b), dendritic cells (Thoma-Uszynski et al. 2000) - and B cells (Roshak et al. 1999). Expression has also been detected in epithelial cells (Cario et al. 2000; Fichorova et al. 2002; Schilling et al. 2001), endothelial cells (Faure et al. 2001) and adipocytes (Lin et al. 2000). Using immortalised human vaginal, ectocervical and endocervical cell lines, TLR1,2,3,5 and 6 mRNA expression has been identified (Fichorova et al. 2002). However these cell lines failed to express TLR4. Recent reports suggest that within the human endometrium, TLR1-6 and CD14 mRNA is identifiable. The endometrial epithelial cell line RL expresses TLR1,2,3,5,6, CD14 and possibly TLR4 (Young et al. 2002). TLR4 expression has been examined through the menstrual cycle, and is present in the proliferative and secretory phases, but not in the menstrual phase. TLR3 is present in all phases with the exception of the late secretory phase (Young et al. 2002).

Bacterial products such as LPS and cytokines including IL-1β, TNFα and IFNγ increase TLR4 expression in human monocytes and neutrophils (Muzio et al. 2000). Endothelial cell expression of TLR2 is induced by LPS, TNFα, and IFNγ while LPS and IFNγ also up-regulated TLR4 in endothelial cells (Faure et al. 2001) and intestinal epithelial cells (Abreu et al. 2002). IL-4 (Staege et al. 2000) and IL-10 (Muzio et al. 2000) have been demonstrated to down regulate TLR4 expression, suggesting that a Th2-type immune response may inhibit TLR activation. An LPS-tolerization phenomenon has been observed and two groups have described a transient decrease in TLR4 mRNA levels on LPS stimulation in mice (Medvedev et al. 2000; Nomura et al. 2000) that may explain this phenomenon. Decreased expression of TLR has been suggested as a protective mechanism in areas like the intestinal mucosa, vagina and cervix where cells are continually being exposed to bacterial LPS (Abreu et al. 2001; Fichorova et al. 2002).
1.4.1.1 TLR4

TLR4 has now been recognised as the predominant receptor for lipopolysaccharide (LPS) of gram negative bacteria. This was originally identified in mice, where a genetic defect resulting in hypo/non-responsiveness to LPS was linked to TLR4 (Poltorak et al. 1998). This has subsequently been confirmed in other systems (Chow et al. 1999; Hoshino et al. 1999; Lien et al. 2000; Rhee and Hwang 2000; Tapping et al. 2000). A constitutively active TLR4 construct drives NF-κB activation, AP-1 activation and cytokine production (Medzhitov et al. 1997). There are a number of other proteins involved in this interaction: LPS binding protein (LBP) was identified in plasma with high affinity binding to the lipid A moiety of LPS (Schumann et al. 1990). Further to this CD14, a differentiation antigen of monocytes, was found to bind complexes of LPS and LBP, and blockade of CD14 with monoclonal antibodies prevented synthesis of TNF-α by whole blood incubated with LPS. (Wright et al. 1990). TLR4 acts with the CD14/LBP/LPS complex to generate a transmembrane signal and cell activation. An additional protein, MD-2, has been identified as being important for signalling via TLR4 (Shimazu et al. 1999; Yang et al. 2000b). MD-2 is a secreted protein that binds to the extracellular portion of TLR4 where it facilitates LPS responsiveness. Work has shown that direct physical contact occurs between LPS, CD14, TLR4 and MD-2 (da Silva Correia et al. 2001; Poltorak et al. 2000), suggesting that for TLR4 a direct recognition of LPS occurs in conjunction with these molecules.

In vitro human β defensin 2 has been up-regulated following stimulation with LPS and bacterial lipoprotein. This has been dependent on CD14 and TLR2 expression respectively (Becker et al. 2000; Birchler et al. 2001; Diamond et al. 1996). Scott et al (Scott et al. 2000) suggest that cationic antimicrobials, including HBD2, may block the binding of LPS to LBP, thus limiting the magnitude of an inflammatory response.

1.4.1.2 CD14
The CD14 molecule, like TLR, has multiple leucine rich repeats extracellularly, but CD14 does not have an intracellular signalling domain. CD14 exists both as a soluble form (Bazil et al. 1989) and as a glycosylphosphatidylinositol (GPI)-linked outer membrane protein (Bazil et al. 1989; Haziot et al. 1988). The soluble form appears to mediate LPS stimulation of cells that do not express membrane bound CD14 (Frey et al. 1992; Pugin et al. 1993). CD14 is predominantly expressed on monocytes, macrophages and neutrophils while expression on endothelial and epithelial cells has not been clearly defined. If present on these latter cells it is at a far lower level of expression (Jersmann et al. 2001). Some work has been carried out looking at the regulation of CD14: LPS (Imai et al. 2000) and PGE₂ (Iwahashi et al. 2000) stimulate CD14 expression, while TGFβ down regulates CD14 expression in mouse macrophages. TNFα and IFNγ increased CD14 expression in human neutrophils (Takeshita et al. 1998). Expression of CD14 in human umbilical vein endothelial cells is up-regulated by LPS, lysophosphatidic acid and fetal calf serum. TNFα, and IFNγ had no effect on CD14 expression in this system, while PMA reduced the expression (Jersmann et al. 2001). Heinemann et al. 1996 (Heinemann et al. 1996) have shown that monocytes infected with C. pneumonia up-regulated CD14 surface expression 8 hours post infection. CD14 has been shown to be an essential receptor for HSP60 and chlamydial HSP60 activation of mononuclear cells (Kol et al. 2000).

**1.4.1.3 TLR9**

Bacterial DNA has been shown to be capable of activating cells in a TLR-dependent manner. Bacterial DNA contains unmethylated CpG (“C followed by a G”) dinucleotides that have inflammatory and adjuvant properties (McCluskie et al. 2001; Zimmermann et al. 1998). In contrast vertebrate DNA CpG dinucleotides occur infrequently and are highly methylated (Krieg 1999). Hemmi et al (Hemmi et al. 2000) demonstrated that TLR9 deficient mice failed to elicit an inflammatory response to CpG DNA in vitro. This has been subsequently confirmed in human cells (Bauer et al. 2001; Hornung et al. 2002; Takeshita et al. 2001). Unmethylated CpG DNA motifs are recognised by macrophages, dendritic cells and B cells, but it is unclear how important
this is in the response to bacteria, considering that the cells will also see a variety of bacterial cell wall products. There may however be a role for the detection of intracellular pathogens. Limited investigation has addressed the regulation of TLR9 expression. Interestingly, LPS stimulated TLR9 gene expression in a mouse macrophage cell line (An et al. 2002).

1.4.2 Natural antimicrobials

Gene encoded natural antimicrobials are secreted proteins of the innate immune response that play a major part in both innate and adaptive immunity. They are widely distributed in nature and represent an ancient mechanism of host defence. Natural antimicrobials have been described in plants (thionins), insects (eg. cecropins, drosomycin and insect defensins), and vertebrates (eg. magainins, protegrins, cathelicidin and defensins) (Hancock and Lehrer 1998; Schroder 1999). Within a single species, there will be a number of different natural antimicrobials. This allows for a wide spectrum of antimicrobial activity and for synergy to occur. Some natural antimicrobials will have additional non-antimicrobial functions (Hancock and Diamond 2000).

The natural antimicrobials are typically cationic (containing excess basic lysine, arginine and histidine residues) hydrophobic molecules composed of 12-45 amino acid residues. They have a variety of 3-D structures and can be divided into four structural classes (Hancock and Diamond 2000); β-sheets, α-helices, extended peptides and loop peptides. Some of these are constitutively expressed while others are inducible.

The predominant mechanism of killing micro-organisms appears to be by permeabilisation of the cell membranes (Risso 2000). The positively charged antimicrobials interact with negatively charged components of bacterial membranes such as LPS, LTA and phospholipids. Their selective toxicity is thought to be due to the high anionic phospholipid content of microbial membranes, in contrast to the zwitterionic phospholipids (which have both a cationic and an anionic region) and the high content of
cholesterol in mammalian cells. Antimicrobial peptides are thought to aggregate, forming multimeric pores in the cytoplasmic membrane. Depolarisation of the cell membrane may also occur. Wu et al (Wu et al. 1999) propose that other mechanisms of action could include stimulation of autolytic enzymes and interference with bacterial DNA and/or protein synthesis.

In humans endogenous antimicrobials under investigation in this thesis include the defensins, secretory leukocyte protease inhibitor (SLPI), and granulysin.

1.4.2.1 Defensins
Mammalian defensins are peptides containing 28 – 42 amino acids, with 6 invariant cysteines, which form triple stranded β sheets. They were first recognised 30 years ago in rabbit macrophages and subsequently in rabbit and guinea pig neutrophils (Zeya and Spitznagel 1966; Zeya and Spitznagel 1968). The defensins have been further characterised and divided into 2 classes α and β, distinguished by structural features at gene and peptide levels. The early peptides identified were of the α defensin sub class. A third, θ defensin, isolated from macaque leukocytes has been described and it has a unique circular structure (Tang et al. 1999). The α defensins consist of 29-35 amino acids and are shorter than the β defensins, which consist of 38-42 residues. The tertiary structures of the α and β defensins are nearly superimposable (Zimmermann et al. 1995), but the position of the cysteine residues in the amino acid sequence and their disulphide motifs differs between the classes as shown below (adapted from (Huttner and Bevins 1999; Raj and Dentino 2002)):

- Position of the cysteine residue in the amino acid sequence:

Human neutrophil α defensins (HNP1-4 and HD-5 and HD-6)
X-C1-X₂-C2-X₄-C3-X₉-C4-X₉-C5-C6
Human β defensins (HBD-1-4)
X-C1-X₆-C2-X₄-C3-X₉-C4-X₆-C5-C6

- Disulphide motif:

α defensins C₁-…-C₆; C₂-…-C₄; C₃-…-C₅
β defensins C₁-…-C₅; C₂-…-C₄; C₃-…-C₆
θ defensin C₁-…-C₆; C₂-…-C₅; C₃-…-C₄

The α defensins have been localised to human (HNP 1-4) and rabbit neutrophils, rabbit lung macrophages, mouse, rabbit and human (HD5 and 6) small intestine Paneth cells (Diamond and Bevins 1998; Lehrer et al. 1993) and more recently to the human female reproductive tract. HD5 has been described in the vagina, ectocervix, endocervix, endometrium, fallopian tubes and chorion (Quayle et al. 1998; Svinarich et al. 1997b). Expression of HD5 in the endometrium was found to be highest in the early secretory phase of the menstrual cycle and similarly in cervico-vaginal lavages highest concentrations were found in the secretory phase (Quayle et al. 1998). The α defensins are produced and stored as pre-propeptides and undergo post translational and enzymatic processing either within the cytoplasmic granules of the neutrophil or in the lumen of the small intestine. In mouse small intestine, matrilysin co-localises with α defensins in paneth cell granules, and in vitro it cleaves the pro segment from α defensin precursors (Wilson et al. 1999). However, this does not appear to be the case in humans. A corresponding enzyme involved in the processing of human defensins has not been clearly identified. Ghosh et al (Ghosh et al. 2002) demonstrate a specific pattern of trypsin isozymes expressed in Paneth cells co-localize with HD5 and that this protease can efficiently cleave HD5 propeptide to forms identical to those isolated in vivo.
defensins were initially described in the bovine tracheal mucosa where levels were found to increase in vitro in response to stimulation with heat killed bacteria and LPS (Diamond et al. 1991). Four human β defensins (HBD1-4) have since been described in a variety of epithelial tissues, some monocytes and alveolar macrophages. HBD1 and 2 were initially identified in skin, plasma, saliva and lung (Bals et al. 1998; Bensch et al. 1995; Harder et al. 1997; Zhao et al. 1996). In the human female reproductive tract HBD1 and 2 have been isolated from the mucosa of the vagina, ectocervix and endocervix, uterus and fallopian tubes, urine and cervical mucus (Jameson 1999; Valore et al. 1998). Cyclical variation has not been investigated. HBD3 has been identified in adult heart, skeletal muscle, placenta, uterus skin, testis, oesophagus, gingival keratinocytes, trachea and fetal thymus (Garcia et al. 2001; Peng Jia et al. 2001). HBD4 expression has been detected during infection in the testis, uterus, thyroid gland, lung and kidney (Garcia et al. 2001). In addition to the above described β defensins a further 28 new human β defensin genes have been identified using a genomics based approach (Schutte et al. 2002), providing new opportunities to examine the functions of these proteins.

All the genes for the human β defensins can be localised to gene clusters on 3 chromosomes; 6, 8, and 20 (Schutte et al. 2002). The 5’-flanking region of HBD1 contains nuclear factor (NF)-interleukin (IL)-6 and IFNγ consensus sites (Raj and Dentino 2002), HBD2 has NF-κB, NF-IL-6, STAT and AP-1 family sites (Tsutsumi-Ishii and Nagaoka 2002), while HBD3 has NF-IL-6, STAT and AP-1 family sites (Jia et al. 2001). These consensus sites suggest that inflammatory mediators regulate the expression of these proteins. Unlike the α defensins in the azurophil granules of neutrophils, the epithelial β defensins are not stored in cytoplasmic granules, and therefore concentrations are governed by synthesis and secretion rates. The β defensins are constitutively expressed and can in some cases, particularly HBD2, be up-regulated upon infection or inflammation (Krisanaprakornkit et al. 2000; Krisanaprakornkit et al. 1998; Liu et al. 1998; Zhao et al. 1996). This can be mimicked in vitro with LPS and
inflammatory cytokines, such as IL-1 and TNFα, in systems such as the lung, gut and gingiva (Diamond et al. 1996; Harder et al. 2000a; Krisanaprakornkit et al. 2000; O'Neil et al. 1999). The inflammatory mediators INFγ, IL-1β and IL-1β in combination with TNFα have been shown to increase HBD2 mRNA in human endometrium (King et al. 2002).

The defensins have been shown to have antimicrobial activity to a wide range of bacteria, fungi and viruses (Ganz et al. 1985; Risso 2000). The in vitro minimal inhibitory concentrations against a panel of microorganisms ranged between 0.5-10μM for most defensin peptides (Risso 2000). Different defensins can have significantly different specificities and microbicidal potencies. For example HNP 1 is a potent antifungal agent against Candida albicans, whereas HNP 3 has minimal activity (Raj and Dentino 2002).

*In vivo* studies show that the defensins are elevated in patients with bacterial infections. For example, in psoriasis where there are constant skin lesions, infection is only a minor problem and this may be due to the fact that there are higher levels of HBD2 than the levels seen in normal skin (Schroder and Harder 1999). A role for defensins in cystic fibrosis has been postulated, where increases in ion concentration inactivate β defensins and Pseudomonas aeruginosa colonises the respiratory epithelium with potentially devastating consequences (Goldman et al. 1997). There is the possibility of a similar role in the female reproductive tract where cyclical changes in the chemical composition (e.g. pH, cytokines) occur through the menstrual cycle.

The defensins have been found to have a number of functions in addition to their antimicrobial activity (Hancock and Diamond 2000; Yang et al. 2001b). Other innate immune functions include the induction of histamine secretion (van Wetering et al. 1999) and the classical complement pathway (van den Berg et al. 1998), and modulation of cytokine production and adhesion molecule expression (Chaly et al. 2000; Perregaux et al. 2002). Evidence is accumulating that the defensins may play a role in the
recruitment of cells of the adaptive immune response. α defensins are chemotactic for monocytes, dendritic cells and T-cells (Chertov et al. 1996; Yang et al. 2000a), while β defensins are chemotactic for dendritic and memory T-cells (Yang et al. 1999a; Yang et al. 2001a; Yang et al. 2001b). α defensins inhibit fibrinolysis and may therefore play a role in thrombosis (Higazi et al. 1996). They can also induce a proteoglycan-dependent catabolism of low-density lipoproteins, possibly contributing to atherosclerosis (Higazi et al. 2000). In vitro neutrophil defensins can interact with ACTH receptors to inhibit ACTH-induced steroidogenesis (Lehrer et al. 1993), potentially decreasing cortisol release during stress from infection. Defensins increase proliferation of epithelial cells and may thus play a role in wound healing (Murphy et al. 1993; van Wetering et al. 1999).

The defensins can be seen to play an important part in the innate immune response, but can also integrate this into the evolving adaptive immune response. In addition other regulatory functions of these molecules are being realised.

1.4.2.2 Secretory Leukocyte Protease Inhibitor (SLPI)

SLPI, also known as antileukoprotease, is an 11.7kDa proteinase inhibitor comprising 107 amino acids with 16 cysteine residues (Seemuller et al. 1986) forming eight intramolecular disulfide bonds. It was first described in bronchial secretions in 1972 and subsequently in human cervical secretions and seminal plasma (Franken et al. 1989). SLPI is produced by neutrophils (Bohm et al. 1992), (Jin et al. 1997) and epithelial cells (Abe et al. 1991).

The presence of SLPI in a variety of human reproductive tissues including the cervical crypts, cervical mucous (Franken et al. 1989), uterine fluid (Casslen et al. 1981), term decidua (Denison et al. 1999c) and amniotic membranes and fluid (Zhang et al. 2001) has been described. Franken et al (Franken et al. 1989) did not find it in the endometrium, ovaries or fallopian tubes or urine. King et al (King et al. 2000) have subsequently described the presence of SLPI in the endometrium, first trimester decidua,
trophoblast and term decidua. SLPI protein levels have been found to increase in the ovulatory phase in human cervical mucosa (Moriyama et al. 1999), and increased endometrial expression occurs within the late secretory phase of the menstrual cycle (King et al. 2000).

SLPI has been characterised as a neutrophil elastase inhibitor in the lung, and has inhibitory effects on other neutrophil proteases, cathepsin G and pancreatic enzymes – trypsin and chymotrypsin (Thompson and Ohlsson 1986). SLPI activity limits the damage that these proteases may have on normal tissue, particularly in chronic inflammatory diseases such as emphysema and cystic fibrosis. SLPI inhibits histamine release from mast cells *in vitro* (Dietze et al. 1990). It suppresses the production of matrix metalloproteinases from monocytes by inhibiting enzymes involved in PGE$_2$ synthesis (Zhang et al. 1997). It may also play a role in wound healing as SLPI null mice show impaired cutaneous wound healing and increased inflammation and elastase activity (Ashcroft et al. 2000). SLPI has been found prevent NK-$\kappa$B activation (Lentsch et al. 1999), a possible mechanism by which it exerts anti-inflammatory effects.

SLPI has another protective role as a protein of the innate immune defence armoury. SLPI has been demonstrated to have bactericidal activity against *Escherichia coli* and *Staphylococcus aureus* *in vitro* (Hiemstra et al. 1996), skin associated bacteria (*P. aeruginosa* and *S. epidermidis* (Wiedow et al. 1998)) and intestinal pathogens such as *Salmonella typhimurium* (Si-Tahar et al. 2000). This suggests that SLPI has activity against both gram negative and gram positive bacteria. Anti-viral effects of SLPI in saliva against HIV has been demonstrated (McNeely et al. 1995). *In vivo* women with lower vaginal fluid concentrations of SLPI had higher concentrations of vaginal HIV-1 and higher transmission rates to their babies (Pillay et al. 2001). Levels of vaginal SLPI are also decreased in women with other STIs such as gonorrhoea, chlamydia and *Trichomonas vaginalis* (Draper et al. 2000). Anti-fungal effects against *Aspergillus fumigatus* and *Candida albicans* have been reported (Tomée et al. 1997; Wiedow et al. 1998). The antimicrobial effects of SLPI are augmented in the presence of other
antimicrobials. Singh et al (Singh et al. 2000) have demonstrated synergistic and additive effects of combinations of antimicrobials.

The gene for SLPI is found on chromosome 20q12 (Kikuchi et al. 1998). The promoter region has not been extensively studied, but early investigations demonstrated AP-1 and AP-2 transcription factors (Abe et al. 1991). Another serine protease inhibitor, elafin has been demonstrated to have a NF-κB promoter site (Bingle et al. 2001), which has not been identified for SLPI. The SLPI protein is composed of two homologous domains, about equal in size. The C terminal domain of the protein is responsible for the protease inhibitory effects (Eisenberg et al. 1990; Kramps et al. 1990), while the N terminal has been shown to effect bacteriocidal functions (Hiemstra et al. 1996).

The mechanism of the antimicrobial action of SLPI is unclear. Like the defensins SLPI is also a cationic protein, and it may be that cationic/anionic interactions with bacterial cell membranes results in permeabilisation as described above. High salt concentrations are known to inhibit the bacteriocidal effects of SLPI (Hiemstra et al. 1996). Another mechanism has been proposed by Miller et al (Miller et al. 1989) who show that SLPI binds to mRNA and DNA interfering with the interaction of RNA-metabolizing enzymes, such as RNase.

Release of SLPI is stimulated by corticosteroids (Abbinante-Nissen et al. 1995), progesterone (Denison et al. 1999a), cytokines including IL-1, TNFα in amniotic cells (Zhang et al. 2001) airway epithelial cells (Sallenave et al. 1994) and intestinal epithelial cells (Si-Tahar et al. 2000), and IL-10 and IL-6 in mouse macrophages (Jin et al. 1998). Jin et al also demonstrated SLPI induction with LPS, LTA in vitro and Pseudomonas aeruginosa in vivo. Neutrophil elastase and defensins results in an increase of cell associated SLPI (van Wetering et al. 2000a; van Wetering et al. 2000b) in primary bronchial epithelial cells. IFNγ (Jin et al. 1997), TGF-β (Jaumann et al. 2000) and PGE2 (Denison et al. 1999a) have been shown to inhibit SLPI release.
1.4.2.3 Granulysin

Granulysin, also known as 519 and NKG5, is a protein present in cytotoxic T lymphocytes (CTL) (Jongstra et al. 1987) and NK cells (Pena et al. 1997; Yabe et al. 1990). It has been localised to the cytotoxic granules of CTL and NK cells that are released upon antigenic stimulation by exocytosis (Smyth et al. 2001) with the capacity to directly kill intracellular microbial pathogens. It is a gene product which is expressed by T lymphocytes 3-5 days after activation (Jongstra et al. 1987) and in this context is therefore a weapon of the adaptive immune response.

Granulysin is a member of the saposin-like protein (SAPLIP) family. SAPLIP molecules contain six conserved cysteines and hydrophobic residues (Munford et al. 1995). The cysteine residues show a disulfide bonding pattern C₁...C₆, C₂...C₅ and C₃...C₄, a similar pattern to that seen for the β defensins. Granulysin contains a tyrosine instead of a cysteine at the first position. The most closely related member of the SAPLIP family to granulysin is porcine NK-lysin which was originally isolated on its antibacterial activity, but in addition kills nucleated target cells (Andersson et al. 1995a). Granulysin shows 43% identity and 66% similarity to NK-lysin in the SAPLIP domain (Krensky 2000). The 5’-flanking region of granulysin contains NF-κB, AP-1 and cAMP-response element promoter sites that may be involved in transcriptional regulation (Manning et al. 1992). Granulysin is initially made as a 15kDa pro-protein which is then processed to a 9kDa protein. It has been proposed that the structural differences between the 15kDa and 9kDa proteins regulate the lytic potential of the molecule, limiting the potential damage it could cause intracellularly post synthesis (Hanson et al. 1999). As seen with the defensins, granulysin is pH sensitive - an acidic environment reducing the lytic potential of the molecule.

Granulysin from cytolytic T lymphocytes has been shown to kill extracellular *Mycobacterium tuberculosis* and decrease the viability of intracellular *M. tuberculosis*
(Stenger et al. 1998). Synergy between granulysin and perforin, a pore-forming protein contained within the same cytolytic granules, was demonstrated. *In vitro* bactericidal activity of recombinant granulysin has been shown against *Escherichia coli*, *Staphlococcus aureus*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Cryptococcus neoformans*, *Candida albicans*, and *Leishmania major* (Stenger et al. 1998). Mackewicz et al (Mackewicz et al. 2000) have shown *in vitro* that granulysin is unable to affect the infectivity of HIV virions or HIV replication. Therefore granulysin has appreciable activity against gram negative and positive bacteria, fungi and parasites, but activity against viruses has yet to be demonstrated. In addition to antimicrobial activities granulysin induces apoptosis of tumour cell lines (Gamen et al. 1998) and stimulates mitogenicity of endothelial cells (Langer et al. 1999).

The mechanisms of antimicrobial activity are not fully elucidated. SAPLIP proteins are known to interact with lipids, activating lipid degrading enzymes, and it has therefore been proposed that granulysin interacts directly with lipids in the bacterial cell wall. Granulysin does not appear to form pores, but it does allow leakage of intracellular dye from liposomes (Krensky 2000). Gamen et al (Gamen et al. 1998) show that apoptosis is induced in Jurkat cells, with ceramide release (which initiates the caspase cascade and induces apoptosis) and mitochondrial perturbations, and these mechanisms may also be involved in the antimicrobial activity of granulysin.
1.5 Sexually transmitted infections (STI)

There are over 25 STIs, few of which are fatal and most of which can be treated. However, if left untreated STIs may cause serious and permanent damage such as infertility in adults, as well as pathology in the fetus and neonate. The damage can be both physical and psychological. The most common of the STIs and their incidence in the UK is shown in Table 5.

<table>
<thead>
<tr>
<th>STI</th>
<th>1998</th>
<th>1999</th>
<th>comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlamydia*</td>
<td>48 956</td>
<td>56 855</td>
<td>including asymptomatic cases</td>
</tr>
<tr>
<td>Gonorrhoea*</td>
<td>13 190</td>
<td>16 470</td>
<td>uncomplicated cases only.</td>
</tr>
<tr>
<td>HIV</td>
<td>2 761</td>
<td>2 942</td>
<td>number of HIV infected individuals</td>
</tr>
<tr>
<td>Genital herpes simplex*</td>
<td>17 098</td>
<td>17 456</td>
<td>first attack</td>
</tr>
<tr>
<td>Genital warts*</td>
<td>70 460</td>
<td>72 233</td>
<td>first attack</td>
</tr>
<tr>
<td>Hepatitis B</td>
<td>861</td>
<td>749</td>
<td>For England Wales and Northern Island</td>
</tr>
<tr>
<td></td>
<td>295</td>
<td>386</td>
<td>For Scotland</td>
</tr>
<tr>
<td>Hepatitis C</td>
<td>4 521</td>
<td>5 768</td>
<td>For England Wales and Northern Island</td>
</tr>
<tr>
<td></td>
<td>2 017</td>
<td>1 954</td>
<td>For Scotland</td>
</tr>
</tbody>
</table>

*New episodes seen in genitourinary medicine clinics, episodes seen can involve the same individual more than once. (Adapted from Morgan 2002)

A review of the effect of the menstrual cycle and contraceptive use on STI acquisition follows. *Chlamydia trachomatis* infection is then considered in further detail.

1.5.1 Sexually transmitted infections and the menstrual cycle

Under the influence of endogenous hormones components of the immune system vary as discussed previously (section 1.3.1). When considering STIs it could be envisaged that these cyclical changes allow certain phases of the menstrual cycle to be more prone to
acquisition of STIs. An increase in chlamydial salpingitis is seen early in the proliferative phase with significantly more cases occurring within seven days of the onset of menstruation (Korn et al. 1998; Sweet et al. 1986), while increased detection of asymptomatic infection occurs in the late secretory phase (Crowley et al. 1997). Anti-chlamydial activity of cervical secretions varies through the menstrual cycle, being greatest in the first three weeks of a five week cycle. This anti-chlamydial effect was more pronounced in women not using hormonal contraceptives when compared to users of hormonal contraceptives (Mahmoud et al. 1994). Acquisition of gonorrhoea has been shown to increase at the time of menstruation (Eschenbach et al. 1977; Sweet et al. 1986). A variation in the detection of HIV RNA levels in HIV positive women according to the phase of the menstrual cycle has been demonstrated; levels falling from the proliferative to the secretory phase (Greenblatt et al. 2000).

1.5.2 Role of contraception in the acquisition of sexually transmitted infections

A number of studies have investigated the role of hormones and contraception in STI acquisition and transmission with conflicting results. The one clear message is that hormones do play a role and the consequences of this need to be considered (Brabin 2001). The review presented here concentrates on the evidence relating to chlamydial infection with some examples of other STIs.

Barrier methods of contraception afford good protection against STIs although this is not absolute due to incorrect or inconsistent usage and rupture. The male condom protects against infections such as Trichomonas vaginalis, C. trachomatis, N. gonorrhoea, and Herpes simplex virus.

The data on hormonal methods of contraception is inconsistent. Animal models (reviewed in (Washington et al. 1985)) show that progesterone suppresses the growth of gonorrhoea but enhances the growth and survival of chlamydia in rats (Kaushic et al. 1998). Oestrogen similarly enhances growth and survival of chlamydia in guinea pigs (Rank et al. 1982) but infection with gonorrhoea is unaffected or slightly increased. In
monkeys, OCP had no effect on the course of chlamydial acute salpingitis (Patton et al. 1994b). In vitro Kleinman et al (Kleinman et al. 1987) present data showing that ethinyl oestradiol, mestranol (a synthetic oestrogen metabolised to ethinyl oestradiol in the body) and medroxyprogesterone acetate (6α methyl 17α hydroxyprogesterone acetate) do not affect chlamydial replication in cultured human endometrium. In contrast, others have shown chlamydial inclusions in epithelial cultures are decreased in the presence of progesterone or progesterone in combination with an oestrogen (Moorman et al. 1986; Wyrick et al. 1989) while oestrogen alone increases attachment of C. trachomatis to epithelial cells (Maslow et al. 1988).

In vivo the OCP has been shown to cause a 70% increase in gonorrhoea and a 3 fold increase if injectable contraceptives are used although other studies have shown no significant association with OCP use (Carlin and Boag 1995). Studies on chlamydia are more consistent, with women on the COCP having higher rates of chlamydial infection of the lower genital tract (Bontis et al. 1994; Cottingham and Hunter 1992; Kinghorn and Waugh 1981). However, the COCP may be protective for upper genital tract infection (Rubin et al. 1982; Washington et al. 1985; Wolner-Hanssen et al. 1990). It has been suggested that thicker cervical mucus and decreased menstrual flow may account for the decrease in pelvic inflammatory disease (PID).

The LNG-IUS has thus far been shown to be protective for PID (Toivonen et al. 1991), in contrast to copper and inert intra uterine devices which have been associated with an increased incidence of infection in the first 20 days following insertion (Farley et al. 1992), and average levels of infection thereafter.

1.5.3 Chlamydia trachomatis

Chlamydia trachomatis is one of the most common causes of sexually transmitted infections (Gerbase et al. 1998; Groseclose et al. 1999; Morgan 2002). Infection can be both acute and chronic with long term morbidity. The following review will examine
the clinical epidemiology, the developmental cycle of the organism and the immune responses to infection. How this relates to the resultant pathology will be discussed.

1.5.3.1 Clinical infection and sequelae

The most important implications of genital chlamydia are the reproductive health sequelae of upper genital tract infection in women. In addition, chlamydia causes genital tract complications in men. Much chlamydial infection goes undiagnosed because it is asymptomatic. If symptoms do appear they usually do so 1-3 weeks after the time of infection. In men the symptoms include dysuria, penile discharge, itchiness and occasionally testicular tenderness, with epididymitis, and proctitis. In men and women, a reactive arthritis can occur. In women the infection is more often asymptomatic but cervicitis, abnormal vaginal bleeding and intermenstrual bleeding, and pelvic pain may occur. Untreated infection can ascend into the fallopian tubes causing a salpingitis and PID (Cates and Wasserheit 1991; McCormack 1994).

Continued infection, inflammation and scarring can lead to chronic pelvic pain, tubal pregnancy, infertility (Buchan et al. 1993; Cates and Wasserheit 1991) and possible adverse events in pregnancy, such as chorioamnionitis, premature rupture of membranes and preterm delivery (Gravett et al. 1986; Martin et al. 1982; Sweet et al. 1987). Evidence suggests that 20% of women with chlamydial lower genital tract infection will develop PID, 4% chronic pelvic pain, 3% infertility and 2% adverse pregnancy outcomes (Paavonen and Eggert-Kruse 1999). After a single episode of PID, the relative risk for tubal factor infertility is approximately 10%, but with repeated episodes this risk doubles rising to almost 40% after 3 or more episodes (Westrom 1994). Women with a history of PID have a 7–10 fold increased risk of tubal pregnancy when compared to women with no history of PID (Westrom et al. 1981). More recently, cervical chlamydial infection has been suggested as an independent risk factor in the development of cervical neoplasia (Anttila et al. 2001; Koskela et al. 2000).
Perinatal transmission of infection to neonates during a vaginal birth occurs in 50%-60% of infants born to infected mothers (Gencay et al. 2001; Schachter et al. 1986). Conjunctivitis and/or chlamydial pneumonia (Beem and Saxon 1977; Gencay et al. 1995; McGregor and French 1991) may develop in 10%-30% of these cases.

Diagnosis of \textit{C. trachomatis} is a routine procedure, with either an ELISA or the more expensive but more sensitive nucleic acid amplification tests (polymerase chain reaction or ligase chain reaction). These more sensitive tests can be carried out on endocervical swabs, as well as first void urine specimens or vaginal or vulval swabs. Tetracyclines or the macrolides – particularly azithromycin – are effective in treating the infection. As \textit{C. trachomatis} is an infection which can be asymptomatic, is detectable and can be treated before expensive complications arise the possibility of population screening is under consideration. At present screening is only carried out in known at risk populations such as those undergoing a termination of pregnancy. Further analysis and review of this topic is ongoing (Pimenta et al. 2000; Pimenta and Fenton 2001).

1.5.3.2 Taxonomy

\textit{Chlamydia} taxonomic classification was updated in 1999 (Everett et al. 1999), and will be briefly reviewed here and in figure 5. The classification is supported by analysis of phenotype, antigenicity, associated disease, host range, biological data and genetic data. Members of the order \textit{Chlamydiales} are obligate intracellular bacteria that have chlamydia-like developmental cycle of replication and have >80% rDNA sequence identity with chlamydial 16S or 23S rRNA genes. The order includes four families; i) \textit{Chlamydiaceae}, with gram negative elementary bodies (EBs), ii) \textit{Simkaniaecae} also with gram negative EBs but with rRNA sequence differences and antigenic differences, iii) \textit{Parachlamydiaceae}, which have variable gram staining EBs iv) an unnamed group containing the strain WSU 86-1044. The family \textit{Chlamydiaceae} contains the two genera; \textit{Chlamydia} and \textit{Chlamydophila}. The genus \textit{Chlamydia} includes the species \textit{Chlamydia trachomatis}, \textit{Chlamydia muridurum} and \textit{Chlamydia suis}, while the genus \textit{Chlamydophila} includes the species \textit{Chlamydophila psittaci}, \textit{Chlamydophila}
pneumoniae, Chlamydophila pecorum, Chlamydophila abortus, Chlamydophila caviae and Chlamydophila felis. Chlamydia trachomatis can be further divided into 18 serovars, clustered into 2 biovars: trachoma and lymphogranuloma venereum (LGV), each serovar being recognised by specific monoclonal antibodies. Trachoma serovars are designated by the letters A-K and Ba, Da and Ia; A-C being primarily associated with endemic trachoma and D-K with sexually transmitted infection. LGV biovar includes the serovars L1, L2, L2a and L3, they are sexually transmitted and infect mononuclear phagocytes.
Figure 5: Taxonomic classification of the order Chlamydiales.
1.5.3.3 Structure and developmental cycle

Chlamydiales are obligate intracellular parasites, but in contrast to viruses they do have an inner cytoplasmic and outer membrane like gram negative bacteria. However, they lack the peptidoglycan layer seen in bacterial cell walls. The outer membrane of the elementary body is instead cross-linked by disulphide bonds between cysteine residues. The predominant components of the outer membrane are the major outer membrane proteins (MOMP), which are 370-375 amino acid proteins. MOMPs make up two thirds of the cell wall weight and are species specific (Caldwell et al. 1981). LPS is also present and is genus specific. Intracellularly RNA and DNA and prokaryotic ribosomes are present. The chlamydiales can synthesis their own proteins, nucleic acids and lipids, but cannot synthesis high energy phosphate compounds such as adenosine triphosphate (ATP) and are therefore reliant on the host cell for energy, hence are known as “energy parasites” (Moulder 1974).

The developmental cycle of chlamydiales sets them apart from other bacteria. It can be divided into several steps: attachment and entry of an elementary body (EB) to the host cell, morphological change from an EB to a reticulate body (RB) with intracellular growth and replication, morphological change from a RB to an EB and release of infectious particles (Figure 6).
Figure 6: The chlamydial developmental cycle. (Adapted from Davis and Wyrick 1997)
1. Infectious elementary body (EB) attaches to the host epithelial cell
2. EB invades cell within a vesicle
3. Vesicles fuse. EBs convert to metabolically active reticulate bodies (RB)
4. RBs grow and divide by binary fission in a membrane bound chlamydial inclusion
5. Non infectious RBs differentiate into infectious EBs and exocytosis or host cell lysis results in release of infectious EBs.
The EB is an infectious spherical particle about 300-400nm in diameter. The initial attachment to the susceptible host cell may involve a specific receptor-ligand interaction, but the exact natures of the structures involved have not been elucidated. There is some evidence suggesting a heparin sulphate like molecule on the surface of *C. trachomatis* may play a role. Attachment may also be charge dependent, as treatment with DEAE-dextran *in vitro* can enhance attachment and infectivity (Kuo et al. 1973). *C. trachomatis* is only able to infect a limited range of cells and these include non-ciliated columnar, cuboidal and transitional epithelium for example the mucous membranes of the urethra, endocervix, endometrium, fallopian tubes, respiratory tract and conjunctivae. Once attached to the cell the EB is rapidly internalised, the mechanisms are controversial but are thought to involve receptor-mediated endocytosis, the receptor involved being unknown. The EB enters the cell in a phagosome and remains in a phagosome through its entire life cycle. Fusion with a lysosome is inhibited by an unknown mechanism (Friis 1972; van Ooij et al. 1997). Vesicles containing *C. trachomatis* EBs fuse and within 6-8 hours the EBs reorganise into RBs. These are larger particles, 800-1000nm in diameter. Their outer cell walls lack the cross-linking of protein and are therefore more osmotically fragile, but this permeability allows uptake of ATP and other nutrients. The RBs are metabolically active and divide by binary fission over the next 18-24 hours within the vesicle, now as an inclusion, which can expand to accommodate 200-1000 organisms. Outer membrane antigens and LPS are shed into the inclusion and can be excreted by the cell (Schachter 1988). Cellular nutrients are required for effective growth, if a cell is starved or depleted of nutrients the chlamydiae can become slow growing and non-productive (Jones et al. 2001b). Cycloheximide can inhibit metabolism of the host cell, but the RB continue to grow, their growth being independent of the host cells metabolism if nutrients are not in short supply. Finally RBs condense and differentiate to infectious EBs, which are released from the host cell either by exocytosis or cell rupture 48-72 hours after initial infection of the cell.
1.5.3.4 Immune Response to *C. trachomatis* in the female reproductive tract

The female reproductive tract with the exception of the vagina is a sterile environment. As detailed previously (section 1.3.1), components of the immune system are present to mount a response if required. In the case of *C. trachomatis* both a non-specific innate immune response and an adaptive immune response are observed.

The histopathological response to *C. trachomatis* (Kiviat et al. 1990) shows epithelial cell degeneration, with intraepithelial lymphocytes present. Beneath the epithelium is a dense infiltration of mononuclear cells and neutrophil exudation occurs through the epithelium into the lumen. Through the stroma further T lymphocytes and plasma cells are identifiable.

Experimental infections in animals are usually self-limiting; indicating that the immune response generated is sufficient to eliminate chlamydiae from the genital tract. It is believed that the pathology following chlamydial infection is as a result of a persistent immune response. The response has not been clearly elucidated in humans, and much of the work has been carried out in animal or *in vitro* models. The following review concentrates on data relating to human infection.

The three components of the innate immune response play a role in early chlamydial infection. The epithelium and mucus secretions provide a physical barrier to infection. *C. trachomatis* overcomes this barrier by inducing the epithelial cells to phagocytose the EB once it is attached to the cell (Moulder 1985). This is unusual, as epithelial cells are not normally phagocytic. The mechanisms involved are unknown. Within the mucus lining the reproductive tract are a number of chemical substances that may play a role in limiting infection. Lactoferrin, lysozyme and zinc are often detailed as having antimicrobial actions. The natural antimicrobials, such as the defensins detailed previously, may also play a role in the initial immune response (Yasin et al. 1996). Spermine and zinc, present in seminal fluid have been reported as having activity against chlamydia (Mardh et al. 1980). Neutrophils can ingest and degrade EBs; they collect in
large numbers in the inflammatory exudate and may play an important part in the early localisation of infection. Epithelial cells themselves will also play a role in the initiation of the immune response through the secretion of proinflammatory cytokines such as IL-1 and IL-8 (Kagnoff and Eckmann 1997; Rasmussen et al. 1997).

Both an antibody mediated and a cell mediated specific immune response occur following chlamydial infection. Antigen specific sIgA and IgG are found in the serum following infection (Cerrone et al. 1991; Watts et al. 1989). MOMP are the principal target for neutralising antibodies, but chlamydial heat shock proteins may also play a part (see below). In vitro these antibodies neutralise infectivity, but do not inhibit adhesion or internalisation of the chlamydia (Caldwell and Perry 1982; Peeling et al. 1984). An inverse correlation between the titre of sIgA in genital secretions and the quantity of chlamydiae isolated from the cervix has been demonstrated (Brunham et al. 1983). Chlamydial heat shock proteins (Chsp), particularly Chsp-60, bears close homology with the human equivalent (48% homology) and may be a major target for immunopathological responses. Heat shock proteins (HSP) were first identified in cells after exposure to elevated temperature. Subsequently HSP have been identified as a critical component of a highly conserved cellular defense mechanism to preserve cell survival under adverse environmental conditions. HSP interact with intracellular polypeptides and prevent their denaturation or incorrect assembly. Chsp-60 immune recognition may result in an autoimmune response through molecular mimicary (Domeika et al. 1998; Lin et al. 1991). In human microvascular endothelial cells and mouse macrophages, recombinant Chsp-60 rapidly activates NF-κB and induces human IL-8 promoter activity (Bulut et al. 2002). These effects were shown to be TLR4/MD2 dependent. Expression of Chsp is up-regulated in IFNγ induced persistent infection (Ward 1995). Antibody to Chsp60 is strongly associated with occluded fallopian tubes, but not with acute C. trachomatis infection (Eckert et al. 1997) and is also associated with ectopic pregnancy (Sziller et al. 1998). The immune response to Chsp in mice has been demonstrated to be genetically determined and it has been speculated that the same may be true of human infection (Cohen and Brunham 1999; Zhong and Brunham 1992).
This could explain why some women are more susceptible to pathology following chlamydial infection.

Another means of eliminating *C. trachomatis* is the cell-mediated immune response. Chlamydia specific T cell clones are generated following infection (Qvigstad et al. 1985). Studies in non-human primates have demonstrated the induction of mRNA for IFNγ, IL-2, IL-6 and IL-10, but not for IL-4 following single or repeated chlamydial infection of salpingeal tissue (Van Voorhis et al. 1997). CD8⁺ T lymphocytes are prominent, although CD4⁺ T lymphocytes are also present. Fibrosis is also observed in some subjects. These findings suggest that Th1 cytokines (e.g. IFNγ) may play a role in intracellular killing of *C. trachomatis*. Through the production of cytokines, particularly IFNγ, infection can be limited (Loomis and Starnbach 2002). *In vitro* IFNγ can inhibit the infection of cell cultures with chlamydia. IFNγ induces an enzyme of tryptophan catabolism, indoleamine 2,3- dioxygenase (IDO), which is responsible for conversion of tryptophan and other indole derivatives to kynurenine (Taylor and Feng 1991). Tryptophan is required for chlamydial growth and without it abnormal growth occurs (Beatty et al. 1993). IL-1 can potentiate this effect of IFNγ (Carlin and Weller 1995). While playing a role in the limitation of infection, the cell-mediated response may also contribute to the delayed, continued immune response thought to be responsible for long term sequelae such as infertility. Studies in macaques have shown that while a single episode of chlamydial salpingitis is usually self limiting, repeated infections will result in tubal scarring (Patton et al. 1987). Delayed hypersensitivity responses to chlamydiae, characterized by a submucosal cellular infiltrate of lymphocytes and macrophages, caused by repeated or persistent infection are thought to be important in the development of the severe scarring sequelae characteristic of chronic salpingitis (Patton et al. 1994a).

Beatty et al (Beatty et al. 1994) suggest the following model for persistent chlamydial development and resultant pathology: Chlamydial infection results in the induction of IFNγ from the stimulated immune system. This leads to IDO induction, tryptophan depletion and aberrant chlamydial growth. The RBs are non-infectious, but viable and
continue to produce Chsp60, but decrease production of MOMP and LPS (Beatty et al. 1993; Shaw et al. 1999). This maintains the antibody response and ensuing tissue damage.

The immune mechanisms following chlamydial infection are not yet fully understood. They are complex, involving the interaction of a number of different systems. The exact role of local or secretory antibody and cell-mediated immune responses in mediating recovery from infection and immunity to reinfection remains unclear. By continuing to unravel these responses, the possibilities for the prevention of chlamydial pathology may become apparent.
1.6 Aims of the Thesis

The human endometrium is continually exposed to a wide selection of pathogens and non-self antigenic stimuli. Wide ranges of immune mechanisms are needed to control the response to these factors. The natural antimicrobials are a recently described innate immune defence mechanism. They have been identified within the human female reproductive tract and in some cases have been found to be cyclically expressed. This suggests that hormonal regulation plays a role in their expression. If this is the case, hormonal methods of contraception, with administration of exogenous sex steroids, may affect their expression.

With components of the immune mechanism within the endometrium altering during the menstrual cycle, the endometrium may be more susceptible to infection at certain stages of the menstrual cycle. Changes in early pregnancy may also result in altered acquisition of infection.

The importance of pattern recognition receptors, in particular Toll-like receptors, in initiation of the immune response is being realised, although the mediators of expression of these receptors and the natural antimicrobials within the human endometrium have not been fully investigated. To elucidate further the innate immune response to *Chlamydia trachomatis*, one of the commonest sexually transmitted infections, these components of the early immune response need to be defined.
Thus the aims of this thesis are:

1. To characterise the natural antimicrobials (HBD1, HBD2, SLPI and granulysin) within the human endometrium through the menstrual cycle. To assess the effect of exogenous sex steroid hormones, in the form of the COCP and LNG-IUS, on this expression.

2. To investigate the expression of the natural antimicrobials (HBD1, HBD2, SLPI and granulysin) in early pregnancy. To assess the effect of chlamydial infection on this expression.

3. To investigate the expression and regulation of components of the innate immune response (natural antimicrobials and Toll like receptors) in vitro.

4. To elucidate the early immune responses to Chlamydis trachomatis infection in vitro.
Chapter 2
General Methods
All materials used in the work described in this thesis are detailed in Appendix 1.

2.1 Sample collection

Endometrium was collected from women undergoing gynaecological procedures for benign conditions either while under investigation in the out patient setting, during a day case procedure or during hysterectomy. All women reported regular menstrual cycles (25-35 days) and had not received any form of hormonal treatment in the three months preceding biopsy (unless otherwise stated). Biopsies were performed with a Pipelle suction curette or, if following hysterectomy, a full thickness endometrial biopsy (lumen to endometrial/myometrial junction) was excised and the endometrium separated from the underlying myometrium. Biopsies were dated from the patient's last menstrual period (LMP); histological dating, according to published criteria (Noyes et al. 1950) was consistent with the date of LMP. Furthermore, circulating sex steroid concentrations were consistent with the histological dating of the biopsy. Serum was separated from venous blood samples at the time of biopsy and frozen at -20°C for subsequent radioimmunoassay of oestradiol and progesterone. The inter-assay coefficients of variation (CV) for these assays were 8.5% and 5.3% respectively; intra-assay CV were 8.6% and 3.5% respectively. Table 6 details the number of endometrial biopsies included in this study.

First trimester decidua (8-11 weeks gestation, as determined by last menstrual period and/or ultrasound scan) was obtained from patients undergoing surgical termination of pregnancy. Prior to the surgical termination, biopsies were collected by curettage of the uterine wall away from the site of implantation. On some occasions trophoblastic villi were also collected during the procedure. Decidua parietalis (without trophoblast) was confirmed by examination of hematoxylin and eosin (H and E) stained sections and
cytokeratin immunolocalization confirmed the absence of infiltrating trophoblast cells. Table 6 details the number of decidual biopsies included in this study.

Saliva and vaginal secretions were collected from patients undergoing surgical termination of pregnancy (8-11 weeks gestation, as determined by last menstrual period and/or ultrasound scan). Prior to surgical termination women were asked to collect saliva using a citric acid salivette as per manufacturers instructions (Sarsted). Immediately prior to surgical termination procedure, the vagina was lavaged with 5ml of phosphate buffered saline (PBS) using a 50ml syringe. Table 6 details the number of saliva and vaginal fluid samples included in this study.

Written informed consent was obtained from all patients prior to biopsy collection and ethical approval was received from Lothian Research Ethics Committee (LREC/2000/6/56, LREC/1994/6/17 and LREC/1993/6/73).

Tissue samples were collected in Roswell Park Memorial Institute (RPMI) 1640 medium. Part of the sample was fixed overnight in 10% neutral buffered formalin (NBF) at 4°C, rinsed and stored in 70% ethanol and thereafter routinely wax embedded. Sections 5µm thick were cut for routine histopathology (H and E). Tissue was also immersed in Tri reagent (a RNA/DNA/protein isolation reagent), homogenised and stored at -70°C for subsequent RNA extraction. Finally tissue portions were used for culture if required, as described in section 2.2.1 and 2.2.2. The saliva and vaginal secretions were centrifuged (450g, 3 minutes) to remove debris. Samples were then stored at -70°C for subsequent enzyme linked immunosorbant assay (ELISA) (see section 2.6).
Table 6: Overview of patient samples analysed in this research project*

<table>
<thead>
<tr>
<th>Cycle Stage</th>
<th>Day of Cycle</th>
<th>Number of Biopsies</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Endometrium</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proliferative</td>
<td>5-13</td>
<td>7</td>
</tr>
<tr>
<td>Early secretory</td>
<td>14-18</td>
<td>3</td>
</tr>
<tr>
<td>Mid secretory</td>
<td>19-23</td>
<td>5</td>
</tr>
<tr>
<td>Late secretory</td>
<td>24-28</td>
<td>3</td>
</tr>
<tr>
<td>Menstrual</td>
<td>1-4</td>
<td>6</td>
</tr>
<tr>
<td>Taking the combined oral contraceptive pill</td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>Levonorgestrel - intrauterine system <em>in situ</em></td>
<td>median length of time <em>in situ</em> 6 months range 3-60 months</td>
<td>13</td>
</tr>
<tr>
<td><strong>Decidua</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7-12 weeks amenorrhoea</td>
<td>19</td>
</tr>
<tr>
<td><strong>Trophoblast</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7-12 weeks amenorrhoea</td>
<td>5</td>
</tr>
<tr>
<td><strong>Saliva/vaginal secretions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7-12 weeks amenorrhoea</td>
<td>10</td>
</tr>
<tr>
<td><strong>Endometrial Explants</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proliferative</td>
<td>5-13</td>
<td>8</td>
</tr>
<tr>
<td>Secretory</td>
<td>14-28</td>
<td>4</td>
</tr>
<tr>
<td><strong>Primary endometrial epithelial cell cultures</strong></td>
<td>Proliferative</td>
<td>1-13</td>
</tr>
<tr>
<td></td>
<td>Secretory</td>
<td>14-28</td>
</tr>
</tbody>
</table>

*Further details of samples in each particular study are given in the relevant data chapter.
2.2 In vitro tissue culture

2.2.1 Explant culture

Explants (approximately 4mm³) of proliferative phase endometrial samples or secretory phase endometrial samples were cultured for 24 hours on sterilised polypropylene capillary matting in RPMI 1640 supplemented with 10% fetal calf serum (FCS), penicillin (100iu/ml), streptomycin (100µg/ml), gentamycin (20µg/ml) and L-glutamine (2mM) (complete (c) RPMI). They were cultured in the presence of oestradiol (10⁻⁸ mol/l) either alone or with treatments as described in the data chapters. The treatments were removed at 24 hours and the culture medium stored at -20°C for subsequent analysis by ELISAs (see section 2.6). Explants were immersed in Tri reagent, homogenised and stored at -70°C for subsequent RNA extraction as detailed in section 2.4. Table 6 details endometrial samples used for explant studies.

2.2.2 Separation of endometrial biopsies into glandular and stromal compartments

This method of separation of glandular and stromal compartments of endometrium was adapted from that of Osteen et al 1989 (Osteen et al. 1989). Several modifications were made and details of the methods used in this study are described. Endometrial biopsies are washed twice in PBS, sliced into small fragments, and immersed in collagenase/DNAase (1mg/ml and 0.1mg/ml) and incubated for 80 minutes at 37°C. After incubation, RPMI 1640 medium was added and tissue disaggregated using a syringe. This yielded single cells and larger glandular fragments. The suspension was centrifuged (450g, 3 minutes) and the resulting cell pellet was resuspended in fresh medium and allowed to separate by density sedimentation. After 5 minutes the supernatant (stromal compartment) was removed leaving 2ml of medium. Fresh medium was added and the density separation step repeated. The remaining 2ml of medium, containing the glandular fraction, was then centrifuged as above. The resulting cell pellet was resuspended in collagenase/DNAase, as above, for 2 hours at 37°C. After incubation
the cell suspension was centrifuged (as above), medium removed and the epithelial cell pellet resuspended in 50% matrigel. A drop of matrigel was added to each well of a 12 well plate, allowed to solidify and medium added as below.

2.2.3 Cell Culture

2.2.3.1 Primary endometrial epithelial cells

Cells were cultured in cRPMI supplemented with epidermal growth factor (25ng/ml), vascular endothelial growth factor (1ng/ml), basic fibroblast growth factor (5ng/ml) and oestradiol (10⁻⁸ mol/l) (epithelial growth medium). Growth factors were included in the culture medium as there is evidence that endometrial epithelial cells express their receptors and hence they are likely to be involved in modulation of cell growth (Li et al. 1994; Meduri et al. 2000; Sangha et al. 1997; Zhang et al. 1995). When subconfluent (7-10 days) the epithelial growth medium was removed and cells were rested for 24 hours in cRPMI supplemented with oestradiol (10⁻⁸ mol/l) with either 10% FCS or 5% human serum as detailed in the data chapters. Treatments were then added as detailed in the data chapters. Immunohistochemical staining (using mouse anti-human cytokeratin antibody, clone MNF116) of representative primary cultures for the epithelial marker cytokeratin (see section 2.5.2), confirmed that at the time of treatment the purity of the epithelial cells was >90% (Figure 7). The majority of the contaminating cells were likely to be of stromal origin and morphologically resembled fibroblasts. Table 6 details the number of endometrial epithelial cell samples included in this study.
Figure 7: Primary endometrial epithelial cells. Cells were grown in matrigel in 12-well plates prior to immunostaining. (a and c) Immunolocalization of cytokeratin in primary endometrial epithelial cells at two different magnifications. Cytokeratin was present in within the cytoplasm of most cells. Arrow denotes area of unstained cells, which morphologically resemble fibroblasts. (b and d) Negative controls. The primary antibody was replaced with equimolar concentrations of an isotype control.
Scale bar = 50 μm
2.2.3.2 Cell Lines

(i) MFE, HES and HeLa Cell Culture
MFE-296 (Hackenberg et al. 1994) and HES (Desai et al. 1994) endometrial epithelial cell lines and HeLa a cervical epithelial cell line, were cultured in 25cm² culture flasks. After passaging cells were seeded at 1x10⁶ cells/flask, and then incubated for 24 hours to allow adherence to the flask. Cells were grown or maintained in cRPMI except that either 10% fetal calf serum or 5% human serum was added as detailed in chapters 3 to 6. Treatments were added and incubated for an appropriate time, as detailed in chapters 3 to 6. Culture supernatants were then removed and stored at -20°C for subsequent analysis by ELISAs (see section 2.6). Cells were collected for RNA extraction by adding Tri reagent directly to the culture flask and then storing the harvest at -70°C for subsequent RNA extraction as detailed in section 2.4.

(ii) HeLa Cell culture for Chlamydia Experiments
The human cervical cell line HeLa was grown or maintained in Iscove’s modified Dulbecco’s medium (IMDM) supplemented with the appropriate concentration of fetal bovine serum (FBS). No antibiotics or other supplements were used unless stated otherwise. The cells were routinely grown in 225cm² vented plastic flasks in IMDM supplemented with 5% FBS.

2.3 Chlamydiae culture
C. trachomatis serovar E was used throughout, propagated in HeLa cell monolayers.

2.3.1 Chlamydial stock cultures
Subconfluent monolayers of HeLa cells in 225cm² flasks were prepared. Medium was discarded. 5ml of Hanks’ Balanced Salt Solution (HBSS) containing 30mg/L DEAE-dextran was added and the cells were left at room temperature for 20 minutes. DEAE-dextran was removed and Chlamydia stock (elementary bodies (EBs)) added. This was allowed to absorb for 1hour at 37°C and the monolayer was then rinsed with HBSS. The
chlamydia infected cells were incubated at 37°C for 72 hours in IMDM supplemented with 5% FBS, gentamycin (25μg/ml) and cyclohexamide (1μg/ml).

### 2.3.2 Purification of *C. Trachomatis*

All media was decanted from the infected flask. Sterile glass beads (approximately 50, 4mm beads) suspended in 10ml IMDM, and 5% FBS were added to the flask. Cells were disrupted mechanically. The suspension was centrifuged at 2000g for 5 minutes at 5°C. The cell pellet obtained was homogenised and added to the supernatant obtained from the initial centrifugation. A further 5 minute centrifugation at 2000g, 5°C was undertaken. The cell debris was discarded and the supernatant ultracentrifuged at 13000g for 15 minutes. The resulting EB pellet was resuspended in chlamydia transport medium and stored at -70°C.

Multiplicity of infection (MOI) (the number of inclusion forming units (ifu) relative to the number of cells) was determined by titration on HeLa cell monolayers and staining with a FITC labelled monoclonal antibody against chlamydial lipopolysaccharide (LPS) (see section 2.5.4). The number of chlamydial inclusions relative to the number of cells at 72 hours is determined. Knowing the volume of infecting media initially used ifu/ml can be deduced and used to calculate the required volume for the MOI finally required.

### 2.3.3 Infection protocol

HeLa cell monolayers were seeded into 24-well plates at a density of 5x10⁴ cells/well, and cultured for 24 hours. Medium was then removed and cells infected with *C. trachomatis* MOI 0.1 or 0.01 diluted in IMDM (200μl/well). Plates were incubated for 1 hour. The inoculum was then aspirated and 1ml of IMDM added. Other treatments included heat-treated organisms (incubated at 65°C in a water bath for 1 hour) and cell lysate. Cell lysate was obtained by the procedure described for the purification of *C. trachomatis* (section 2.3.2), however a mock infected flask of HeLa cells was purified so that the resultant pellet contained no *C. trachomatis*. Further treatments and incubation times are as detailed in Chapter 6. At each time point supernatant was collected and
stored at \(-70^\circ\text{C}\) for subsequent ELISAs (see section 2.6); cells were lysed in Tri reagent and stored at \(-70^\circ\text{C}\) for subsequent mRNA extraction (see section 2.4).
2.4 RNA extraction and quantitative reverse transcription-polymerase chain reaction (Q-RT-PCR)

2.4.1 RNA extraction

Tissue samples were immersed in 1ml Tri reagent, homogenised and RNA extracted as follows. Samples were transferred into eppendorfs containing phase lock gel (Eppendorf AG) and incubated for 5 minutes at 4°C. 200μl of chloroform was then added and the sample mixed by inversion. After a further 5 minute incubation at 4°C, samples were centrifuged at 12000g for 20 minutes at 4°C, allowing separation into three phases. Upon centrifugation the phase lock gel migrates to form a tight seal between phases of an aqueous/organic extraction. The upper aqueous phase above the phase lock gel contained RNA with the remaining phases containing DNA and protein. The aqueous layer was pipetted to a fresh eppendorf tube and 500μl of 100% isopropanol was added. Samples were mixed thoroughly and then incubated at 4°C for at least 60 minutes. Samples were then centrifuged at 12000g for 15 minutes. The resulting supernatant was removed leaving a pellet of RNA. This was washed in 1ml of 70% ethanol by vortexing briefly and then centrifugation at 12000g for 5 minutes at 4°C. Ethanol was removed and the pellet resuspended in RNA storage solution.

The optical densities of the RNA samples were measured at 260nm and 280nm to determine the purity and the concentration of RNA present. A 260:280 value of approximately 1.8 indicates a pure RNA sample. An optical density value of 1 at 260nm is equivalent to 40μg/ml of mRNA.

Concentrations of RNA were determined using the following formula:

\[
260\text{nm value} \times \text{dilution of RNA} \times 40 = \text{RNA concentration (ng/μl)}
\]

For use in RT-PCR all RNA samples were diluted with TE buffer to 100ng/μl. Samples were then stored at -70°C for subsequent use.
2.4.2 Reverse transcription

RNA samples were reverse transcribed using random primers with MgCl₂ (5.5 mmol/l), dNTPs (1 mmol/l), random hexamers (2.5 μmol/l), RNAase inhibitor (0.4 IU/μl) and Multiscribe reverse transcriptase (1.25 IU/μl). The mix was divided into aliquots in individual tubes (16μl/tube) and template RNA was added (4μl/tube of 100ng/μl RNA). Mineral oil was added and samples were incubated for 20 minutes at 25°C, 60 minutes at 42°C and then at 95°C for 5 minutes. cDNA was diluted 2.5x with TE buffer.

The possibility of contamination of RNA samples with genomic DNA was excluded by detection of β-actin signal in RNA samples (without reverse transcriptase). All samples included for analysis in this thesis had a β-actin signal that was above a previously defined, arbitrary level of 27 cycles, which was 3 standard deviations from a mean of samples (King et al. 2000) indicating that there was no major genomic DNA contamination (see figure 8).
Figure 8: β-actin levels in mRNA samples that were subsequently used for RT-PCR. All endometrial and decidual samples were checked. The control samples for all explant, primary epithelial cell experiments and cell line experiments were checked. The cycle number is the value at which fluorescence release reached a threshold value. Any sample with a β-actin measurement below 27 (dashed line) was excluded from this thesis. One sample was excluded on this basis.
2.4.3 Real time quantitative polymerase chain reaction (Taqman; Q-RT-PCR)

This PCR method allows the measurement of PCR product via the detection of released fluorescent reporter dye (see Bustin et al (Bustin 2000) for a comparative review of conventional RT-PCR and Q-RT-PCR). Forward and reverse primers are used which recognise the sequence of target DNA. Concurrently a probe is used which recognises a sequence present between the annealing sequences of the two primers. The probe is labelled with two fluorescent dyes: a 5 prime reporter dye (all amplicons except 18S use FAM; 6-carboxyfluorescein) and a 3 prime quencher dye (TAMRA; 6-carboxytetramethylrhodamine). The reporter dye on 18S is VIC (chemical name is unavailable). When annealed to the target sequence the quencher dye suppresses the fluorescence of the reporter dye as they are in close proximity. But as the target sequence is amplified during a PCR reaction the probe is cleaved by the endonuclease activity of taq polymerase. This results in separation of the two dyes so the quencher is no longer suppressed and fluorescence is therefore increased and can be measured (see figure 9). This increase in fluorescence is detected only if the target sequence for the probe is amplified during the reaction, thus preventing the detection of non-specific amplification. The amount of specific amplicon is related to ribosomal 18S (constant to the relative amount of cDNA present) and subsequently to an internal control. Two controls were used; either the control for the particular experiment being undertaken or when whole tissue samples were being compared a mixture of leukocyte mRNA. Concurrent measurement of ribosomal 18S and specific amplicons in the PCR well is possible as the reporter dyes of each emit different wavelengths.
Figure 9: Quantitative polymerase chain reaction (Taqman).

Stage 1 shows initiation of polymerisation. The probe has annealed to the desired sequence and the reporter (FAM) and quencher (TAMRA) dye are in close proximity, decreasing the amount of fluorescence detected.

Stage 2 shows the forward primer extending along the template displacing the reporter dye of the probe.

Acting as an endonuclease, Taq polymerase then cleaves the probe, stage 3, breaking the link between the reporter and quencher dyes resulting in increased ‘free’ reporter dye - FAM. The reporter dye is no longer in close proximity to the quencher dye and therefore fluorescence increases.

Finally, stage 4, polymerisation is completed. The amount of fluorescence generated will be proportional to the amount of PCR product generated, which in turn will reflect the initial quantities of the desired sequence.

Adapted from the Taqman PCR Reagent Kit Protocol.
Stage 1: Polymerization

Stage 2: Strand displacement

Stage 3: Cleavage

Stage 4: Polymerization complete

--- Forward Primer
--- Reverse Primer
--- Probe
A reaction mix was made from a Stratagene kit containing Taqman buffer, MgCl₂ (3.6 mmol/l), dNTPs (0.8 mmol/l), “sure start” taq polymerase (0.025U/μl), reference dye (0.03%), to which was added ribosomal 18S forward and reverse primers and probe (all at 50nmol/l), and the primers and probe of the RNA message under investigation (300nmol/l). The mixture was divided into aliquots in separate tubes for each cDNA sample. 2.5μl/replicate of cDNA was added to each tube. After mixing 24μl of sample was added to the wells on a PCR plate. Each sample was added in at least duplicate. A no template control (containing water) and a positive control (containing mixed leukocyte mRNA) were run on each PCR plate. Wells were sealed with optical caps and the PCR run on ABI Prism 7700 using standard conditions.

PCR data was analysed using the formula $2^{\Delta\Delta C_t}$ (Ct is the cycle number at which the PCR fluorescent signal crosses a threshold; ΔCt is the difference between Ct values for the specific amplicon and 18S; ΔΔCt relates the ΔCt value of each sample to the internal control). This normalises the amount of target mRNA to the amount of 18S RNA and then relates this to the internal control, finally giving a value showing the fold difference in amount of amplicon in relation to the control.

Primers and probes for quantitative PCR were designed using the PRIMER express program (PE Biosystems). The sequences for the primers and probes used are shown in Table 7. BLAST (Basic Local Alignment Tool) searches (www.ncbi.nlm.nih.gov) were used to determine the presence of sequences in the scientific databases that are similar to those amplified by each set of primers. The results of these searches showed that, in all cases, the primer and probe sets used were unlikely to amplify an inappropriate template. The results of BLAST searches gives an expected (E) value which shows the number of hits expected by chance when searching the data base (Table 7). The lower the E value the less likely another sequence matches the sequence of interest by chance.
**Table 7**: Sequences and accession numbers of quantitative polymerase reaction (PCR) primers and probes investigated. Within-assay variation of PCR measurements for each set of primers and probes is shown. BLAST search E values for each set of primers and probes is also shown.

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe</th>
<th>Within assay precision</th>
<th>Accession Number (mRNA sequence)</th>
<th>E value from blast search</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribosomal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18S*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β actin</td>
<td>TCA CCC ACT GTG</td>
<td>GCC CAT TCA GA</td>
<td>CAT GGT GAC ACT GTG</td>
<td>CAG GGA ACG GGT</td>
<td>ATGCC CCCC CAT</td>
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</tr>
<tr>
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<td>GCC GCC AGG ATG</td>
<td>TGG GAC ACT CGA</td>
<td>GGC CAT CTC ACT</td>
<td>CAG GGG GAC CC</td>
<td>TCC AGG GCC CTT GA</td>
<td>1.60%</td>
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<tr>
<td>CII TA</td>
<td>GCT GTT GTG AC</td>
<td>TGG AGT CTC TGA</td>
<td>AGA CAT ACT GT</td>
<td>ACG CAT AC</td>
<td>ACT CTA CTA C</td>
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<tr>
<td>Cox-2</td>
<td>GTT GTG AC A TCA</td>
<td>GAG AAC GTT CCC</td>
<td>AGC TTT GT GA</td>
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<td>ACT TAA TAC CTT GA</td>
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<tr>
<td>Granulysin</td>
<td>CAG GGT GTG AA AG</td>
<td>GGC CAT GTG CTG</td>
<td>AAG GA CAC ATG</td>
<td>AAG GC CAA ACC</td>
<td>CAC CCT CAC C</td>
<td>2.00%</td>
</tr>
<tr>
<td>HBD1</td>
<td>TCG AGG ACT GAG</td>
<td>GGT CTC ACT TGA</td>
<td>TCT TAT TCT GCT</td>
<td>CAG CAC CCT TCA A</td>
<td>AAG TCC ACT TAC</td>
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<tr>
<td>HBD2</td>
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<td>CTG GAT CAC AT A</td>
<td>GGC CAG GTC A</td>
<td>AAG CAC GGT AA</td>
<td>CAG TCC A CT TCA A</td>
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<td>HBD3</td>
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<td>CTG GTG CAC GTG</td>
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<td>TCA TGA</td>
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<td>IκBα</td>
<td>CAT CTG T</td>
<td>TGA GGA</td>
<td>GGT A AG</td>
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<td>2E 30</td>
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<tr>
<td>Amplicon</td>
<td>Forward Primer</td>
<td>Reverse Primer</td>
<td>Probe</td>
<td>Within assay precision</td>
<td>Accession Number</td>
<td>E value from blast search</td>
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<td>IL-1α</td>
<td>TGTATGACTGACTGC</td>
<td>CCAAGATGAA</td>
<td>CAAGCATCTCGTCTGTTGC</td>
<td>2.00%</td>
<td>BC 013142</td>
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<tr>
<td>IL-8</td>
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<td>CAAAACCT</td>
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<td>4.00%</td>
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<td>MAL</td>
<td>CCCTGATGGTGGGCCTTGT</td>
<td>TTTGGGATGTTACAT</td>
<td>CAAGTCAAGAAGACTGCTGGT</td>
<td>2.29%</td>
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<tr>
<td>MCP-1</td>
<td>GTCG TGATCTCTCAA</td>
<td>CCACCTTCTGTTACAT</td>
<td>CAAGGAGATCTGCTGCTG</td>
<td>11.40%</td>
<td>S 71513</td>
<td>3E−05</td>
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<tr>
<td>MyD88</td>
<td>TTGGGCTGCTTTTCATTCC</td>
<td>CTGCTGCTGCTGCTGCTG</td>
<td>CACCTTCTGTTACAT</td>
<td>1.91%</td>
<td>NM 002468</td>
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<tr>
<td>PGDH</td>
<td>GCGGACATATTACATATAGTCA</td>
<td>CGCTGCTGCTGCTGCTGCTG</td>
<td>CACCTTCTGTTACAT</td>
<td>1.40%</td>
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<td>PGES</td>
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<td>ATGAGTCGCTGCTGCTGCTG</td>
<td>CAAGGAGATCTGCTGCTG</td>
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<td>NM 000660.1</td>
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<td>XM 057452</td>
<td>3E−04</td>
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<tr>
<td>TLR9</td>
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<td>CCAAGGATCTGCTGCTGCTGCTG</td>
<td>CGCTGCTGCTGCTGCTGCTG</td>
<td>5.70%</td>
<td>AF 245704</td>
<td>2E−07</td>
</tr>
</tbody>
</table>

* Sequences for the VIC labelled 18S probe are unavailable. Within assay precision for 18S cannot be provided as it is not possible to correct this data relative to the amount of cDNA present.
Linearity of the response was validated using a serial dilution of a standard pool of RNA. The log of total RNA in ng was plotted against ΔCt value and a regression line obtained. Slope of the regression line was <0.1. Representative validation plots are shown in Figure 10. Within assay variation of the PCR measurement of specific amplicon in cDNA was calculated from 6 replicates (relative standard deviation) (Table 7).
Figure 10: Validation of (a) Toll-like receptor 9 (TLR9) and (b) Granulysin PCR primers and probe. Validation was performed for each set of primers and probe used in this thesis. This figure shows two representative graphs. ΔCt is the difference between the cycle number at which fluorescence reaches a threshold value for the probe of interest and 18S. To validate the gradient of the line of best fit is <0.1.
2.5 Immunohistochemistry

Immunohistochemistry was used to detect SLPI and cytokeratin proteins in formalin fixed paraffin embedded tissue biopsies (5μm). The protocols were optimised to determine appropriate conditions for maximum immunostaining. Immunohistochemistry for HBD2 was undertaken on both paraffin fixed tissue biopsies (5μm) and frozen tissue biopsies (5μm) using two different commercially available antibodies. Staining obtained was not satisfactory and therefore HBD2 protein analysis was not carried out. No antibodies are available commercially for granulysin, so once again protein analysis was not carried out. Immunostaining was used to determine the presence of *C. trachomatis* LPS in HeLa cells.

2.5.1 SLPI

SLPI immunohistochemistry was performed on cultured primary epithelial cells as well as on fixed tissue sections. This method has been previously described (King et al. 2002). Matrigel was removed from primary epithelial cells and they were fixed in NBF for 10 minutes at room temperature and subsequently permeabilised with Triton X100 (0.05%). Tissue sections were dewaxed in histoclear and rehydrated in descending grades of ethanol to distilled water. The sections were then washed twice in 0.01M PBS for 5 minutes. Non-specific endogenous peroxidase activity was blocked in the sections and cells by treatment with 3% hydrogen peroxide in distilled water for 10 minutes at room temperature. Sections/cells were again washed. A non-immune block of diluted horse serum for 20 minutes in a humidified chamber at room temperature was used to reduce non-specific binding. This was then carefully removed and the primary antibody, SLPI IgG1 (diluted 1:200 in horse serum), was applied. Slides/cells were incubated overnight at 4°C. After the primary antibody incubation, sections/cells were washed for 10 minutes between each stage with PBS + Tween 20. Antibody binding was detected by the sequential application of biotinylated horse anti-mouse IgG and an avidin-biotin-peroxidase complex (Elite ABC), both for 60 minutes at room temperature. The peroxidase substrate diaminobenzidine (DAB), which forms a brown precipitate on
contact with the antigen-antibody complex, was used to identify positive staining. Finally sections were counter stained with Harris’ hematoxylin, dehydrated in ascending grades of ethanol and mounted in Pertex. Cells were counter stained with Harris’ hematoxylin and then stored in ethanol.

Negative controls were included for the SLPI immunohistochemistry, where the primary antibody was substituted with equimolar concentrations of mouse immunoglobulin (IgG1) to exclude the possibility of non-specific binding. Human first trimester decidua acted as a positive control.

2.5.2 Cytokeratin
Cytokeratin immunolocalisation was performed to assess the purity of epithelial cell cultures and was also used to confirm the absence of infiltrating trophoblast cells in first trimester decidual biopsies. Cytokeratin is present in endometrial epithelial cells, but in decidua any additional immunoreactivity indicates the presence of trophoblast cells. Any decidual biopsies with trophoblast cells were excluded from analysis.

To assess epithelial cell purity primary epithelial cells were cultured as described above. Matrigel was then removed and the cells fixed in acetone for 10 minutes at room temperature. Following a wash in PBS, cells were permeabilised with TritonX100 (0.05%) in PBS for 20 minutes at 37°C. The protocol then followed that of SLPI (section 2.5.1). The primary antibody was anti-cytokeratin IgG1 (diluted 1:60 in horse serum) and the incubation with this primary antibody was for 1 hour at 37°C.

The protocol for paraffin sections of decidua was identical to that of SLPI (section 2.5.1), with the exception that the primary antibody was anti-cytokeratin IgG1 (diluted 1:60 in horse serum) and the incubation with this primary antibody was for 1 hour at 37°C.
2.5.3 HBD2

HBD2 immunohistochemistry was performed on both paraffin and frozen endometrial tissue sections and paraffin placental tissue sections. Two different commercially available antibodies were used. Despite the variation in protocol detailed in appendix III, we continued to get high background and non-specific staining. Therefore HBD2 protein analysis was not carried out.

2.5.4 C. trachomatis LPS

HeLa cells were grown on slides and infected with C. trachomatis or other treatments as detailed elsewhere. After 72 hours medium was removed and slides fixed in acetone for 10 minutes. After washing in PBS, primary anti-chlamydial LPS antibody (monoclonal antibody 13/4 (Graham et al. 1995), diluted 1:200 in PBS) was applied. Negative controls were incubated with isotype matched IgG. Slides were incubated for 30 minutes at room temperature. There after the slides were washed 3 x 5 minutes in PBS. Antibody binding was detected with a 30 minute incubation with FITC conjugated rabbit anti-mouse antibody (diluted 1:50 under sterile conditions in PBS). Cell nuclei were labelled for 30 seconds with propidium iodide (diluted 1:2000 in PBS). Slides were finally washed with PBS and fixed with glycerol/PBS. Use of a fluorescent microscope allowed determination of staining. Slides were kept at 4°C, with light excluded, following staining.
2.5.5 Scoring of immunohistochemistry

Location and intensity of immunostaining was measured using a semi-quantitative scoring system. Sections were scored blind by two observers (blind to the stage of the menstrual cycle and to the others results). This scoring system is a standard method used in previous studies. (Critchley et al. 1998b; Jones et al. 1997; Wang et al. 1998). A high correlation has been demonstrated between objectively measured immunoreactivity (measured by computerised image analysis) and subjective semi-quantitative scoring of immunostaining patterns (Wang et al. 1998).

0 = no immunoreactivity
1 = faint immunoreactivity
2 = moderate immunoreactivity
3 = strong immunoreactivity
2.6 Enzyme Linked immunosorbant assays (ELISA)

SLPI, PGE\textsubscript{2} and IL-8 protein concentrations in culture supernatants were determined by ELISA. This technique allows the determination of the concentration of a given substance in solution by comparing it to a standard curve created from solutions of known concentration.

SLPI and IL-8 ELISAs are two site (double antibody) sandwich ELISAs, the PGE\textsubscript{2} ELISA is a polyclonal competition displacement assay (see Figure 11 and 12).

2.6.1 SLPI ELISA

This is a two site sandwich ELISA assay. Throughout the ELISA all dilutions were made in SLPI ELISA buffer. 96 well assay plates were coated overnight at 4°C with 200\mu{l}/well of goat anti-SLPI (2\mu{g}/ml) and then blocked for 30 minutes with 200\mu{l}/well of blocking/protecting solution. Plates were washed with wash buffer and subsequently, 200\mu{l} of standard/sample were added to each well and incubated for 2 hours at room temperature on a plate shaker. Two non-specific binding wells (200\mu{l} buffer only) were included on each plate. Standards were added in duplicate and their concentration range was from 0.024-25ng/ml (recombinant SLPI). Plates were washed and then incubated for 1 hour with 200\mu{l}/well of biotinylated mouse anti-human SLPI (1:10000) as above. After further washing, 200\mu{l}/well of streptavidin peroxidase (1:4000) was added and incubation was for 20 minutes as above. Plates were washed again and then 200\mu{l}/well of ELISA substrate were added. After 10 minutes wells were quenched with 50\mu{l}/well of 2N sulphuric acid. Plates were read in a plate reader at 450nm. Intra- and interassay coefficients of variation were 12.80% and 14.76% respectively.

2.6.2 PGE\textsubscript{2} ELISA

This ELISA has been detailed previously (Denison et al. 1999a; Greystoke et al. 2000) and will be described only briefly here. On collection samples were treated 1:1 with methyloximating solution. It is a polyclonal competition displacement assay. The
standard concentration range was from 10pg/ml – 5120pg/ml. Plates were coated with purified donkey anti-rabbit serum and blocked with bovine serum albumin. Samples and standards were incubated overnight at 4°C in the presence of antisera and a biotin labelled link. After washing followed a 20 minute incubation with streptavidin peroxidase (100μl/well) at room temperature. Finally ELISA plates were washed and ELISA substrate (200μl/well) was added for 10 minutes. Subsequently, plates were quenched with 2N sulphuric acid at 50μl/well and absorption was read at 450nm.

The ELISA measures a stable oximated derivative. The specificities and cross reactivities of these antibodies have been detailed previously (Kelly and Critchley 1997; Kelly and Smith 1987). Inter-assay coefficients of variation were 15.0% and the intra-assay coefficients of variation were 7.8% for the PGE₂ ELISA.

2.6.3 IL-8 ELISA

The protocol for this ELISA has been previously described (Denison et al. 1997). This is a two site sandwich ELISA assay. Briefly, plates were coated overnight at 4°C with 100 μl/well of capture antibody (2μg/ml). After incubation, plates were washed in water and 100μl/well of blocking solution was added. After 30 min at room temperature the solution was flicked out, plates were air-dried and stored at 4°C. Standards were diluted in ELISA buffer plus tween – standard concentration range 1000pg/ml – 3.9pg/ml. 100μl/well of standard or sample was added. Plates were sealed and incubated overnight at 4°C. Plates were washed 4 times in wash buffer and then detection antibody (50ng/ml) was added at 100μl/well. A 90 minute incubation on a plate shaker, at room temperature then followed. Plates were washed a further 4 times. 100μl/well of streptavidin peroxidase (1:1000 dilution) was added and plates were incubated for 20 minutes on a plate shaker at room temperature. 4 further washes were followed by addition of 200μl/well of ELISA substrate. Plates were left for 20 minutes, quenched with 50μl/well of 2N sulphuric acid and then read on a plate reader at 450nm. Intra- and inter-assay coefficients of variation were 9.1% and 22.1% respectively.
Figure 11: Two site sandwich ELISA
Figure 12: Competition displacement ELISA for PGE2. Higher levels of PGE2 in sample result in increased displacement of the biotin labelled link and a lower concentration of coloured measurable product.
2.7 Flow cytometric analysis

Flow cytometry is a method for quantitating components or structural features of cells primarily by optical means. It makes measurements on one cell at a time, but can process thousands of cells in a few seconds. A single cell suspension of cells is passed through a laser beam. Each cell scatters some of the laser light and also emits fluorescent light excited by the laser. A flow cytometer then measures several parameters simultaneously for each cell:

- low angle forward scatter intensity, approximately proportional to the cell diameter
- 90 degree scatter intensity, approximately proportional to the granularity of the cell
- fluorescence intensities at several wavelengths – fluorescent probes can be used to report the quantities of specific components of the cells such as surface receptors

2.7.1 CD14 expression, analysed by flow cytometry

Cells infected with *C. trachomatis* were harvested with acutase. Activated U937 cells, a monocyte cell line, were used as a positive control. Cells were incubated with 25μl of a non-specific isotype control antibody (VPM 20) for 15 minutes at room temperature. Cells were washed and then labelled with a PC5-conjugated anti-CD14 antibody for 30 minutes on ice. After further washing cells were resuspended and fixed in 1% paraformaldehyde, and analysed by flow cytometry using a Becton Dickenson FACScan (Mountain View, CA).
2.8 Statistical analysis

Significant differences of PCR and ELISA results were determined by Student’s t test or analysis of variance (ANOVA; Statview 3.0). Fisher’s protected least significant difference (PLSD) was used to assign individual differences (see chapters 3-6 for details).

The immunohistochemistry data, which are discrete and non-continuous, were analysed by non-parametric Kruskal-Wallis analysis, using Dunns Multiple Comparisons Test to assign significance (Instat 2.03).

Statistical differences are indicated on graphs by the use of letters above the relevant bars. P values relating to the letters used are detailed in the figure legends. P<0.05 was taken as being significant.
Chapter 3
Natural Antimicrobial gene transcription through the Menstrual cycle and the effect of Hormonal Contraception
3.1 Introduction

The female reproductive tract is potentially exposed to a wide range of pathogens, providing a need for an effective immune response. The innate response is crucial in initial limitation of infection and is comprised of mechanical barriers, secretions and phagocytic or natural killer cells. The natural antimicrobials, which are a part of the innate immune defence system, are small cationic peptides produced by leukocytes as well as by epithelial cells at mucosal surfaces (Hancock and Diamond 2000; Huttner and Bevins 1999; Ryley 2001). This study investigates four of these molecules: Secretory leukocyte protease inhibitor (SLPI), human β defensins 1 and 2 (HBD1 and HBD2) and granulysin.

SLPI is a neutrophil elastase inhibitor, which has also been shown to have antimicrobial activity (Hiemstra et al. 1996; McNeely et al. 1995; Tomee et al. 1997). It has been detected in human endometrium and is maximal in the secretory phase (King et al. 2000). The defensins and granulysin have been shown to have anti-bacterial, anti-viral and anti-fungal activity in vitro (Krensky 2000; Lehrer et al. 1993; Stenger et al. 1998). HBD1 and HBD2 are secreted from epithelial cells at mucosal surfaces. They have been identified in endometrium, but their cyclical variations have not been described (Bals et al. 1998; Valore et al. 1998; Zhao et al. 1996). Granulysin is a protein secreted from cytolytic T lymphocytes and natural killer (NK) cells (Andersson et al. 1995b; Krensky 2000). Uterine NK cells (CD56+) are the predominant leukocyte found in the human endometrium, being phenotypically distinct from peripheral NK cells. They increase in number in the secretory phase of the menstrual cycle (King et al. 1989; Starkey et al. 1991).

Sexually transmitted infections (STI) in the female reproductive tract can have acute and chronic sequelae and include *Chlamydia trachomatis*, gonorrhoea, herpes simplex virus, human papilloma virus and human immunodeficiency virus (HIV). *C. trachomatis* is the most commonly diagnosed bacterial sexually transmitted infection in the developed
world and a leading cause of pelvic inflammatory disease (PID) (Groseclose et al. 1999; Morgan 2002). Studies suggest a link between the occurrence of chlamydial infection and the menstrual cycle. An increase in chlamydial salpingitis is seen early in the proliferative phase and significantly more cases occur within seven days of the onset of menstruation (Sweet et al. 1986), while increased detection of asymptomatic infection occurs in the late secretory phase. There are a number of possible reasons for the changes in susceptibility to infection through the menstrual cycle, such as changes in composition and consistency of cervical mucus and cervical ectopy.

Anti-chlamydial activity of cervical secretions varies cyclically, and this is modulated by hormonal contraceptives (Mahmoud et al. 1994). Women on the COCP have higher rates of chlamydial infection of the lower genital tract (Avonts et al. 1990; Bontis et al. 1994; Kinghorn and Waugh 1981), but the COCP may be protective for upper genital tract infection (Rubin et al. 1982; Washington et al. 1985; Wolner-Hanssen et al. 1990). The LNG-IUS has thus far been shown to be protective for PID (Toivonen et al. 1991), which contrasts with copper and inert intra uterine devices which have been associated with an increased incidence of infection in the first 20 days following insertion (Farley et al. 1992).

The aim of this study is to investigate natural antimicrobial expression in the human endometrium through the menstrual cycle. How this might be related to the acquisition of infection can be speculated upon. In addition the effects of two methods of hormonal contraception, the COCP and the LNG-IUS, with systemic and local hormone delivery respectively, on natural antimicrobial expression have been examined.
3.2 Methods

3.2.1 Tissue collection

Endometrium was collected from three groups of women undergoing gynaecological procedures for benign conditions either while under investigation in the outpatient setting, during a day case procedure or during hysterectomy. Group 1 had not used any method of contraception other than barrier methods in the 3 months preceding biopsy (n=25). Group 2 had been on the COCP for at least 3 months prior to biopsy (n=20) and could be at any day of the menstrual cycle including a day in the pill free week (day 1 taken as first day of menses). Group 3 had a LNG-IUS in situ for at least 3 months prior to biopsy (n=17). All women reported regular menstrual cycles (25-35 days) unless they had a LNG-IUS in situ in which case cycles were variable. Biopsies from patients in group 1 were dated as described in section 2.1. A hematoxylin and eosin section of each sample was examined for signs of infection such as acute or chronic inflammatory changes, in particular the presence of a plasma cell infiltrate. One sample from group 1, no samples from group 2 and 4 samples from group 3 were excluded on these grounds. Final numbers of samples in each group were therefore; group 1 n=24, group 2 n=20 and group 3 n=13. Five subgroups of group 1 were further defined as: (i) proliferative phase samples (day 5-13), n=7; (ii) early secretory (day 14-18), n=3; (iii) mid secretory (day 19-23), n=5; (iv) late secretory (day 24-28) n=3; (vi) menstrual phase samples (day 1-4), n=6. Figure 13a shows significant differences between serum oestradiol levels in the proliferative and menstrual phases of the menstrual cycle in women who provided endometrial samples. Progesterone levels (figure 13b) were significantly higher in the early and mid secretory phases compared to the other phases of the menstrual cycle.

Written informed consent was obtained from all patients prior to biopsy collection and ethical approval was received from Lothian Research Ethics Committee (LERC/2000/6/56, LREC/1994/6/17 and LREC/1993/6/73). See Table 8 for details of endometrial samples analysed in this chapter.
Table 8: Endometrial samples analysed in this chapter

<table>
<thead>
<tr>
<th>Cycle Stage</th>
<th>Day of Cycle</th>
<th>Number of Biopsies</th>
<th>Age of patient (years; median/range)</th>
<th>Serum oestradiol concentrations (pmol/L; median/range)</th>
<th>Serum progesterone concentrations (nmol/L; median/range)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WHOLE BIOPSIES</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proliferative</td>
<td>5-13</td>
<td>7</td>
<td>45 (26-49)</td>
<td>640 (245-1010)</td>
<td>4.3 (1.4-6.6)</td>
</tr>
<tr>
<td>Early secretory</td>
<td>14-18</td>
<td>3</td>
<td>30 (24-33)</td>
<td>293 (265-358)</td>
<td>21.2 (15.4-63.3)</td>
</tr>
<tr>
<td>Mid secretory</td>
<td>19-23</td>
<td>5</td>
<td>39 (32-43)</td>
<td>398 (120-931)</td>
<td>28.7 (19.7-71.3)</td>
</tr>
<tr>
<td>Late secretory</td>
<td>24-28</td>
<td>3</td>
<td>36 (30-36)</td>
<td>255 (140-369)</td>
<td>5.7 (3-8.4)</td>
</tr>
<tr>
<td>Menstrual</td>
<td>1-4</td>
<td>6</td>
<td>39.5 (33-43)</td>
<td>103 (57-189)</td>
<td>2 (1.6-4.4)</td>
</tr>
<tr>
<td>COCP</td>
<td>20</td>
<td></td>
<td>36.5 (26-42)</td>
<td>82 (&lt;50-1702)</td>
<td>2 (4.9-0.4)</td>
</tr>
<tr>
<td>LNG-IUS</td>
<td>13</td>
<td></td>
<td>42 (32-52)</td>
<td>Not available</td>
<td>Not available</td>
</tr>
<tr>
<td><strong>EXPLANTS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proliferative</td>
<td>5-13</td>
<td>8</td>
<td>41 (39-47)</td>
<td>505 (361-1235)</td>
<td>2.2 (1.3-8.27)</td>
</tr>
<tr>
<td>Secretory</td>
<td>14-28</td>
<td>4</td>
<td>34.5 (31-40)</td>
<td>367.5 (224-468)</td>
<td>17.6 (10.9-29.4)</td>
</tr>
</tbody>
</table>

Details the endometrial samples used when analysing the natural antimicrobials through the menstrual cycle and the effect of hormonal contraception.
3.2.2 Tissue culture

Endometrial explants of approximately 4mm³ were obtained. Proliferative endometrial samples (n=8) and secretory endometrial samples (n=4) were cultured for 24 hours on sterilised polypropylene capillary matting as described in section 2.2.1. They were cultured in the presence of oestradiol (10⁻⁸ mol/l) either alone or with progesterone (10⁻⁶ mol/l), with the anti-progestin Ru486/mifepristone (10⁻⁶ mol/l), or with progesterone (10⁻⁶ mol/l) and Ru486/mifepristone (10⁻⁶ mol/l) in combination. The treatments were removed at 24 hours and the explants immersed in Tri reagent, homogenised and stored at −70°C for subsequent RNA extraction. See Table 8 for details of endometrial explant tissue samples used in this chapter and figure 14 showing significantly higher serum progesterone levels in the women in the secretory phase of the menstrual cycle.
Figure 13: (a) Oestradiol (pmol/l) and (b) progesterone (nmol/l) serum measurements from women who provided endometrial samples analysed in this chapter. Data represents mean ± s.e.m. Paired letters indicate significance. Note the significant fall in progesterone serum levels in the late secretory and menstrual phases.

(a) a and b: P=0.02
(b) a: P=0.0002, b: P=0.004, c: P=0.0001, d: P<0.0001, e, g and h: P<0.0001,
f: P=0.0008
Figure 14: (a) Oestradiol (pmol/l) and (b) progesterone (nmol/l) serum measurements from women who provided endometrial explant samples analysed in this chapter. Data represents mean ± s.e.m. Paired letters indicate significance. a: P=0.0003
3.2.3 RNA extraction and Q-RT-PCR
Tissue samples or explants (following treatment) were immersed in Tri reagent and cDNA prepared. Amounts of HBD1, HBD2, SLPI and granulysin mRNA were determined using quantitative real time PCR. (See section 2.4 for details of method)

3.2.4 Immunohistochemistry
To localise the expression of SLPI protein in the primary endometrial samples, immunohistochemistry was performed as described in section 2.5. Three components were assessed subjectively: cellular staining intensity, luminal secretions staining intensity and the volume of luminal secretions.

3.2.5 Statistical analysis
Significant difference of PCR results obtained for whole endometrium was determined by analysis of variance (ANOVA; Statview 3.0). Fisher’s protected least significant difference (PLSD) was used to assign individual differences. The data on explant samples were analysed using unpaired student t test (Statview 3.0). There were no significant differences between levels of antimicrobial mRNA investigated in samples from women on the COCP collected during the proliferative, secretory or menstrual phases as determined by day of cycle. They were therefore treated as one group for statistical analysis. Similarly length of time since insertion of LNG-IUS showed no significant differences between levels of antimicrobial mRNA investigated and they were treated as one group for statistical analysis.

The immunohistochemistry data, which are discrete and non-continuous, were analysed by non-parametric Kruskal-Wallis analysis, using Dunns Multiple Comparisons Test to assign significance (Instat 2.03).
3.3 Results
3.3.1 Q-RT-PCR
SLPI mRNA expression increased at least 11 fold in the late secretory and menstrual phase when compared to both the proliferative and early secretory phases of the menstrual cycle. (Figure 15a). The COCP and the LNG-IUS induced at least a 7 fold increase of SLPI mRNA, over the proliferative and early secretory phases. (Table 9)

HBD1 mRNA expression was maximal in the mid secretory phase, 21 fold higher than the levels observed in the proliferative phase (P<0.0001) and at least twice the levels in the early secretory (P=0.0004) and menstrual phases (P=0.008) (Figure 15b). Mid secretory levels of HBD-1 mRNA were at least 6 fold higher than those in women on the COCP (P<0.0001) and with the LNG-IUS in situ (P<0.0001). Levels of HBD1 mRNA in woman using hormonal methods of contraception under investigation were not significantly different to the other phases of the menstrual cycle (Figure 15b). (Table 9)

At least a 40 fold increase of HBD2 mRNA was observed in the menstrual phase when compared to all other phases (P<0.001) of the menstrual cycle and compared to women using the COCP (P<0.0001) or with a LNG-IUS in situ (P=0.001) (Figure 15c).(Table 9)

Granulysin mRNA expression was maximal in the late secretory phase being 76, 1910 and 31 fold increased above the proliferative and early and mid secretory phases respectively. Granulysin mRNA expression in the late secretory phase was 5 and 8 fold higher than those levels seen in women on the COCP or with a LNG-IUS in situ respectively. Women on the COCP or with a LNG-IUS in situ had increased expression of granulysin mRNA compared to women in the proliferative phase of the menstrual cycle (not significant) (Figure 15d). (Table 9)
Figure 15: Natural antimicrobial mRNA expression within the endometrium through the menstrual cycle, and level of expression in women on the COCP or with a LNG-IUS in situ. 'n' numbers are shown below the x-axis. Data are presented as the fold changes in mRNA expression relative to a control (mixed leukocyte mRNA), given a nominal value of 1, mean ± s.e.m. Paired letters indicate statistical significance. Note the difference in y axis scale for figure 15d.

(a). SLPI expression. (b). HBD1 expression. a,b,c,d,e: P<0.008. (c). HBD2 expression. a,b,c,d,e,f: P<0.001. (d). Granulysin expression a,b,c,d,e,f: P<0.001.

Abbreviations: Prolif, proliferative phase, ES, early secretory, MS, mid secretory phase, LS, late secretory phase, Menst, menstrual phase, COCP, combined oral contraceptive pill, LNG-IUS, levonorgestrel intrauterine system.
a) Secretory Leukocyte Protease Inhibitor

b) Human β Defensin 1

c) Human β Defensin 2

d) Granulysin
Table 9: mRNA fold changes through the menstrual cycle and under the influence of the combined oral contraceptive pill or levonorgestrel intrauterine device

<table>
<thead>
<tr>
<th></th>
<th>Proliferative</th>
<th>Early Secretory</th>
<th>Mid Secretory</th>
<th>Late Secretory</th>
<th>Menstrual</th>
<th>COCP</th>
<th>LNG-IUS</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLPI</td>
<td>1.0</td>
<td>0.05</td>
<td>4.7</td>
<td><strong>12.6</strong></td>
<td><strong>11.0</strong></td>
<td>8.1</td>
<td>7.0</td>
</tr>
<tr>
<td>HBD1</td>
<td>0.3</td>
<td>0.02</td>
<td><strong>6.9</strong></td>
<td>3.3</td>
<td>2.8</td>
<td>1.1</td>
<td>0.4</td>
</tr>
<tr>
<td>HBD2</td>
<td>0.4</td>
<td>0.005</td>
<td>0.2</td>
<td>0.02</td>
<td><strong>15.32</strong></td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Granulysin</td>
<td>2.0</td>
<td>0.08</td>
<td>5.3</td>
<td><strong>153.1</strong></td>
<td>14.4</td>
<td>31.9</td>
<td>18.4</td>
</tr>
</tbody>
</table>

Values are presented as the mean fold change of the mRNA related to an internal control with a value of 1. Values in bold represent the phase of the menstrual cycle during which each antimicrobial is maximally expressed.

COCP, combined oral contraceptive pill; LNG-IUS, levonorgestrel intrauterine device
3.3.2 Immunohistochemistry SLPI

Expression of SLPI was limited to the glandular epithelium and secretions. No staining was observed in the stromal compartment (Figure 17a). There was significantly more staining of luminal secretions in the secretory phase when compared to the proliferative phase or from women on the COCP (P<0.05) (Figure 16b). No significant differences were observed in the glandular epithelial staining in the proliferative and secretory phases of the menstrual cycle and from women on the COCP, although the trends were the same as those observed in the staining of luminal secretions (Figure 16a). Samples from women on the COCP or from the proliferative phase of the cycle had significantly less secretions within the glands when compared to those in the secretory phase (P<0.05) (Figure 16c, Figure 17).
Figure 16: Box and whisker plots showing relative amounts of (a) SLPI immunostaining in the glandular epithelial compartment, (b) SLPI immunostaining in the glandular, luminal secretions or (c) secretions within the glands of the endometrium, of women in the proliferative or secretory phase of the menstrual cycle, or women on the COCP, as determined by scoring of immunohistochemistry slides. n=5 in each group.

Paired letters indicate statistical significance. The box extends from the 25th percentile (bottom line) to the 75th percentile (top line). The horizontal line represents the median, which sometimes falls at the 25th or 75th percentile. Whiskers are drawn to the 10th or 90th centiles.

(a) Significantly more SLPI was observed in the luminal secretions of women in the secretory phase of the menstrual cycle. a,b: P<0.05. (c) Significantly more secretions were observed in women in the secretory phase of the menstrual cycle a,b: P<0.05.
Figure 17: Photomicrographs of Secretory Leukocyte Protease Inhibitor (SLPI) immunostaining in human endometrium. (a) and (b) Mid secretory endometrium. Immunoreactivity is present in the epithelial cells and secretions of the glands. (c) and (d) Negative control (primary antibody replaced with mouse immunoglobulin at equimolar concentrations). (e) Endometrium of a woman on the COCP, with secretions within the glands, and strong immunoreactivity for SLPI. (f) Inactive endometrium of a woman on the COCP, with few secretions within the glands, and little immunoreactivity for SLPI.

Scale bar = 100μm
3.3.3 Endometrial explants

Progesterone treatment (10^6 mol/l) of proliferative endometrial explants for 24 hours significantly increased the expression of SLPI mRNA (P=0.04) while significantly decreasing the expression of HBD1 and 2 mRNA (P<0.01). No significant differences were seen in the expression of granulysin mRNA. (Figure 4). Anti-progestin treatment (RU 486/mifepristone) of secretory endometrial explants for 24 hours decreased granulysin mRNA expression (P=0.03) while having no significant effects on the expression of SLPI, HBD1 or HBD2 when compared to samples treated with progesterone alone (Figure 18).
Figure 18: Regulation of antimicrobial mRNA expression by progesterone and progesterone withdrawal in endometrial explants. Data are presented as the fold changes in mRNA expression relative to the control, mean ± s.e.m. Paired letters indicate statistical significance.

Proliferative endometrial explants (n=8) were treated with progesterone (10⁻⁶mol/l) and compared to untreated controls. a,b,c: P<0.04. Secretory explants (n=4) were treated with progesterone (10⁻⁶mol/l) and the anti-progestin RU486/mifepristone (10⁻⁶mol/l) in combination and compared to progesterone (10⁻⁶mol/l) treated control. d: P=0.03.
3.4 Discussion

The presence of natural antimicrobial mRNA in the human endometrium has been demonstrated. This study also shows variation through the menstrual cycle of HBD1, HBD2 and granulysin, which has not been previously demonstrated. All the antimicrobials investigated showed an increase in mRNA expression in the mid secretory, late secretory or menstrual phase when compared to the proliferative or early secretory phase. Furthermore, as well as being regulated by endogenous hormones they are also modulated by exogenous delivery of sex steroid hormones.

The patterns of mRNA expression through the menstrual cycle are similar, all being maximal in the latter half of the cycle. It has been shown that the antimicrobials can have additive and synergistic effects (Singh et al. 2000) when present in combination as is observed here. The menstrual and early proliferative phases of the menstrual cycle have been linked to an increased susceptibility to sexually transmitted infections (STIs) (Crowley et al. 1997; Sweet et al. 1986). Menstruation is a time when the endometrial surface is exposed due to the disruption of the epithelial barrier hence, there is a potential enhanced risk of infection. Similar observations have been made in the eye where HBD2 is up-regulated during re-epithelialization of the cornea after injury, in keeping with the defensins acting as antimicrobial peptides (McDermott et al. 2001). It may be that the increase in antimicrobials during menstruation is an attempt to limit infection, and without this observed increase, infection rates would be even higher. Additionally, the secretory phase of the menstrual cycle is a time when the maternal immune system undergoes changes and implantation of an allogeneic fetus occurs.

Previous work on the defensins has described HBD1 as being constitutively expressed (Krisanaprapornkit et al. 1998; O'Neil et al. 2000). In the endometrium HBD1 is maximal at a time of high circulating progesterone concentrations. In vitro studies did not support a direct effect of progesterone, where progesterone in fact decreased levels of HBD1 mRNA. It may be that by affecting endometrial morphology and secretions,
progesterone induces the changes in gene expression indirectly (O'Neil et al. 1999; Zhao et al. 1996). HBD2 has been shown to be inducible by inflammatory mediators such as IL-1 and TNFα and mimics of infection such as lipopolysaccharide (Diamond et al. 2000; King et al. 2002; Krisanaprakornkit et al. 2000; Russell et al. 1996). We show that HBD2 is maximal in the menstrual phase and it is likely that the inflammatory mediators, which are present at this time such as IL-1 and TNFα, will be influencing HBD2 expression (Hunt et al. 1992; Simon et al. 1993a; Tabibzadeh and Sun 1992). This is probably an indirect effect of progesterone, with progesterone withdrawal resulting in up-regulation of the inflammatory mediators (Critchley et al. 1999). Granulysin is a protein produced by NK cells, which proliferate within the endometrium or infiltrate the endometrium and differentiate there during the mid/late secretory phase of the menstrual cycle (King et al. 1989) presumably under the influence of progesterone (albeit probably indirectly). Increased numbers and differentiation of NK cells may result in the rise in granulysin mRNA levels, enhancing the innate immune capacity of the endometrium.

In vitro, progesterone inhibits HBD2 mRNA expression and increases SLPI mRNA expression as is seen in the menstrual cycle. However, progesterone down-regulated HBD1 expression in vitro. This may be due to time of exposure to progesterone not mimicking the in vivo cyclical nature of exposure. Furthermore, as suggested previously, in vivo progesterone may be acting indirectly to increase HBD1 levels. Treatment of proliferative phase explants of endometrium with progesterone did not mimic the in vivo increase in granulysin, because of a lack of NK cells within the endometrium during the proliferative phase. In secretory phase explants granulysin expression was decreased by anti-progestin (RU486/mifepristone) whereas the opposite is observed with progesterone withdrawal in the late secretory phase in vivo (see Figure 15d). Once again this may be due to time of exposure to progesterone not mimicking the in vivo cyclical nature of exposure or due to the absence or presence of other undefined factors in vivo. Addition of an anti-progestin (RU486/mifepristone) negated the effects of progesterone alone on HBD1 and HBD2 mRNA expression in secretory phase explants.
The endometrium of women on the COCP shows a histological pattern with poorly developed glands and stroma, which with prolonged COCP use becomes inactive (Dallenbach-Hellweg 1981; Deligdisch 2000). The effect of the COCP on HBD1, HBD2 and granulysin mRNA expression is to suppress the increase in mRNA levels seen in mid secretory, late secretory and menstrual phases. The lower levels of gene transcription of the defensins and granulysin may be secondary to the reduced amounts of cervical mucus observed in the endometrium of women on the COCP. Studies have shown that women on the COCP have higher rates of chlamydial and HIV infection (Carlin and Boag 1995; Kinghorn and Waugh 1981) and it may be that the loss of the increased levels of antimicrobials pre and perimenstrually contribute to this susceptibility to infection. The incidence of PID has been shown to be lower in women on the COCP (Rubin et al. 1982; Washington et al. 1985; Wolner-Hanssen et al. 1990) and mechanical factors such as thicker secretions and decreased menstrual flow in these women may result in decreased ascension of infection into the upper genital tract. The data regarding whether the effects of contraceptive steroids on infection acquisition are direct or indirect is conflicting. With regards to Chlamydia, Kleinman et al (Kleinman et al. 1987) present data showing that ethinyl oestradiol, mestranol and medroxyprogesterone acetate (6α methyl 17α hydroxyprogesterone acetate) do not affect chlamydial replication in cultured human endometrium, thus implying that the effects of the contraceptive steroids in chlamydial infections are indirect. In contrast, others have shown chlamydial inclusions in epithelial cultures are decreased in the presence of progesterone or progesterone in combination with an oestrogen (Moorman et al. 1986; Wyrick et al. 1989) while oestrogen alone increases attachment of C. trachomatis to epithelial cells in vitro (Maslow et al. 1988). Work on other STIs has similarly been inconclusive thus far.

Immunohistochemistry results concur with the cyclical data showing increased levels of SLPI in the secretions of the secretory phase of the cycle. However, these levels were not maintained in the COCP samples, and this may be due to the fact that these samples
have significantly fewer secretions, probably due to their inactive glandular morphology. In one sample where glands containing secretions and an arrested histological growth was seen, the secretions stained strongly for SLPI (Figure 17). Commercially available HBD2 polyclonal antibodies lacked sensitivity in a western blot of human endometrium and immunohistochemistry was also unsuccessful (see appendix III). Using a genomics approach 28 additional new β defensin genes have been identified with close homology (Schutte et al. 2002). Preliminary analysis suggests that 26 of these are transcribed, potentially making sensitive/specific antibody production difficult. Lack of commercially available antibodies to granulysin meant that protein data were not obtained.

The effects of locally delivered high dose progestogen (LNG-IUS) are similar to that of the COCP. mRNA levels of the antimicrobials, with the exception of SLPI, are suppressed by the LNG-IUS. A local high dose of levonorgestrel will cause extensive endometrial gland atrophy, similar to the effects observed after long term COCP use. Other mechanisms, such as the uterine NK cells or thicker cervical secretions would explain reported protective effects of the LNG-IUS.

Thus, it has been shown here that natural antimicrobial gene transcription varies through the menstrual cycle and can be suppressed by hormonal contraceptives. Currently, a priority in the development of new contraceptives is that they should have an anti-infective component and in this context the expression of natural antimicrobials needs to be assessed. New strategies should be sought to up-regulate the innate defence mechanisms of the female reproductive tract.
Chapter 4
Natural antimicrobials in early pregnancy and the effect of *Chlamydia trachomatis* infection
4.1 Introduction

Secretory leukocyte protease inhibitor (SLPI), the defensins and granulysin all have natural antimicrobial activity in vitro (Hiemstra et al. 1996; Krensky 2000; Lehrer et al. 1993; McNeely et al. 1995; Stenger et al. 1998; Tomee et al. 1997). It has therefore been suggested that these molecules play a role in the defence against infection within the human body, and particularly at mucosal surfaces (Franken et al. 1989). SLPI, HBD1 and HBD2 have been identified in the human endometrium (Bals et al. 1998; King et al. 2000; Valore et al. 1998; Zhao et al. 1996). The cyclical activity through the menstrual cycle has been described for SLPI (King et al. 2000) and within this thesis for HBD1, HBD2 and granulysin (Chapter 3). SLPI expression during pregnancy has been well characterised, levels of SLPI mRNA and protein are higher in decidual tissue than in secretory phase endometrium (King et al. 2000). SLPI levels increase through pregnancy and at term SLPI is found in large decidualised stromal cells, amnion and amniotic fluid (Denison et al. 1999c; Zhang et al. 2001). mRNA for α (neutrophil) defensins has been demonstrated in the placenta, amnion and chorion at term (Svinarich et al. 1997a). The β defensins have not been characterised in pregnancy. Granulysin has been identified in the γδ cytotoxic T cells in decidual samples at 8-14 weeks gestation (Mincheva-Nilsson et al. 2000).

The role of infection in pregnancy is controversial. There is a large body of evidence suggesting that preterm rupture of fetal membranes and preterm delivery (<37 weeks gestation) are associated with clinical or subclinical infection (Cram et al. 2002; Gibbs 2001; Yost and Cox 2000). It is hypothesised that infection leads to release of inflammatory cytokines such as IL-1 and IFNγ and collagenases such as the matrix metalloproteinases (MMPs). The micro-organisms may degrade fetal membranes directly, via the MMPs or by induction of leukocyte invasion. Cytokines in combination with the initial infection result in increased levels of prostaglandins, which can then initiate labour (Gibb 1998). Bacterial vaginosis, Ureaplasma urealyticum, Chlamydia trachomatis and Neisseria gonorrhoeae have been more clearly associated with preterm
labour (Cram et al. 2002; Gravett et al. 1986: McGregor, 1991 #521; Purwar et al. 2001), while the roles of other infections such as group B streptococci, *Escherichia coli*, and *Mycoplasma hominis* are less certain. The use of antibiotic treatment has therefore been suggested to prevent these adverse outcomes. Randomised controlled trials, ORACLE I and II, have been carried out (Kenyon et al. 2001b; Kenyon et al. 2001c) and show that following preterm, prelabour rupture of fetal membranes erythromycin administration is associated with a range of health benefits for the neonate. A Cochrane review also suggests a significant reduction in maternal morbidity if antibiotics are administered following preterm, prelabour rupture of fetal membranes (Kenyon et al. 2001a). No significant improvement in neonatal outcome was found when antibiotics were given to women in spontaneous preterm labour in the absence of clinical infection. The role of infection in adverse pregnancy outcome thus remains uncertain, perhaps playing a greater role in preterm, prelabour rupture of fetal membranes than in spontaneous preterm labour.

It is proposed that a relative deficiency in gene-encoded natural antimicrobial expression might be a predisposing factor in premature rupture of fetal membranes or premature labour. This could combine with other factors such as a shortened cervix or the prominence of a particular pathogenic organism in the vagina. The aim of this study was to look at the expression of natural antimicrobials in early pregnancy and to assess the effect of asymptomatic infection with *C. trachomatis* on the mRNA expression of these molecules. The oral cavity provides a mucosal surface that might mimic the immune character of other mucosal surfaces such as the mucosa within the female reproductive tract. Preliminary work was undertaken to investigate the possibility of using salivary measurements of natural antimicrobials as non-invasive surrogate markers of natural antimicrobial secretion in the genital tract.
4.2 Methods

4.2.1 Sample Collection

Endometrium was collected from women undergoing gynaecological procedures for benign conditions either during a day case procedure or during hysterectomy, n=24 (as described in sections 2.1 and 3.2.1).

First trimester decidua (8-11 weeks gestation, as determined by last menstrual period and/or ultrasound scan) was obtained from patients undergoing surgical termination of pregnancy, n=19 (as described in section 2.1). On some occasions trophoblastic villi were also collected during the procedure, n=5. Prior to the termination chlamydia status of the patient had been routinely assessed and 11 patients were found to be chlamydia uninfected and 8 patients chlamydia infected. All the trophoblast samples were from chlamydia uninfected patients. Patients who were chlamydia positive were treated with antibiotics after the decidual sample had been taken and the termination procedure completed. All samples were examined histologically to exclude or confirm the presence of trophoblast. No samples had clear evidence of an inflammatory response with a plasma cell infiltrate.

Saliva and vaginal secretions were collected from patients undergoing first trimester surgical termination of pregnancy (8-11 weeks gestation), n=10 (as described in section 2.1). None of these patients were infected with C. trachomatis. Prior to the termination women were asked to collect saliva using a citric acid salivette. Immediately prior to the termination procedure, the vagina was lavaged with 5ml of phosphate buffered saline (PBS) using a 50ml syringe.

Written informed consent was obtained from all patients prior to biopsy collection and ethical approval was received from Lothian Research Ethics Committee (LREC/2000/6/56 and LREC/1993/6/73). See Tables 10 and 11 for details of endometrial, decidual, trophoblast, saliva and vaginal samples analysed in this chapter.
Table 10: Endometrial samples analysed in this chapter (as chapter 3)

<table>
<thead>
<tr>
<th>Cycle Stage</th>
<th>Day of Cycle</th>
<th>Number of Biopsies</th>
<th>Age of Patient (years; median/range)</th>
<th>Serum oestradiol concentrations (pmol/L; median/range)</th>
<th>Serum progesterone concentrations (nmol/L; median/range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferative</td>
<td>5-13</td>
<td>7</td>
<td>45 (26-49)</td>
<td>640 (245-1010)</td>
<td>4.3 (1.4-6.6)</td>
</tr>
<tr>
<td>Early secretory</td>
<td>14-18</td>
<td>3</td>
<td>30 (24-33)</td>
<td>293 (265-358)</td>
<td>21.2 (15.4-63.3)</td>
</tr>
<tr>
<td>Mid secretory</td>
<td>19-23</td>
<td>5</td>
<td>39 (32-43)</td>
<td>398 (120-931)</td>
<td>28.7 (19.7-71.3)</td>
</tr>
<tr>
<td>Late secretory</td>
<td>24-28</td>
<td>3</td>
<td>36 (30-36)</td>
<td>255 (140-369)</td>
<td>5.7 (3-8.4)</td>
</tr>
<tr>
<td>Menstrual</td>
<td>1-4</td>
<td>6</td>
<td>39.5 (33-43)</td>
<td>103 (57-189)</td>
<td>2 (1.6-4.4)</td>
</tr>
</tbody>
</table>

Table 11: Early pregnancy samples analysed in this chapter

<table>
<thead>
<tr>
<th>Tissue sample</th>
<th>Chlamydia status</th>
<th>Misoprostol use (Yes/No)</th>
<th>Number of samples (week+days; median/range)</th>
<th>Gestation* (weeks+days; patient age (years; median/range))</th>
<th>Parous/Nulliparous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decidua</td>
<td>Infected</td>
<td>Yes</td>
<td>8</td>
<td>10+1.5 (7-11+6)</td>
<td>19 (16-29)</td>
</tr>
<tr>
<td>Decidua</td>
<td>Uninfected</td>
<td>Yes</td>
<td>5</td>
<td>9+3 (9-11)</td>
<td>21 (17-24)</td>
</tr>
<tr>
<td>Decidua</td>
<td>Uninfected</td>
<td>No</td>
<td>6</td>
<td>8+4.5 (7+2-9+4)</td>
<td>34 (31-39)</td>
</tr>
<tr>
<td>Trophoblast</td>
<td>Uninfected</td>
<td>For the nulliparous women</td>
<td>5</td>
<td>8 (8-11)</td>
<td>27 (25-36)</td>
</tr>
<tr>
<td>Saliva, vaginal secretions</td>
<td>Uninfected</td>
<td>No</td>
<td>10</td>
<td>9+.55 (7-11+6)</td>
<td>28 (26-45)</td>
</tr>
</tbody>
</table>

*Gestation determined by last menstrual period and/or ultrasound
4.2.2 RNA extraction and Q-RT-PCR
Tissue samples were immersed in Tri reagent and cDNA prepared. Amounts of HBD1, HBD2, SLPI and granulysin mRNA were determined using quantitative real time PCR. (See section 2.4 for details of method)

4.2.3 SLPI ELISA
SLPI protein concentrations in saliva and vaginal samples were determined by ELISA, using a commercially available kit (R & D systems).

3.2.5 Statistical analysis
Significant difference of PCR results obtained for whole endometrium was determined by analysis of variance (ANOVA; Statview 3.0). Fisher’s protected least significant difference (PLSD) was used to assign individual differences.

The data on samples from chlamydia uninfected and chlamydia infected patients was analysed using unpaired student t-test (Statview 3.0). Use of misoprostol in the chlamydia uninfected group did not statistically alter the expression of the natural antimicrobials, so the women not infected with chlamydia were analysed as one group.

SLPI protein levels in saliva and vaginal samples were analysed for a correlation between the two (Statview 3.0).
4.3 Results

4.3.1 Natural antimicrobial mRNA levels in early pregnancy as compared to levels through the normal menstrual cycle.

SLPI levels are highest in the late secretory and menstrual phases of the menstrual cycle as determined previously (section 3.3.1). mRNA levels of SLPI in decidual tissue are approximately half the levels seen in the late secretory and menstrual phases (p=0.047 and p=0.05 respectively). See Figure 19a.

Decidual levels of HBD1 mRNA were approximately 4.6 fold lower than mRNA levels in the mid secretory phase (when levels of mRNA HBD1 are at their highest), p=0.002. See Figure 19b.

Granulysin mRNA levels were 21 fold higher in the late secretory phase when compared to mRNA levels detected in the decidual tissue (p=<0.0001). See Figure 19d.

HBD2 mRNA was undetectable in decidua. See Figure 19c.

mRNA levels of all the antimicrobials investigated, SLPI, HBD1, HBD2 and granulysin, were undetectable in trophoblast tissue. See Figure 19.
Figure 19: Natural antimicrobial mRNA expression in decidual and trophoblast tissue compared to mRNA levels within the endometrium through the menstrual cycle. Data represents mean ± s.e.m for decidual and trophoblast samples. Data through the menstrual cycle is as that presented in chapter 3 and only the mean of the data is presented in these graphs. All values are compared to an internal control (mixed leukocyte mRNA) given a nominal value of one. Paired letters indicate statistical significance. Note differences in y axis scales.

(a). SLPI mRNA expression a,b,c: p<0.05.
(b). HBD1 expression. a,b: p<0.002.
(c). HBD2 expression. a,b: P<0.003.
(d). Granulysin expression a,b: P<0.0001.

Decidua n=11, trophoblast n=5, with the exception of SLPI where n=4, proliferative phase n=7, early secretory phase n=3, mid secretory phase n=5, late secretory phase n=3, menstrual phase n=6.
SLPI mRNA expression in early pregnancy
- SLPI mRNA expression through the menstrual cycle

HBD1 mRNA expression in early pregnancy
- HBD1 mRNA expression through the menstrual cycle

HBD2 mRNA expression in early pregnancy
- HBD2 mRNA expression through the menstrual cycle

Granulysin mRNA expression in early pregnancy
- Granulysin mRNA expression through the menstrual cycle
4.3.2 Natural antimicrobial levels in decidual samples from women with known chlamydial infection.
The effect of chlamydial infection on natural antimicrobial mRNA expression in first trimester pregnancy was determined. No significant changes were observed between decidual samples from infected and uninfected women. See Figure 20.

**Figure 20:** Natural antimicrobial mRNA expression in decidual tissue from women infected with *Chlamydia trachomatis* (n=8) and woman without *C. trachomatis* infection (n=11). Data represents mean ± s.e.m.
4.3.3 Correlation between SLPI protein levels in saliva samples when compared to paired vaginal fluid samples.

Samples from women with acute infections of the genital tract are difficult to obtain due to pain and the risk of ascending infection. A pilot study was therefore undertaken to determine whether levels of the antimicrobial SLPI in saliva could be correlated to levels in vaginal fluid. A correlation would allow saliva samples to be collected instead of vaginal samples.

The median value of SLPI in saliva was 801.5ng/ml (range 377 – 2515ng/ml). Median value of SLPI in vaginal fluids was 211.2ng/ml (range 5.4 – 5266.1ng/ml).

No significant correlation was found between levels of SLPI in the saliva or in the vaginal fluids (correlation coefficient 0.76). See Figure 21.
Figure 21. Correlation graph of vaginal fluid and salivary levels of SLPI (ng/ml). Correlation coefficient 0.76.
4.4 Discussion

This study confirms the presence of the natural antimicrobials SLPI, HBD1 and granulysin in first trimester decidua, where they may play an anti-infective role. Preterm labour represents only approximately 10% of all labours, but results in more than 70% of neonatal morbidity and mortality (Yost and Cox 2000). There is evidence that infection plays a part in the aetiology of these events although the exact role that it plays is debated.

In this study levels of mRNA for SLPI in decidua are approximately half the levels seen in the late secretory and menstrual phases. In contrast, levels of SLPI have been reported as being higher in decidual tissue than those levels observed in secretory phase endometrium (King et al. 2000). However, the secretory phase had been examined as a whole and the different stages of the secretory phase were not examined as has been done in the present study. A role for progesterone in the control of SLPI expression either directly or indirectly via changes in endometrial morphology and cytokine expression, has been suggested by King et al (King et al. 2000). At a time of high circulating progesterone concentrations the levels of SLPI expression are lower than those seen as progesterone concentrations decrease in the late secretory and menstrual phases. SLPI expression is decreased in three further circumstances, supporting a role for progesterone in the control of SLPI expression:

a) when there are high circulating levels of progesterone in early pregnancy
b) under the influence of high progesterone levels locally (levonorgestrel intrauterine device (LNG-IUS) in situ) (see section 3.3.1)
c) in vitro when proliferative phase endometrial explants were treated with progesterone for 24 hours (see section 3.3.3)

In these circumstances of chronic exposure to progesterone, progesterone receptors will be down regulated (Critchley et al. 1998b; Perrot-Applanat et al. 1994) and this may be important in the expression patterns of the antimicrobials observed.
HBD1 expression is highest in the mid secretory phase of the menstrual cycle suggesting a positive effect of progesterone. However, when high levels of circulating progesterone are present in early pregnancy, HBD1 mRNA expression in the decidua is not as high as levels seen in the mid secretory phase. Data presented in section 3.3.1 similarly show that when high levels of progesterone are present locally in the form of the LNG-IUS the expression of HBD1 mRNA is low. It may be that this is a consequence of the prolonged exposure to progesterone rather than the cyclical changes observed through the menstrual cycle. HBD2 mRNA was not detected in the decidua. Levels of HBD2 can be up-regulated by inflammatory mediators such as IL-1 and TNFα and mimics of infection such as lipopolysaccharide (Diamond et al. 2000; King et al. 2002; Krisanaprakornkit et al. 2000; Russell et al. 1996). Work is ongoing to determine whether the same is true of expression of HBD2 in pregnancy derived tissue. Granulysin mRNA is expressed in low levels in decidua, correlating with similar levels that are observed under the influence of the LNG-IUS. It is probable that the source of granulysin is the immune cells present at this time such as uterine NK cells and γδ cytotoxic T cells (Mincheva-Nilsson et al. 2000). The mRNA levels of granulysin observed in early pregnancy were far lower than those seen in the late secretory phase of the menstrual cycle, when uNK cells are abundant. It may be that in pregnancy the uNK cells have completed differentiation and the turn over of mRNA is no longer as high. However, the mRNA expression of granulysin might increase if the tissue becomes acutely infected. Trophoblastic tissue did not express any of the natural antimicrobials examined suggesting that at this stage in pregnancy the fetally derived trophoblast does not have a role in this aspect of innate immune defence.

There is evidence that the antimicrobials under discussion play a role in control of genital tract infection. The production of cysteine proteases by *Trichomonas vaginalis*, which can degrade recombinant SLPI suggests that the presence of compounds such as SLPI may be detrimental to this microbe (Draper et al. 1998). Vaginal levels of SLPI are decreased in women with sexually transmitted infections (STI) (Draper et al. 2000). Furthermore, high levels of SLPI in the vagina have been associated with lower rates of
perinatal HIV transmission (Pillay et al. 2001). Draper et al. (Draper et al. 1998) suggest that the lower levels of SLPI in women with STI may predispose those women to HIV infection. Within the vagina a complex mixture of organisms co-exist, with Lactobacillus playing a prominent role maintaining a low vaginal pH. If the balance of microbes is disturbed, bacterial vaginosis can occur with a concomitant increase in vaginal pH. This increase in pH might reduce the natural antimicrobial secretions, predisposing to acquisition of infection. In vitro Yasin et al have demonstrated that neutrophil defensins can protect McCoy cells from C. trachomatis infection (Yasin et al. 1996). An increase in neutrophil defensins in the amniotic fluid of women with intrauterine infection in labour has been observed (Heine et al. 1998). The levels of defensins in the amniotic fluid correlated with increasing severity of histologic chorioamnionitis.

This study examined the effect of infection with C. trachomatis (detected prior to first trimester termination of pregnancy) on the mRNA expression of SLPI, HBD, HBD2 and granulysin and no significant differences were observed. The natural antimicrobials are part of the innate immune response and the women we obtained samples from will most likely have had the infection for a prolonged period of time. Any initial response to the organism will no longer be detectable. It was known that all these women had chlamydia infection detected by an endocervical swab, but whether there was infection in the sample of decidua analysed is debatable. No obvious histological evidence of infection was present. A further study looking at women acutely infected with C. trachomatis may provide further information.

There are limitations to carrying out a study of uterine tissue in women with acute sexually transmitted infections, hence this study was limited to asymptomatic infection. During an acute infection women may find examination very uncomfortable and not tolerate an endometrial sample being taken. Often these women are nulliparous and even if the procedure can be attempted, obtaining the sample can be technically difficult. A preliminary study was therefore undertaken to examine the feasibility of using a saliva
sample as a non-invasive surrogate marker of natural antimicrobial secretion in the genital tract. Associations between oral and genital tract infections and possible adverse outcomes in pregnancy have been postulated. Hill et al suggest an association between maternal periodontal disease with *Fusobacterium nucleatum*, a common oral species, and preterm labour. *Fusobacterium nucleatum* is a frequently isolated species from amniotic fluid cultures among women with preterm labour and intact membranes (Hill 1998). In the present study SLPI protein levels in paired saliva and vaginal fluid samples were examined. As yet commercial ELISAs for β defensins and granulysin are unavailable. Median levels of SLPI in saliva samples were comparable to levels described in other studies (Wahl et al. 1997). SLPI levels in vaginal fluids were slightly higher than those described in other studies. Two groups describe levels of SLPI in vaginal fluids of approximately 60ng/ml, decreasing in women with STIs (Draper et al. 2000) and increasing to levels of approximately 550ng/ml in women with HIV (Pillay et al. 2001). The present study excluded chlamydial infection, but did not look for the presence of other infections that might affect SLPI levels. A paired analysis was carried out and each woman was therefore acting as her own control, with the role of infection not being assessed. This study showed no clear correlation between levels of SLPI in the saliva and in vaginal fluids. Thus these data show that saliva is not a good surrogate marker of SLPI secretion in the vagina. It may be that in the presence of infection greater variability of results would be observed and a correlation between salivary and vaginal fluid levels might become apparent. It would be of interest to determine whether either saliva or vaginal measurements of SLPI were a good marker for adverse events in pregnancy, such as premature rupture of membranes or preterm labour.

This study has determined the presence of natural antimicrobial peptides in early pregnancy and further work will need to be undertaken to define in more detail the role of these molecules. Measurements of natural antimicrobials may still be shown to be useful in tracking the risk of adverse outcomes in pregnancy.
Chapter 5
Mediators of natural antimicrobial and Toll-like receptor gene transcription in the endometrium
5.1 Introduction

Infection of the female genital tract can have both short and long term sequelae. Rapid detection of pathogens and induction of an effective immune response are crucial to limiting these sequelae. Infection in the non-pregnant state can result in pain and discomfort leading to, in some cases if untreated, infertility and chronic pain (Cates and Wasserheit 1991). Implantation may be compromised in the presence of infection and if pregnancy is established preterm rupture of fetal membranes, premature labour and infection of the fetus can occur (Gravett et al. 1986; Martin et al. 1982). The innate immune system of the genital mucosal surface is likely to play a role in early elimination of pathogens and activation of the subsequent adaptive immune responses (Kagnoff and Eckmann 1997). Two components of this early response are the receptors involved in pathogen recognition, the Toll-like receptors (TLR) and the natural antimicrobials.

This study investigates the role of bacterial (lipopolysaccharide (LPS) and lipoteichoic (LTA)) and viral (polyinosinic-polycytidylic acid (poly I:C)) mimics of infection and some local inflammatory mediators, including prostaglandins and IL-1β, in the regulation of natural antimicrobials in human endometrial epithelial cells (Part A). The work is then expanded upon in Part B where the regulation of receptors involved in pathogen recognition, the Toll-like receptors and a co-factor for TLR4, CD14, are investigated.
Part 5A

Mediators of natural antimicrobial gene transcription
in the endometrium
5.2 Introduction

Several natural antimicrobials have been detected in the endometrium, including HBD1, human defensin 5, and SLPI: SLPI having maximal expression in the secretory phase of the menstrual cycle (King et al. 2000; Quayle et al. 1998; Valore et al. 1998). HBD1, 2, 3 and 4 have been detected in the uterus (Garcia et al. 2001; Jameson 1999; Valore et al. 1998). This thesis demonstrates the presence of HBD1 and HBD2 within the endometrium, showing that both vary in expression through the menstrual cycle (see chapter 3). The presence of these molecules, which have antimicrobial functions, in the endometrium suggests that they play a role in protection from infection.

Within the endometrium, as in other systems, inflammatory mediators - IFNγ, IL-1β and IL-1β + TNFα - have been shown to up-regulate mRNA expression of HBD2 (King et al. 2002). In other systems such as the lung, gut and gingiva, LPS - mimicking Gram-negative infection- has been shown to up-regulate the expression of the defensins and SLPI (Diamond et al. 1996; Jin et al. 1998; Krisanaprakornkit et al. 2000). LTA, a mimic of Gram positive bacterial infection, has been shown to up-regulate SLPI expression (Jin et al. 1998). It has been demonstrated that intracellular double-stranded RNA (dsRNA) from a virally infected cell induces gene expression that can be mimicked by exogenous dsRNA (Field et al. 1967). Polyinosinic-polycytidylic acid (poly I:C) is a synthetic dsRNA that has been used to simulate a viral-infected state in cells. The effect of this molecule on natural antimicrobial regulation has not been examined previously.

This part of the study investigates the role of bacterial (LPS and LTA) and viral (poly I:C) mimics of infection and some local inflammatory mediators, including prostaglandins and IL-1β, in the regulation of natural antimicrobials in human endometrial epithelial cells.
5.3 Methods

5.3.1 Primary epithelial cell tissue collection and endometrial epithelial cell lines

Endometrial biopsies (n=42) were collected as detailed in section 2.1. Tissue was separated into glandular and stromal compartments for cell culture. Biopsies used in this chapter are detailed in Table 12. MFE-296 (Hackenberg et al. 1994) and Hes (Desai et al. 1994) endometrial epithelial cell lines were used in these studies.

<table>
<thead>
<tr>
<th>Cycle Stage</th>
<th>Day of Cycle</th>
<th>Number of Biopsies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferative</td>
<td>1-13</td>
<td>28</td>
</tr>
<tr>
<td>Secretory</td>
<td>14-28</td>
<td>14</td>
</tr>
</tbody>
</table>

5.3.2 Cell culture and treatments

Epithelial cells were grown to near confluence (7-10 days) in 12 well plates in epithelial growth medium. Cell lines were seeded at 1x10⁶ cells per 25cm² flask and allowed to adhere over night. Both primary endometrial epithelial cells and endometrial epithelial cell lines were then treated with mimics of infection (LPS, LTA and poly I:C), inflammatory mediators (PGE₂, PGF₂α and IL-1β) or combinations of these. See Tables 13-15 for details of treatments. These molecules were chosen as they have been reported to influence antimicrobial expression in the human endometrium (IL-1β, TNFα and IFNγ) (King et al. 2002) and in other systems (Alexopoulou et al. 2001; Diamond et al. 1996; Hao et al. 2001; Harder et al. 2000a; Jin et al. 1998; Krisanaprakornkit et al. 2000; Sallenave et al. 1994). Rolipram is a phosphodiesterase inhibitor, maintaining cAMP levels and therefore potentiating the effect of prostaglandins acting through the EP2/EP4 receptor.

Experiments were done in the presence of fetal calf serum or human serum as recognition of LPS requires co-factors such as LPS binding protein and CD14 which may be more abundant and specific in human serum (Meszaros et al. 1995).
Combinations of the treatments were used to mimic multiple infections with Gram negative, Gram positive and/or viral infection and also in combination with the resultant expression of inflammatory mediators as might be occurring *in vivo*. All treatments were carried out in the presence of oestradiol (10^{-8} mol/l) to maintain cell growth. Controls (cells grown in medium with serum and oestradiol only) were included throughout.

**Table 13**: Treatments for primary endometrial epithelial cells (in the presence of fetal calf serum). Results detailed in section 5.4.1.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Treatment a mimic of:</th>
<th>Concentration</th>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E.coli</em> Serotype 026:B6 lipopolysaccharide (LPS)</td>
<td>Infection with a Gram negative bacteria</td>
<td>1 ng/ml</td>
<td>24 hours</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 ng/ml</td>
<td>24 hours</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1000 ng/ml</td>
<td>4 hours</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8 hours</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>24 hours</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> lipoteichoic acid (LTA)</td>
<td>Infection with a Gram positive bacteria</td>
<td>5 μg/ml</td>
<td>4 hours</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8 hours</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>24 hours</td>
</tr>
<tr>
<td>Polyinosinic-polycytidylic acid (poly I:C)</td>
<td>Viral infection</td>
<td>50 μg/ml</td>
<td>8 hours</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>24 hours</td>
</tr>
<tr>
<td>LPS + LTA</td>
<td>Combination of Gram negative and positive bacterial infection</td>
<td>1 μg/ml + 5 μg/ml</td>
<td>24 hours</td>
</tr>
<tr>
<td>LPS + Poly I:C</td>
<td>Combination of Gram negative bacterial and viral infection</td>
<td>1 μg/ml + 50 μg/ml</td>
<td>24 hours</td>
</tr>
<tr>
<td>LTA + Poly I:C</td>
<td>Combination of Gram positive bacterial and viral infection</td>
<td>5 μg/ml + 50 μg/ml</td>
<td>24 hours</td>
</tr>
<tr>
<td>LPS + IL-1β</td>
<td>Combination of Gram negative bacterial infection and inflammatory cytokine</td>
<td>1 μg/ml + 1 ng/ml</td>
<td>24 hours</td>
</tr>
</tbody>
</table>
### Table 14: Treatments for primary endometrial epithelial cells (in the presence of human serum). Results detailed in section 5.4.2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Treatment a mimic of:</th>
<th>Concentration</th>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS (E.coli Serotype 026:B6)</td>
<td>Infection with a Gram negative bacteria</td>
<td>1000ng/ml</td>
<td>30 minutes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGE₂</td>
<td>Local inflammatory mediator</td>
<td>10⁻⁶ M</td>
<td>4 hours</td>
</tr>
<tr>
<td>PGE₂ + LPS</td>
<td>Combination of Gram negative bacterial infection and inflammatory mediator</td>
<td>10⁻⁶ M +1μg/ml</td>
<td>4 hours</td>
</tr>
<tr>
<td>PGF₂α</td>
<td>Local inflammatory mediator</td>
<td>1.5x10⁻⁶ M</td>
<td>4 hours</td>
</tr>
<tr>
<td>PGF₂α + IL-1β</td>
<td>Combination local inflammatory mediator and cytokine</td>
<td>1.5x10⁻⁶ M + 1ng/ml</td>
<td>4 hours</td>
</tr>
</tbody>
</table>
Table 15: Treatments for endometrial epithelial cell lines. Results detailed in section 5.4.3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Treatment a mimic of:</th>
<th>Cell Line</th>
<th>Concentration</th>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS (E.coli Serotype 026:B6)</td>
<td>Infection with a Gram negative bacteria</td>
<td>Hes and MFE</td>
<td>1000ng/ml</td>
<td>30 minutes$^5$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MFE</td>
<td></td>
<td>1 hour$^5$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hes</td>
<td></td>
<td>2 hours$^5$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MFE</td>
<td></td>
<td>2 hours*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hes</td>
<td></td>
<td>4 hours$^5$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MFE</td>
<td></td>
<td>4 hours*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MFE</td>
<td></td>
<td>10 hours*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MFE</td>
<td></td>
<td>24 hours*</td>
</tr>
<tr>
<td>LTA (S. aureus)</td>
<td>Infection with a Gram positive bacteria</td>
<td>MFE</td>
<td>5μg/ml</td>
<td>2 hours*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MFE</td>
<td></td>
<td>4 hours*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MFE</td>
<td></td>
<td>10 hours*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MFE</td>
<td></td>
<td>24 hours*</td>
</tr>
<tr>
<td>Poly I:C</td>
<td>Viral infection</td>
<td>MFE</td>
<td>50μg/ml</td>
<td>2 hours*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MFE</td>
<td></td>
<td>4 hours*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MFE</td>
<td></td>
<td>10 hours*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MFE</td>
<td></td>
<td>24 hours*</td>
</tr>
<tr>
<td>PGE$_2$</td>
<td>Local inflammatory mediator</td>
<td>Hes</td>
<td>$10^6$ M</td>
<td>4 hours$^5$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MFE</td>
<td></td>
<td>4 hours$^5$</td>
</tr>
<tr>
<td>PGE$_2$+LPS</td>
<td>Combination of Gram negative bacterial infection and inflammatory mediator</td>
<td>Hes</td>
<td>$10^6$ M+1μg/ml</td>
<td>4 hours$^5$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MFE</td>
<td></td>
<td>4 hours$^5$</td>
</tr>
<tr>
<td>PGE$_2$+ Rolipram</td>
<td>Combination of inflammatory mediator and phosphodiesterase inhibitor (to maximise cAMP)</td>
<td>Hes</td>
<td>$10^6$ M + 10μg/ml</td>
<td>4 hours$^5$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MFE</td>
<td></td>
<td>4 hours$^5$</td>
</tr>
<tr>
<td>PGF$_2$α</td>
<td>Local inflammatory mediator</td>
<td>Hes</td>
<td>$1.5\times10^6$ M</td>
<td>4 hours$^5$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MFE</td>
<td></td>
<td>4 hours$^5$</td>
</tr>
</tbody>
</table>

$^5$Experiments done in the presence of human serum

* Experiments done in the presence of fetal calf serum
5.3.3 RNA extraction and Q-RT-PCR
After treatment, cells were harvested in Tri reagent and cDNA prepared. Amounts of HBD1, HBD2 and SLPI were determined using quantitative real time PCR. (See section 2.4 for details of method)

5.3.4 Immunohistochemistry
To localise the expression of SLPI protein from the primary epithelial cell cultures, immunohistochemistry was performed as described in section 2.5.

5.2.5 SLPI ELISA
SLPI protein concentrations in culture supernatants were determined by ELISA as detailed in section 2.6.

5.3.6 Statistical analysis
Significant differences of PCR and ELISA results were determined by analysis of variance (ANOVA; Statview 3.0). Fisher’s protected least significant difference (PLSD) was used to assign individual differences. \( P<0.05 \) was considered significant.

No attempt was made to analyse experiments involving epithelial cells from proliferative and secretory endometrium separately. However, SLPI mRNA expression in control epithelial cells originating from the proliferative and secretory phases, grown in the presence of epithelial growth medium, fetal calf serum and oestradiol until near confluent were compared. There was no significant difference (as determined by unpaired t-test) between SLPI levels derived from the two different phases of the menstrual cycle (Figure 22). Fahey et al (Fahey and Wira 2002) similarly find no differences between SLPI secretion from different phases of the menstrual cycle. \( \text{In vivo} \) SLPI is expressed only in secretory endometrium (see section 3.3.1). This suggests that after 7-10 days in culture the cells do not exhibit characteristics specific to their original menstrual cycle phase and it is therefore appropriate to treat them as one group for statistical analysis.
**Figure 22:** SLPI mRNA expression in primary endometrial epithelial cells. Cells were grown in matrigel in 12-well plates for 7-10 days in the presence of epithelial growth medium, containing fetal calf serum and oestradiol, prior to quantification of SLPI mRNA expression. Samples from the proliferative (n=14) and secretory phases (n=11) of the menstrual cycle were compared. Data is represented as the mean and s.e.m. No significant differences (as determined by unpaired t-test) were seen between the two groups.
5.4 Results

Results are presented in 3 sections with details of the treatments shown in Tables 13-15:

- **5.4.1** Treatment of primary epithelial cells in the presence of fetal calf serum with:
  a) mimics of infection: LPS – Gram negative mimic, LTA- Gram positive mimic and poly I:C – viral mimic
  b) combinations of mimics of infection
  c) combinations of mimics of infection and local inflammatory mediators

- **5.4.2** Treatment of primary epithelial cells in the presence of human calf serum with:
  a) LPS – Gram negative mimic
  b) local inflammatory mediators
  c) combinations of mimics of infection and local inflammatory mediators

- **5.4.3** Treatment of endometrial epithelial cell lines
  a) mimics of infection: LPS – Gram negative mimic, LTA- Gram positive mimic and poly I:C – viral mimic
  b) local inflammatory mediators
  c) combinations of mimics of infection and local inflammatory mediators
5.4.1 Results (1)
See Table 13 for details of treatment.

5.4.1.1 LPS and Poly I:C but not LTA affect expression of the natural antimicrobials HBD1, HBD2 and SLPI

Treatment with LPS resulted in significant changes in the mRNA expression of all three natural antimicrobials under investigation (Figure 23a, 23b, 23c). HBD1 mRNA expression was increased 11 fold after 8 hours of treatment and was higher than levels observed after 4 hours and 24 hours of treatment (Figure 23a). HBD2 mRNA expression was not significantly different to the control, but levels after 24 hours of treatment were significantly higher than those observed at 4 and 8 hours post treatment (Figure 23b). SLPI mRNA expression was increased at 8 hours over levels seen at 4 and 24 hours, and was significantly decreased when compared to the control after 24 hours of treatment. Treatment with 1ng/ml of LPS (data not shown) or 100ng/ml of LPS resulted in no significant changes compared to the control. The mRNA expression of SLPI at 24 hours was significantly higher after treatment with100ng/ml LPS compared to treatment with1000ng/ml LPS (Figure 23c).

Primary epithelial cells treated with poly I:C 50µg/ml for 8 and 24 hours resulted in a significant 7-fold increase in HBD1 mRNA expression at 24 hours (Figure 24a). SLPI mRNA expression was down-regulated after both 8 hours and 24 hours of treatment with poly I:C (Figure 24a).

No significant differences were seen in the mRNA expression of HBD1, HBD2 or SLPI after treatment with LTA 5µg/ml for 4, 8 or 24 hours (Figure 24b).
Figure 23: Effect of LPS on HBD1, HBD2 and SLPI expression in primary epithelial cells. Cells were treated with LPS 100ng/ml for 24 hours (n=4) and LPS 1000ng/ml for 4 hours (n=5), 8 hours (n=3) and 24 hours (n=5) in the presence of fetal calf serum (FCS). mRNA expression of (a) HBD1, (b) HBD2 and (c) SLPI were determined. Data are presented as the fold changes in mRNA expression relative to the control, mean ± s.e.m. Paired letters indicate statistical significance.

(a) a: P=0.01, b: P=0.01, c: P=0.01 (b) a: P=0.03, b: P=0.03 (c) a: P=0.003, b: P=0.04, c: P=0.03, d: P=0.0008
Figure 24: Effect of the mimics of infection, poly I:C and LTA, on HBD1, HBD2 and SLPI expression in primary epithelial cells. Data are presented as the fold changes in mRNA expression relative to the control, mean ± s.e.m. Paired letters indicate statistical significance.

Cells were treated as below and expression of SLPI, HBD1 and HBD2 mRNA were determined.

(a) Poly I:C 50μg/ml for 8 hours (n=3) and 24 hours (n=5)
   a: P=0.01, b: P=0.005, c: P=0.04

(b) LTA 5μg/ml for 4 hours (n=5), 8 hours (n=3) and 24 hours (n=6)
5.4.1.2 *SLPI* protein expression is localised within the primary epithelial cells, but is unchanged by treatments with mimics of infection.

SLPI protein was localised to the cytoplasm of the primary epithelial cells using immunohistochemistry (Figure 25).

SLPI protein concentration in cell culture supernatants as determined by ELISA was not significantly changed following treatment with LPS, LTA or poly I:C (Figure 26a, 26b, 26c).
Figure 25: Primary endometrial epithelial cells, immunostained for SLPI. Cells were grown in matrigel in 12 well plates until subconfluent (7-10 days) prior to immunostaining. (a) Immunolocalisation of SLPI in primary endometrial epithelial cells. SLPI is present in the cell cytoplasm and in the secretions from the cells (diffuse staining). (b) Negative control. The primary antibody was replaced with equimolar concentrations of an isotype control (IgGl). Note absence of immunostaining. Scale bar = 50μm.
**Figure 26:** SLPI protein expression was determined in the media from the same experiments carried out to determine mRNA expression. Data are presented as ng/ml, mean ± s.e.m. No significant differences in SLPI protein secretion were observed.

Cells were treated with:

(a) LPS 100ng/ml for 24 hours (n=3) and LPS 1000ng/ml for 4 hours (n=5), 8 hours (n=3) and 24 hours (n=5)

(b) LTA 5µg/ml for 4 hours (n=5), 8 hours (n=3) and 24 hours (n=4)

(c) Poly I:C 50µg/ml of 8 hours (n=3) and 24 hours (n=5)
5.4.1.3 Combinations of treatments result in increases in mRNA expression of HBD2 and HBD1

Mimics of infection cause small changes in natural antimicrobial expression. The hypothesis proposed was that combinations of infection, or infection in the presence of inflammatory mediators, as is observed in vivo, might increase levels further. Primary epithelial cells were treated with combinations of LPS 1000ng + LTA 5µg/ml, LPS 1000ng + poly I:C 50µg/ml, LTA 5µg/ml + poly I:C 50µg/ml or LPS 1000ng + IL-1β 1ng/ml for 24 hours (Figure 27).

Poly I:C had increased HBD1 mRNA expression 7 fold at 24 hours, and this effect was maintained with the addition of LPS, where a 14 fold increase in HBD1 was observed. This was significantly higher than the levels seen after treatment with LPS alone (Figure 27a).

HBD2 expression was increased 3.5 fold when treated in combination with LPS and IL-1β (Figure 27b).

SLPI mRNA expression was significantly decreased after 24 hours of treatment with LPS 1000ng/ml and this decrease was observed when treatment with LPS was combined with IL-1β, LTA or poly I:C (Figure 27c). No significant changes were observed with SLPI protein expression (Figure 27d).
Figure 27: Effect of combinations of treatments on HBD1, HBD2 and SLPI expression in primary epithelial cells. Cells were treated for 24 hours with LPS 1000ng/ml (n=5), LTA 5μg/ml (n=6) or poly I:C 50μg/ml (n=5), or at the same doses but in combination, LPS+IL-1β 1ng/ml (n=3), LPS+LTA (n=3), LPS+poly I:C (n=3) or LTA+poly I:C (HBD2 and SLPI n=3). mRNA expression of (a) HBD1, (b) HBD2 and (c) SLPI were determined. Data are presented as the fold changes in mRNA expression relative to the control, mean ± s.e.m. Paired letters indicate statistical significance.

(a) a: P=0.05, b: P=0.002, c: P=0.01 (b) a: P=0.01
(c) a: P=0.003, b: P=0.008, c: P=0.005, d: P=0.03, e: P=0.001

SLPI protein expression was determined in the media from the same experiments carried out to determine mRNA expression. Data are presented as ng/ml, mean ± s.e.m. Cells were treated for 24 hours with LPS 1000ng/ml (n=5), LTA 5μg/ml (n=4) or poly I:C 50μg/ml (n=5), or at the same doses but in combination, LPS+IL-1β 1ng/ml (n=3), LPS+LTA (n=3), LPS+poly I:C (n=3) or LTA+poly I:C (n=3) (d).
### Table 16: Summary of significant effects of mimics of infection on natural antimicrobial mRNA expression in primary endometrial epithelial cells in the presence of fetal calf serum

<table>
<thead>
<tr>
<th>Natural antimicrobial</th>
<th>Mimic of Infection</th>
<th>Polynosinic-polycytidylic acid (poly I:C – viral mimic)</th>
<th>Lipo-teichoic acid (LTA- Gram negative bacterial mimic)</th>
<th>Combinations of mimics or mimics + inflammatory mediators</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipopolysaccharide (Gram negative bacterial mimic)</td>
<td>1000ng/ml <strong>increases</strong> mRNA expression at 8 hours</td>
<td>50ug/ml <strong>increases</strong> mRNA expression at 24 hours</td>
<td>No effect</td>
<td>LPS and Poly I:C <strong>increase</strong> mRNA expression at 24 hours</td>
</tr>
<tr>
<td>HBD1</td>
<td>1000ng/ml <strong>increases</strong> mRNA expression at 24 hours when compared to 4 and 8 hours treatment</td>
<td>No effect</td>
<td>No effect</td>
<td>LPS and IL-1 <strong>increase</strong> mRNA expression at 24 hours</td>
</tr>
<tr>
<td>SLPI</td>
<td>1000ng/ml <strong>decreases</strong> mRNA expression at 24 hour. mRNA levels at 8 hours are higher than those observed at 4 and 24 hours. mRNA levels are higher at 24 hours when treated with 100ng/ml LPS than when compared to 1000ng/ml</td>
<td>50ug/ml <strong>decreases</strong> mRNA expression at 8 and 24 hours</td>
<td>No effect</td>
<td>LPS in combination with: IL-1, LTA or poly I:C <strong>decreases</strong> mRNA expression of SLPI.</td>
</tr>
</tbody>
</table>
5.4.2 Results (2)

Treatment of primary epithelial cells in the presence of human calf serum with:

a) LPS – Gram negative mimic
b) local inflammatory mediators
c) combinations of mimics of infection and local inflammatory mediators

See Table 14 for details of treatments.

5.4.2.1 Presence of human serum increases the levels of natural antimicrobial mRNA expression after treatment with LPS

LPS detection and resultant effects require the presence of LPS binding protein and cofactors, including CD14. Fetal calf serum may not have sufficient or appropriate soluble CD14 to allow maximal effects after treatment with LPS. Experiments were therefore repeated in the presence of human serum, treating primary epithelial cells with 1000ng/ml LPS for 30 minutes, 2 and 4 hours.

The mRNA expression of HBD1, HBD2 and SLPI were significantly higher at 4 hours when treated in the presence of human serum (Figure 28a, 28b, 28c).

SLPI mRNA expression increased approximately 2 fold at 4 hours compared to the control and expression was significantly increased at 2 hours and 4 hours when compared to levels at 30 minutes (Figure 28a). This difference was not observed in the SLPI protein secretion, although there were significantly higher levels of SLPI in the media collected from cells treated in the presence of human serum as opposed to fetal calf serum (Figure 28b).

Neither HBD1 nor HBD2 mRNA expression was significantly different to the control at any time point. HBD1 mRNA expression was significantly higher at 2 and 4 hours when compared to the levels at 30 minutes (Figure 28c). Similarly HBD2 mRNA expression was significantly increased at 4 hours when compared to levels at 30 minutes (Figure 28d).
Figure 28: Effect of LPS treatment in the presence of human serum, on HBD1, HBD2 and SLPI expression in primary epithelial cells. Cells were treated with LPS 1000ng/ml for 30 minutes (n=4 SLPI and HBD2, n=3 HBD1), 2 hours (n=3) and 4 hours (n=10 SLPI and HBD2, n=9 HBD1) in the presence of human serum. This is compared to treatment with LPS 1000ng/ml for 4 hours (n=5), 8 hours (n=3) and 24 hours (n=5) in the presence of fetal calf serum. mRNA expression of (a) SLPI, (c) HBD1 and (d) HBD2 were determined. Data are presented as the fold changes in mRNA expression relative to the control, mean ± s.e.m. Paired letters indicate statistical significance.
(a) a: P=0.005, b: P=0.03, c: P=0.01, d: P=0.01 (c) a: P=0.04, b: P=0.07, c: P=0.03
(d) a: P=0.04, b: P=0.03

SLPI protein expression was determined in the media from the same experiments carried out to determine mRNA expression. Data are presented as ng/ml, mean ± s.e.m. (b). Cells were treated with LPS 1000ng/ml for 30 minutes (n=4), 2 hours (n=3) and 4 hours (n=10) in the presence of human serum. This is compared to treatment with LPS 1000ng/ml for 4 hours (n=5), 8 hours (n=3) and 24 hours (n=5) in the presence of fetal calf serum. Paired letters indicate statistical significance.
(b) a: P=0.004, b: P=0.006
3n

Treatment + 5% human serum
Treatment + 10% fetal calf serum

CD3n

HBD1

Treatment + 5% human serum
Treatment + 10% fetal calf serum

Control 5% human serum
LPS 1000ng/ml + 5% human serum
Control + 10% fetal calf serum
LPS 1000ng/ml + 10% fetal calf serum

Control
LPS 0.5 hour
LPS 2 hour
LPS 4 hour
LPS 24 hour

SLPI mRNA
SLPI mRNATreatment + 5% human serum
Treatment + 10% fetal calf serum

SLPI Protein
LPS 1000ng/ml + 5% human serum

Control + 10% fetal calf serum
LPS 1000ng/ml + 10% fetal calf serum

0.5 hours
2 hours
4 hours
8 hours
24 hours

SLPI 1ng/ml

HBD1

HBD2

Treatment +5% human serum
Treatment + 10% fetal calf serum
5.4.2.2 Prostaglandins do not alter the expression of natural antimicrobial mRNA

Some inflammatory cytokines have been shown to up-regulate natural antimicrobial mRNA expression. The effect of another group of inflammatory modulators, the prostaglandins was therefore examined.

Primary epithelial cells were treated for 4 hours in the presence of human serum with PGE$_2$ $10^{-6}$ M alone or in combination with LPS 1000ng/ml, PGF$_2$α 1.5$x10^{-6}$ M alone or in combination with IL-1β 1ng/ml. As shown previously (section 5.4.2.1) LPS increased SLPI mRNA expression significantly over the control after 4 hours of treatment and over treatments with PGE$_2$ alone. PGE$_2$ inhibits the effect of LPS on SLPI mRNA expression when the treatments are given in combination (Figure 29a). No significant changes in SLPI protein expression compared to the control were observed (Figure 29b).
Figure 29: Effect of mediators of inflammation on HBD1, HBD2 and SLPI expression in primary epithelial cells.

(a) Primary epithelial cells were treated for 4 hours with: LPS 1000ng/ml (n=10 SLPI and HBD2, n=9 HBD1), PGE$_2$10$^{-6}$ M (n=7), a combination of the two treatments (n=3), PGF$_2$α 1.5x10$^{-6}$ M (n=7) and PGF$_2$α 1.5x10$^{-6}$ M + IL-1β 1ng/ml (n=3) in the presence of human serum. mRNA expression of HBD1, HBD2 and SLPI were determined. Data are presented as the fold changes in mRNA expression relative to the control, mean ± s.e.m. Paired letters indicate statistical significance. a: P=0.002, b: P=0.01, c: P=0.05

(b) SLPI protein expression was determined in the media of primary epithelial cells treated for 4 hours with; LPS 1000ng/ml (n=10), PGE$_2$10$^{-6}$ M (n=7), a combination of the two treatments (n=3) and PGF$_2$α 1.5x10$^{-6}$ M (n=7) in the presence of human serum. Data are presented as ng/ml, mean ± s.e.m.
5.4.2.3 Summary of results for section 4.4.2

Treatment of primary endometrial epithelial cells with LPS 1000ng/ml in presence of human serum results in:

- higher levels of SLPI, HBD1 and HBD2 mRNA expression and SLPI protein expression at 4 hours after treatment when compared to experiments carried out in presence of fetal calf serum.
- an increase of SLPI mRNA at 4 hours compared to the control.
- an increase of HBD1 and HBD2 mRNA at 2-4 hours when compared to levels at 30 minutes.
- PGE₂ and PGF₂α do not alter SLPI, HBD1 or HBD2 mRNA expression in primary endometrial epithelial cells.
5.4.3 Results (3): Use of endometrial epithelial cell lines as a model for antimicrobial expression.

As some interesting results had been obtained with primary epithelial cell cultures, two epithelial cell lines were treated with mimics of infection or prostaglandins to assess effect on natural antimicrobial expression. Treatments are detailed in Table 15.

SLPI expression was detectable but was not significantly increased by any of the treatments, LTA, poly I:C, LPS (in presence of fetal calf serum and human serum), PGE$_2$ or PGF$_2\alpha$ (data not shown).

Levels of the defensins were too low to detect, and they were not increased to a detectable level by any of the treatments (data not shown).

MFE cells release very little SLPI into culture supernatants and it was not possible to determine the regulation of protein release (data not shown).
5.4.4 Summary results for section 5.4

*In vitro* studies of primary endometrial epithelial cells and two endometrial epithelial cell lines have investigated the expression of the natural antimicrobials, SLPI, HBD1 and HBD2. The role of bacterial (Gram negative - LPS and Gram positive – LTA) and viral (poly I:C) mimics of infection and some inflammatory mediators, including prostaglandins and IL-1β in the regulation of natural antimicrobials in human endometrial epithelial cells has been determined. LPS and poly I:C increased the mRNA expression of HBD1 while decreasing the mRNA expression of SLPI. LTA had no significant effect on the expression of the natural antimicrobials. The response to treatment with LPS was augmented in the presence of human serum, which may be providing required co-factors such as CD14 or undefined cytokines. The prostaglandins, PGE$_2$ and PGF$_2$α, did not alter natural antimicrobial expression in primary endometrial epithelial cells.
5.5 Discussion

This study demonstrates the presence and regulation of the natural antimicrobials HBD1, HBD2 and SLPI in endometrial epithelial cells. These cells are a first line of defence against infection and as such play a fundamental role in detection of pathogens and initiation of an immune response. Three mimics of infection were investigated: LPS as a mimic of Gram negative infection, LTA as a mimic of Gram positive infection and poly I:C as a mimic of viral infection. The female reproductive tract is susceptible to infection with organisms from each of these groups of pathogens. These include Gram negative *Neisseria gonorrhoea* and *Chlamydia trachomatis*, Gram positive streptococcus Group B infection (normally a commensal organism, potentially causing morbidity in the neonate post delivery), Gram positive anaerobic *Peptococcus* and human immunodeficiency virus and herpes simplex virus.

*In vitro* treatment with LPS and poly I:C treatment lead to increased expression of HBD1 mRNA in primary endometrial epithelial cells. A combination of treatment with LPS and poly I:C resulted in a further increase in HBD1 mRNA expression. Poly I:C did not alter the expression of HBD2 mRNA while expression of SLPI mRNA was decreased. HBD1 is generally regarded as being constitutively expressed in gingival tissues (Krisanaprakornkit et al. 1998) the intestine (O’Neil et al. 1999) and the lung (Zhao et al. 1996), as expression has not been up-regulated by inflammatory mediators in these systems. While work has shown that viral infection can be inhibited by HBD1 (Daher et al. 1986; Gropp et al. 1999; Nakashima et al. 1993), the effects of viral infection on the expression of natural antimicrobials have not been examined. It could be that HBD1, having particular antiviral properties, is unaffected by other mediators and is selectively up-regulated by viral infection. It may also be that differences in defensin regulation exist between epithelial cells from distinct sites. Further work is underway to look at the effects of viral infection on natural antimicrobial expression in cervical epithelial cells.
LTA had no significant effect on the mRNA expression of HBD1, HBD2 or SLPI in vitro. It may be that Gram positive bacteria are uncommon pathogens of the endometrium and therefore a response to LTA is limited. It may also be that higher or lower doses of LTA would have resulted in a response.

HBD2 has been detected in the uterus (Bals et al. 1998), although the site of expression within the uterus was not investigated. However, HBD2 is present at other epithelial surfaces (Harder et al. 2000b; Krisanaprakornkit et al. 2002; Krisanaprakornkit et al. 2000). These investigators detail the up-regulation of HBD2 by inflammatory mediators in respiratory and gingival epithelial cells respectively. King et al have demonstrated up-regulation of HBD2 by IL-1β, TNFα and IFNγ in endometrial epithelial cells (King et al. 2002). The HBD2 promoter is reported to have several consensus sites for the proinflammatory transcription factors NF-κB and AP-1, accounting for its induction by these mediators (Harder et al. 2000b). Previous studies have shown HBD2 to be up-regulated by LPS (Becker et al. 2000; McNamara et al. 1999) while others report little effect of LPS, but have found an increased HBD2 expression upon infection with pathogenic bacteria (Harder et al. 2000b; O'Neil et al. 1999). This suggests that components of the bacteria other than LPS are responsible for stimulating defensin expression. It also suggests that LPS actions may depend on the cell type investigated. While this study did not show an increase of HBD2 over the control after treatment with LPS at different doses and time point, it did show an increase of HBD2 at 24 hours when compared to the levels at 4 hours and 8 hours. A combination of LPS and IL-1β resulted in significant up-regulation of HBD2, but whether this is a synergistic or additive effect can not be determined from these studies. This study used E. coli LPS and it might be that endometrial epithelial cells would respond to LPS from more common genital tract pathogens, such as C. trachomatis, more effectively.

SLPI has been reported to be an LPS inducible gene in mouse macrophages (Jin et al. 1998). However, this study found that SLPI mRNA expression decreased after treatment with LPS for 24 hours. Combinations of treatments with other mimics of infection or IL-
1β did not alter the low levels of SLPI expression. Poly I:C also down-regulated levels of SLPI 24 hours post treatment. This may indicate that, as expected, innate immune responses to LPS occur over a shorter time period. Following initiation of the adaptive immune response high levels of a leukocyte protease inhibitor may be inappropriate, hence the observed down regulation. Experiments carried out over a shorter time course, but in the presence of human serum (not fetal calf serum), did result in increased mRNA expression of SLPI mRNA 4 hours post treatment. These changes in mRNA expression were not observed in SLPI protein secretion. This may be because there is a lag time between changes in mRNA expression and protein secretion, and in each case the experiments were terminated at the time points where significant changes in mRNA expression were observed. Furthermore, SLPI secretion has been demonstrated to remained associated with the tissue under investigation in culture and increased recovery observed with mild acid extraction. This is in agreement with the high isoelectric point (pI) for SLPI, associated with high solubility at low pH (King et al. 2000). In the present study there were no significant differences between SLPI secretion from control and treated samples (figures 26a and 27d), and this may be because SLPI remained associated with the tissue.

LPS activity requires the presence of co-factors such as LPS binding protein and CD14. CD14 can be either soluble in the serum or membrane bound. It has been suggested that CD14 of epithelial cell origin mediates the LPS induction of an antibiotic peptide gene in tracheal epithelial cells (Diamond et al. 1996). Becker at al (Becker et al. 2000) have demonstrated that the up-regulation of HBD2 after LPS stimulation is CD14 dependent in human tracheobronchial epithelium. CD14 is expressed on non-professional antigen presenting cells, such as epithelial cells, but expression is generally low (Funda et al. 2001; Song et al. 2001). There may therefore be greater reliance on sCD14 in the serum (Frey et al. 1992). The initial experiments in this study were carried out in the presence of fetal calf serum. It was hypothesised that human serum provides species specific CD14, therefore resulting in more effective response to LPS and hence improved defense strategies. This was indeed the case, with significantly greater mRNA
expression of HBD1, HBD2 and SLPI at 4 hours after treatment with LPS when treated in the presence of human serum when compared to those samples treated in the presence of fetal calf serum. This increase was also observed in the secretion of SLPI protein. Although HBD1 and HBD2 mRNA expression were not significantly increased when compared to the control, they were both increased at 4 hours after treatment when compared to levels at 30 minutes after treatment. SLPI mRNA expression was significantly increased when compared to the control at 4 hours after treatment. It is possible that CD14 in human serum plays a role in the response to LPS, although the role of other factors that differ in the human serum, such as increased amounts of cytokines, cannot be excluded.

Use of endometrial cell lines to examine further the regulation of the natural antimicrobials is limited, as they do not express high levels of mRNA for HBD1 and HBD2, and the expression of these was not stimulated by any treatments in this study. Furthermore SLPI, which was expressed, was not modulated by the treatments in this study. These cell lines are therefore not good models for continued work looking at the effects of mimics of infection on antimicrobial expression.

The role of all these molecules in the elimination of infection has been concentrated upon. It should be borne in mind that the natural antimicrobials have several other actions that may be important. The defensins have been shown to be chemotactic for T cells and dendritic cells (Chertov et al. 1996; Yang et al. 1999a) and may play a part in linking and activating the adaptive immune response. SLPI has generalized anti-inflammatory actions including inhibition of neutrophil elastase and cathepsin G (Thompson and Ohlsson 1986) and inhibition of NFkB (Lentsch et al. 1999). It therefore plays an important part in limiting the damage that can occur as a result of inflammatory responses. A combination of these functions and the antimicrobial activity of these molecules probably contribute to the immune responses in vivo.
In summary, the natural antimicrobials HBD1, HBD2 and SLPI have been detected in endometrial epithelial cells. Their regulation by mimics of infection (LPS, LTA and poly I:C) and prostaglandins was examined. LPS was observed to have the widest effect on natural antimicrobial mRNA expression and up-stream components of the response to LPS, the Toll-like receptors and CD14, have therefore been examined further in the following section, Part 5B.
Part 5B
Mediators of Toll-like receptor
gene transcription in the endometrium
5.6 Introduction

The mimics of infection including LPS and LTA, discussed in Part 5A, are described as pathogen associated molecular patterns (PAMPS). They are conserved elements of pathogens, which can be recognized by defence systems. The receptors involved in their recognition (pathogen recognition receptors, PRR) include toll like receptors (TLRs) and their associated co factors such as CD14. Ten TLRs have been described to date (TLR 1-10). Ligands have been described for 5 of these TLRs (Table 4); TLR2 (bacterial lipoproteins and peptidoglycans), TLR3 (double-stranded RNA), TLR4 (lipopolysaccharide (LPS) and HSP60), TLR5 (flagellin), and TLR9 ( unmethylated CpG DNA motifs found in bacterial DNA (vertebrate DNA CpG dinucleotides occur infrequently and are highly methylated)) (Underhill and Ozinsky 2002). The most relevant of these to Gram negative bacterial recognition appear to be TLR4 and TLR9.

TLR4 expression has been demonstrated in the endometrium (Young et al. 2002) and the regulation of TLR4 has been examined in a variety of systems, but not in the endometrium. LPS and IFNγ induce TLR 2 and TLR 4 expression in human endothelial cells (Faure et al. 2001), in intestinal epithelial cells (Abreu et al. 2002) and in monocytes and polymorphonuclear leukocytes (Muzio et al. 2000). Monocyte-like THP-1 cells regulate TLR mRNA levels in response to a variety of stimuli including phorbol esters, LPS, bacterial lipoproteins, live bacteria, and cytokines (Zarember and Godowski 2002). TLR9 has not been demonstrated in the endometrium and its regulation has not been examined.

CD14 acts as a co factor in the recognition of LPS and can either be present in the serum as soluble CD14 or as a membrane bound form on leukocytes (particularly monocytes and macrophages) and epithelial cells. The expression of mCD14 on epithelial cells is far lower than that seen on leukocytes, but the regulation of CD14 on epithelial cells might influence the effects following recognition of LPS. LPS has been shown to up-regulate the expression of CD14 on endothelial cells (Jersmann et al. 2001). Another
group has examined the effect of prostaglandins on CD14 expression in mouse macrophages. PGE₁ and PGE₂ among the prostaglandins tested strongly stimulated the expression of the CD14 gene in the cells (Iwahashi et al. 2000). No published work has examined the expression of CD14 within the epithelial cells of the endometrium.

This study investigates the role of a bacterial mimic of infection (LPS) and some local inflammatory mediators, including prostaglandins, IFNγ, and combinations of IL-1β and TNFα, in the regulation of TLR 4 and 9 and CD14 in human endometrial epithelial cells.
5.7 Methods

5.7.1 Primary epithelial cell tissue collection and endometrial epithelial cell lines
Endometrial biopsies were collected as detailed in sections 2.1 and 5.2.1. Tissue was separated into glandular and stromal compartments for cell culture. MFE-296 (Hackenberg et al. 1994) and Hes (Desai et al. 1994) endometrial epithelial cell lines were used in these studies.

5.7.2 Cell culture and treatments
Epithelial cells were cultured as described in section 5.2.2. Both primary endometrial epithelial cells and endometrial epithelial cell lines were then treated with; LPS as a mimic of infection, inflammatory mediators (PGE$_2$, PGF$_2$α, IL-1β, TNFα and IFNγ) or combinations of these as section 5.2.2. See Tables 17 and 18 for details.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Treatment a mimic of:</th>
<th>Concentration</th>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS (E.coli Serotype 026:B6)</td>
<td>Infection with a Gram negative bacteria</td>
<td>1000ng/ml</td>
<td>30 minutes$^5$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 hours$^5$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 hours$^5$</td>
</tr>
<tr>
<td>PGE$_2$</td>
<td>Local inflammatory mediator</td>
<td>10$^{-6}$ M</td>
<td>4 hours$^5$</td>
</tr>
<tr>
<td>PGE$_2$ + LPS</td>
<td>Combination of Gram negative bacterial infection and inflammatory mediator</td>
<td>10$^{-6}$ M + 1µg/ml</td>
<td>4 hours$^5$</td>
</tr>
<tr>
<td>PGE$_2$ + Rolipram</td>
<td>Local inflammatory mediator and phosphodiesterase inhibitor</td>
<td>10$^{-6}$ M + 10µg/ml</td>
<td>4 hours$^5$</td>
</tr>
<tr>
<td>PGF$_2\alpha$</td>
<td>Combination local inflammatory mediator and cytokine</td>
<td>1.5x10$^{-6}$ M</td>
<td>4 hours$^5$</td>
</tr>
<tr>
<td>IL-1β + TNFα</td>
<td>Local inflammatory cytokines</td>
<td>1ng/ml + 2ng/ml</td>
<td>4 hours$^*$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1ng/ml + 2ng/ml</td>
<td>24 hours$^*$</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Local inflammatory cytokine</td>
<td>10ng/ml</td>
<td>4 hours$^*$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10ng/ml</td>
<td>24 hours$^*$</td>
</tr>
</tbody>
</table>

$^*$ Experiments carried out in the presence of fetal calf serum
$^+$ Experiments carried out in the presence of human serum
Table 18: Treatments for primary endometrial epithelial cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Treatment a mimic of:</th>
<th>Concentration</th>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS (E.coli Serotype 026:B6)</td>
<td>Infection with a Gram negative bacteria</td>
<td>1000ng/ml</td>
<td>30 minutes$^5$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1000ng/ml</td>
<td>4 hours$^5$</td>
</tr>
<tr>
<td>PGE$_2$</td>
<td>Local inflammatory mediator</td>
<td>$10^{-6}$M</td>
<td>4 hours$^5$</td>
</tr>
<tr>
<td>PGE$_2$ + LPS</td>
<td>Combination of Gram negative bacterial infection and inflammatory mediator</td>
<td>$10^{-6}$M +1μg/ml</td>
<td>4 hours$^5$</td>
</tr>
<tr>
<td>PGF$_2\alpha$</td>
<td>Local inflammatory mediator</td>
<td>$1.5x10^{-6}$M</td>
<td>4 hours$^5$</td>
</tr>
<tr>
<td>IL-1β + TNFα</td>
<td>Local inflammatory cytokines</td>
<td>1ng/ml + 2ng/ml</td>
<td>24 hours$^*$</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Local inflammatory cytokine</td>
<td>10ng/ml</td>
<td>24 hours$^*$</td>
</tr>
</tbody>
</table>

$^*$ Experiments carried out in the presence of fetal calf serum

$^5$ Experiments carried out in the presence of human serum
5.7.3 RNA extraction and Q-RT-PCR

After treatment, cells were harvested in Tri reagent and cDNA prepared. Amounts of CD14, TLR4 and TLR9 mRNA were determined using quantitative real time PCR. (See section 2.4 for details of method)

5.7.4 Statistical analysis

Significant differences were determined by analysis of variance (ANOVA; Statview 3.0). Fisher’s protected least significant difference (PLSD) was used to assign individual differences. P<0.05 was considered significant.

No attempt was made to analyse experiments involving epithelial cells from proliferative and secretory endometrium separately (see section 5.2.6).
5.8 Results

5.8.1 LPS affects expression of CD14 in an endometrial epithelial cell line, but has no affect on primary epithelial cell CD14 mRNA expression.

To further examine the components of the pathways involved up stream of natural antimicrobial expression, the effects of LPS 1000ng/ml, PGE₂ 10⁻⁶ M, PGF₂α 1.5x10⁻⁶ M, IFNγ 10ng/ml and IL-1β 1ng/ml + TNFα 2ng/ml on CD14 mRNA expression was determined (Figure 30). See Tables 17 and 18 for details of treatment.

LPS increased the expression of CD14 mRNA in Hes cells 2.2 fold over the control at 2 hours, which was significantly higher than levels seen after 4 hours of treatment (Figure 30a).

CD14 mRNA expression in MFE cells was significantly decreased at 2 hours when compared to levels at 30 minutes after treatment with LPS (Figure 30a).

No other treatments had an effect on CD 14 mRNA expression (Figure 30a).

Treatment of primary endometrial epithelial cells with the same treatments resulted in no significant changes in CD14 mRNA expression (Figure 30b)
Figure 30: CD14 expression in (a) endometrial epithelial cell lines (MFE and Hes) and (b) primary endometrial epithelial cells in the presence of human serum. Data are presented as the fold changes in mRNA expression relative to the control, mean ± s.e.m. Paired letters indicate statistical significance.

(a) MFE and Hes cells were treated with LPS 1000ng/ml for 30 minutes (n=5), 2 hours (n=5) and 4 hours (n=5).
4 hour treatment with, PGE$_2$ 10$^{-6}$ M (n=5), LPS 1000ng/ml+PGE$_2$ 10$^{-6}$ M (n=5), PGE$_2$10$^{-6}$ M+ rolipram (a phosphodiesterase inhibitor) (MFE n=5, Hes n=4) and PGF$_2$α 1.5x10$^{-6}$ M (n=5).
IFNγ 10ng/ml at 4 hours (n=3) and 24 hours (n=3) and IL-1β 1ng/ml + TNFα 2ng/ml at 4 hours (n=3) and 24 hours (n=3) was also carried out.
a: P=0.002, b: P=0.04, c: P=0.001, d: P=0.001

(b) Primary endometrial epithelial cells were treated with LPS 1000ng/ml for 30 minutes (n=4), and 4 hours (n=10). 4 hour treatment with; PGE$_2$ 10$^{-6}$ M (n=7), LPS 1000ng/ml+PGE$_2$ 10$^{-6}$ M (n=3), PGF$_2$α 1.5x10$^{-6}$ M (n=6). IFNγ 10ng/ml at 24 hours (n=3) and IL-1β 1ng/ml + TNFα 2ng/ml at 24 hours (n=3) was also carried out.
CD14 Fold change over control

- MFE
- Hes

### a
- LPS 0.5 hours
- LPS 2 hours
- LPS 4 hours
- PGE 4 hours
- PGE+Rho1144 4 hours
- IFN-γ 4 hours
- IFN-γ 24 hours
- IL-1 + TNFα 4 hours
- IL-1 + TNFα 24 hours

### b
- LPS 0.5 hours
- LPS 4 hours
- PGE 4 hours
- PGF2α 4 hours
- IFN-γ 4 hours
- IFN-γ 24 hours
- IL-1 + TNFα 4 hours
- IL-1 + TNFα 24 hours

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5.8.2 Inflammatory mediators, PGE₂, IFNγ and IL-1⁺TNFα increase expression of TLR4 mRNA in an endometrial epithelial cell line, but have no effect on TLR9 mRNA.

The TLR are pathogen recognition receptors, TLR4 particularly for LPS and TLR9 for unmethylated CpG DNA motifs. Regulators of their mRNA expression were examined. Treatments are detailed in Tables 17 and 18.

PGE₂ increased the mRNA expression of TLR4 in Hes cells 1.5 fold over the control at 4 hours and levels were significantly higher than after treatment with LPS alone or PGE₂ in combination with LPS or rolipram (a phosphodiesterase inhibitor, which will increase cAMP levels) (Figure 31a). Expression of TLR4 mRNA was increased by PGE₂ in primary epithelial cells but was not significant due to wide variations in the results (Figure 31b).

IFNγ increased TLR4 mRNA expression 6.5 fold at 24 hours in Hes cells and IL-1β⁺TNFα increased TLR4 mRNA expression 4.8 fold in MFE cells (Figure 31a). The effect of IFNγ and IL-1β⁺TNFα were not observed in the primary epithelial cells (Figure 31b). LPS 1000ng/ml and PGF₂α had no effect on TLR4 mRNA expression.

PGE₂, PGF₂α, IFNγ and IL-1β⁺TNFα had no effect on TLR9 mRNA expression in the cell lines examined or in the primary epithelial cells (data not shown).
Figure 31: TLR4 mRNA expression in endometrial epithelial cell lines (MFE and Hes) (a) and primary endometrial epithelial cells (b) in the presence of human serum. Data are presented as the fold changes in mRNA expression relative to the control, mean ± s.e.m. Paired letters indicate statistical significance.

(a) MFE and Hes cells were treated with LPS 1000ng/ml for 30 minutes (n=5), 2 hours (n=5) and 4 hours (n=5). 4 hour treatments with LPS 1000ng/ml+PGE₂ 10⁻⁶ M (n=5), PGE₂ 10⁻⁶ M (n=5), PGE₂10⁻⁶ M + rolipram (MFE n=5, Hes n=4), PGF₂α 1.5×10⁻⁶ M (n=5), IFNγ 10ng/ml (n=3), IL-1β 1ng/ml + TNFα 2ng/ml (n=3) were also carried out. MFE and Hes cells were treated for 24 hours with IFNγ 10ng/ml (n=3) and IL-1β 1ng/ml + TNFα 2ng/ml (n=3).

a: P=0.0003, b: P=0.007, c: P=0.04, d: P=0.004, e: P=0.005, f: P=0.0002, g: P=0.008

(b) Primary endometrial epithelial cells were treated with LPS 1000ng/ml for 30 minutes (n=4), and 4 hours (n=10). 4 hour treatment with; LPS 1000ng/ml + PGE₂ 10⁻⁶ M (n=3), PGE₂ 10⁻⁶ M (n=7), PGF₂α 1.5×10⁻⁶ M (n=6), IFNγ 10ng/ml at 24 hours (n=3) and IL-1β 1ng/ml + TNFα 2ng/ml at 24 hours (n=3) was also carried out.
### 5.8.3 Summary of results

**Table:** Summary of significant effects of treatments on CD14 and TLR4 mRNA expression in endometrial epithelial cell lines MFE and Hes*

<table>
<thead>
<tr>
<th>Treatment**</th>
<th>CD14</th>
<th>TLR4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MFE</td>
<td>Hes</td>
</tr>
<tr>
<td>LPS</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>PGE2</td>
<td>no effect</td>
<td>no effect</td>
</tr>
<tr>
<td>PGF2α</td>
<td>no effect</td>
<td>no effect</td>
</tr>
<tr>
<td>IFNγ</td>
<td>no effect</td>
<td>no effect</td>
</tr>
<tr>
<td>TNFα + IL-1β</td>
<td>no effect</td>
<td>no effect</td>
</tr>
</tbody>
</table>

*CD14, TLR4 and TLR9 mRNA expression in primary endometrial epithelial cell was unaffected by any of these treatments

**TLR9 mRNA expression was unaffected by any of these treatments
5.9 Discussion

This study demonstrates the presence and regulation of CD14 and TLR4 and TLR9 mRNA in endometrial epithelial cells. If CD14 does indeed play a role in detection of LPS, its regulation is of interest. This has not been examined in the endometrium.

Fichorova et al (Fichorova et al. 2002) have examined immortalized vaginal and cervical epithelial cells and were unable to demonstrate mRNA expression of TLR4. These cells were unresponsive to LPS but did respond to whole Gram negative bacteria and bacterial lysates. They demonstrated the presence of soluble (s)CD14 and propose that sCD14 can act as a co-receptor for non-TLR4 ligands. Jersmann et al show an up-regulation of CD14 on endothelial cells following treatment with LPS (Jersmann et al. 2001). In contrast Cario et al (Cario et al. 2000) did not detect CD14 expression in 3 intestinal epithelial cell lines while Funda et al (Funda et al. 2001) who did detect CD14 expression, showed no modulation of CD14 expression in 3 intestinal epithelial cell lines following treatment with LPS. The results in this study were endometrial cell line dependent. Hes cells showed an increased CD14 mRNA expression, while LPS treatment of MFE cells resulted in decreased CD14 mRNA expression. No effect was demonstrated in primary endometrial epithelial cells. It is unclear why such differing results were obtained. Hes is reported to be an immortalized endometrial cell line, derived from the endometrial lining of hysterectomy specimen removed due to leiomyomata (Desai et al. 1994). In contrast, MFE is a glandular derived endometrial carcinoma epithelial cell line (Hackenberg et al. 1994). It may be that the underlying different pathological origins of these epithelial cells results in differing responses to microbial mimics.

In other systems the role of inflammatory mediators in the expression of CD14 have been investigated. PGE$_2$ has been shown to stimulate CD14 expression in mouse macrophages (Iwahashi et al. 2000). IFN$\gamma$ and TNF$\alpha$ enhance LPS binding to neutrophils via increased expression of CD14 (Takeshita et al. 1998), while TNF$\alpha$
increases murine CD14 expression in epithelial cells but not myeloid cells (Fearns and Loskutoff 1997). This latter group suggest that the changes in CD14 seen after treatment with LPS may an indirect effect, with inflammatory mediators playing a role in CD14 expression. The present study found no effect of PGE₂, PGF₂α, IFNγ or IL-1β + TNFα on the expression of CD14 in primary endometrial epithelial cells or endometrial epithelial cell lines. The effects of LPS on CD14 expression may of course still be indirect via another mediator not examined, or the effect may be a direct effect of signaling events following LPS binding. The functional significance of changes in CD14 mRNA expression needs to be determined. Levels of CD14 expression on epithelial cells are low, if present at all. If CD14 is present on the membrane surface at low levels small increases in expression could magnify the resultant responses.

LPS is believed to bind to cells via a complex, which includes TLR4 (Chow et al. 1999). The signaling pathway is homologous to the IL-1 signalling pathway, resulting in NFκB activation (Gay and Keith 1991; Medzhitov et al. 1997). It has been proposed that TLR9 is a receptor for CpG DNA motifs found within bacterial DNA (Bauer et al. 2001; Hemmi et al. 2000). Thus far TLR9 expression has not been described in endometrial epithelial cells and its regulation in endometrial cells has not been addressed. The regulation of TLR4 has been examined in myeloid cells (Muzio et al. 2000), endothelial cells (Faure et al. 2000) and intestinal epithelial cells (Abreu et al. 2002) where inflammatory mediators, IFNγ, TNFα and LPS have been shown to increase TLR4 expression. This study confirmed these findings in endometrial epithelial cell lines where mRNA expression was increased by PGE₂ and IFNγ in Hes cells, and by IL-1β + TNFα in MFE cells. In contrast primary endometrial epithelial cell TLR4 expression remained unchanged after treatment with the same mediators. This may represent differences in the regulation of TLR4 between the primary cells and cell lines such as differences in the numbers of receptors on the cell surfaces for IFNγ, IL-1β or TNFα. TLR4 expression was unaltered by treatment with LPS. In other systems such as the gut it has been suggested that TLR4 down regulation may be a protective mechanism where cells are continually exposed to LPS and a constant inflammatory response would be
deleterious (Abreu et al. 2001). Within the endometrium, which is normally sterile, this protective effect is not required. TLR9 mRNA was expressed in both the primary endometrial epithelial cells and the endometrial epithelial cell lines, but expression was unaltered by treatments of PGE_2, PGF_2α, IFNγ or IL-1β + TNFα.

In summary CD14, TLR4 and TLR9 mRNA has been detected in endometrial epithelial cells. CD14, TLR4 and TLR9 were found to be differentially regulated: CD14 by LPS either directly or indirectly via an unknown mediator, TLR4 by inflammatory molecules and TLR9 by none of the mediators examined in this study. It is likely that all of these molecules will play a role in protecting the endometrium from infection.
Toll-like receptors are an important component of the pathway involved in the recognition of pathogens. Previous work has demonstrated a down-stream activation pathway resulting from TLR ligation that leads to activation of NFκB (Alexopoulou et al. 2001; Cario et al. 2000; Chow et al. 1999) and can stimulate the expression of natural antimicrobials (Becker et al. 2000; Krutzik et al. 2001). This chapter has examined local mediators that might modulate some of the natural antimicrobials and TLRs. Part A showed an increase in natural antimicrobial mRNA expression following treatment of endometrial epithelial cells with LPS. The mechanism of this response may be through TLR4, the expression of which has been shown to be modulated by some local inflammatory mediators. One can see how a complex set of interactions evolves in the presence of infection as the target organ tries to limit pathogenic sequelae. Figure 32 for summarizes possible events at the endometrial mucosal surface as defined by this chapter. Future studies will continue to delineate the roles of the natural antmicrobials and TLRs more clearly.
**Figure 32**: Possible role of Toll-like receptors and natural antimicrobials at the mucosal surface of the female reproductive tract. Toll-like receptors are activated on contact with a ligand such as LPS, LTA or CpG motifs. This may or may not be in the presence of co-factors such as CD14. A cascade of events follows culminating in translocation of NFkB to the nucleus and up-regulation in gene expression of effector molecules such as the natural antimicrobials. These effector molecules will go on to have direct or indirect effects, contributing to the potential elimination of pathogens.
Chapter 6

Early immune responses to *Chlamydia trachomatis* in a cervical epithelial cell line
6.1 Introduction

The early recognition of infection by the host is a fundamental component of the immune response, providing an early line of defence against pathogens (Janeway 1992b). However, activation of innate defence mechanisms by pathogens not only provides early protection for the host, but can also trigger cytokine and chemokine cascades that shape the specific acquired immune response (Fearon and Locksley 1996). It is now recognised that there is more specificity in the activation of the innate immune system than was initially believed. This has been attributed to the variety of pattern recognition receptors (PRRs) expressed on host cells that recognise highly conserved motifs that are defined as pathogen-associated molecular patterns (PAMPs).

To come in contact with a professional antigen presenting cell (APC), pathogens first have to gain access to the body, often via epithelial surfaces, such as the respiratory tract, gastrointestinal tract and reproductive tract. *Chlamydia trachomatis* infection is one of the most common sexually transmitted infections (Gerbase et al. 1998; Morgan 2002): Serovars D and E being the commonest serovars associated with human genital infection (Workowski et al. 1994). Within the female reproductive tract *C. trachomatis* can colonise epithelial cells without disseminating (Shaw et al. 2001) and initiate an inflammatory response. It is therefore important for the host to generate an appropriate response at the site of infection that controls the organism but does not cause damage.

Commensal organisms are abundant at many epithelial sites in the body but do not cause disease. It has been postulated that pathogens are capable of activating cells via PRRs in ways that commensal organisms do not (Svanborg et al. 1999). This could provide the ‘danger’ signals that activate innate immune components and subsequently drive the acquired immune response (Matzinger 1994). Expression of PRRs by epithelial cells and the triggering of an inflammatory response are central to this concept.
The Toll-like receptors (TLR) are a family of PRR. TLRs play a fundamental role in mediating the innate immune response, recognising PAMPs such as LPS, lipoteichoic acid and unmethylated CpG DNA motifs. Downstream signalling events are thought to involve pathways resulting in the activation of nuclear factor κ B (NF-κB) (Cario et al. 2000; Kopp and Medzhitov 1999; Medzhitov et al. 1997; Muzio et al. 1998). Activation of the adaptive immune response then requires MHC class II expression and antigen presentation.

Following recognition of a pathogen, a cascade of events occurs as the host attempts to control infection. This includes the secretion of chemokines and cytokines such as IL-8, GM-CSF and IL-6 following in vitro infection of epithelial cells with C. trachomatis (Rasmussen et al. 1997). Natural antimicrobials are effectors of the innate immune system that have been shown to have antimicrobial activity (Bals et al. 1998; Hiemstra et al. 1996; Lehrer et al. 1993; McNeely et al. 1995; Tomee et al. 1997). The role of natural antimicrobials in the response to genital tract chlamydial infection has not been characterised.

This study uses a cervical epithelial cell line (HeLa) to characterise the mediators of early events in the innate and adaptive immune responses following infection with C. trachomatis serovar E. The expression of components of the TLR pathway, MHC class II activation, natural antimicrobials, chemokines and cytokines involved in cell recruitment and inflammation are described. By building up a pathway of events that occur in response to C. trachomatis infection, new areas to target therapeutically may become apparent.
6.2 Methods

6.2.1 Cell culture

The human cervical cell line HeLa, was grown or maintained in Iscove’s modified Dulbecco’s (IMDM) medium supplemented with the appropriate concentration of fetal bovine serum. No antibiotics or other supplements were used unless stated otherwise.

6.2.2 Chlamydiae

*C. trachomatis* serovar E was used throughout, propagated in HeLa cell monolayers (see section 2.3 for details).

6.2.3 Infection protocol

HeLa cells were treated with *C. trachomatis* serovar E (MOI 0.1 and 0.01), heat killed *C. trachomatis* (MOI 0.1), and LPS (*Salmonella minnesota* R595) (1000ng/ml and 100ng/ml). As a further control, cells were treated with a cell lysate; control uninfected HeLa cells were processed in the same way as infected HeLa cells when harvesting *C. trachomatis*. The subsequent cell lysate from the uninfected HeLa cells acted as a control for cellular components, which may have been stimulating the innate immune response. Cultures were incubated at 37°C for 2 hours, 4 hours, 8 hours, 24 hours or 48 hours. At each time point supernatant was collected and stored at -70°C for subsequent sandwich ELISA; cells were lysed in Tri reagent and stored at -70°C for subsequent mRNA extraction. Experiments were performed three times.

To confirm the infectivity of *C. trachomatis*, HeLa cells were grown in 8 well chamber slides and treated as above. 72 hours after treatment slides were fixed in acetone and stained for chlamydial LPS as described in section 2.5.4. (see Figure 33)
Figure 33: Detection of chlamydial LPS in HeLa cells. HeLa cells were treated as follows:

a) untreated control, medium alone
b) cell lysate
c) heat killed *C. trachomatis* (MOI 0.1)
d) LPS (*Salmonella minnesota* R595), 1000ng/ml
e) and (f) *C. trachomatis* (MOI 0.1)

Scale bar = 50 μm.
Scale is the same for a – e, with a higher magnification of e shown in f.

72 hours post inoculation, cells were stained with fluorescent (FITC - green) antibody to chlamydial LPS. No staining is seen in untreated cells (a) and cells treated with cell lysate (b). Cells treated with *S. minnesota* LPS show minimal staining (c), probably due to cross reactivity with the antibody. Cells treated with heat killed *C. trachomatis* also show a small amount of staining (d), as chlamydial LPS will survive the heating process. Clear staining is seen in those cells treated with live *C. trachomatis* (e and f). *C. trachomatis* has a biphasic growth cycle. Infectious elementary bodies (EBs) enter the host cells and differentiate into larger reticulate bodies (RBs). The RBs divide within an expanding endosome resulting in an intracellular chlamydial inclusion. After a period of growth RBs reorganise into new infectious EBs that are released by cell lysis or exocytosis. A higher magnification of e is shown in f. Cell nuclei have been stained with propidium iodide. Two inclusions are seen, with a third inclusion possibly releasing EBs.
6.2.4. RNA extraction and Q-RT-PCR

After treatment cells were harvested in Tri reagent and cDNA prepared. Fold changes in amounts of mRNA for IL-8, CD14, TLR4, TLR9, HBD1, HBD2, HBD3, SLPI, MCP1, IL-1α, TGFβ, COX 2, PGDH, PGES, PGFS, MAL, MyD88, IκBα, HLA and CIITA were determined using quantitative real time PCR. (See section 2.4 for details of method). There were no significant differences between the 18S levels in the control or chlamydia infected samples (Figure 34a). When the mRNA expression of a ubiquitous nuclear gene, β actin, was compared to the mRNA expression for 18S there were no significant differences between the control and chlamydia infected samples (Figure 34b).
Figure 34: (a) No significant differences between 18S mRNA Ct values of control and chlamydia (MOI 0.1) infected cells as determined by Q-RT-PCR. Data represents the mean and s.e.m of 33 experiments (11 different primer/probe sets analysed). (b) No significant differences between expression of β actin and 18S mRNA were observed. Data represents the mean and s.e.m of three experiments.
6.2.5 SLPI ELISA
SLPI protein concentrations in cell culture supernatants were determined by ELISA as detailed in section 2.6.1.

6.2.6 PGE₂ ELISA
PGE₂ was measured in cell culture supernatants by ELISA as detailed in section 2.6.2.

6.2.7 IL-8 ELISA
IL-8 was measured in cell culture supernatants by ELISA as detailed in section 2.6.3.

6.2.8 Flow cytometric analysis
HeLa cells infected with *C. trachomatis* and uninfected control cells were harvested and labelled with a PC5-conjugated anti-CD14 antibody as described in section 2.7. Activated U937 cells, a monocyte cell line, were used as a positive control. Activation of U937 cells was achieved following treatment with TPA 100ng/ml for 72 hours. CD14 expression was analysed by flow cytometry using a Becton Dickenson FACScan (Mountain View, CA). Data is presented as forward scatter versus FL-3.

6.2.9 Statistical analysis
Significant differences of PCR and ELISA results were determined by analysis of variance (ANOVA; Statview 3.0). Fisher’s protected least significant difference (PLSD) was used to assign individual differences. P<0.05 was considered significant. Mean and s.e.m are presented.
6.3 Results

6.3.1 Up-regulation of IL-8 expression and components initiating the response following stimulation of HeLa cells

Treatment with *C. trachomatis* increased IL-8 mRNA expression and secretion of mature protein in a time- and dose-dependent manner (Figure 35a). Following infection at MOI of 0.1 the mRNA expression was maximal at 8 hours post infection, increased 200 fold (P<0.0001) and then decreased over the following 40 hours. MOI of 0.01 resulted in a lower and delayed response at 24 hours, with an 80-fold increase (P=0.0002). A parallel increase of IL-8 was seen in the supernatant with levels of 1590pg/ml at 48 hours after infection at MOI 0.1 (P<0.0001) and 831pg/ml after infection at MOI 0.01 (P<0.0001) (Figure 35b). Heat killed *C. trachomatis* and cell lysate also induced IL-8 mRNA expression and secretion of mature protein, but the response was of a smaller magnitude (Figure 36a and 36b). LPS had a non significant effect on IL-8 mRNA expression and protein secretion (Figure 36a and 36b).
Figure 35: Kinetics of IL-8 expression after infection with *C. trachomatis* serovar E (MOI 0.1 and 0.01), heat killed chlamydia (MOI 0.1), uninfected cell lysate and LPS (1000ng/ml and 100ng/ml). Cells were exposed to treatments for the indicated periods of time. Thereafter, the culture supernatant was stored, cells lysed and mRNA extracted. Data represents the mean and s.e.m of three experiments. (a) shows IL-8 mRNA expression. Each time point represents the fold change compared to the uninfected control sample (medium alone) at that time point. (b) shows IL-8 protein secretion into the culture supernatant of *C. trachomatis* infected cells as measured by ELISA. Letters indicate significant increases over control samples.

(a) a: P<0.0001, b: P<0.0001, c: P<0.0001, d: P=0.019, e: P=0.002
(b) a: P<0.0001, b: P<0.0001, c: P<0.0001, d: P<0.0001
**Figure 36:** Kinetics of IL-8 expression after treatment with heat killed chlamydia (MOI 0.1), uninfected cell lysate and LPS (1000ng/ml). Cells were exposed to treatments for the indicated periods of time. Thereafter, the culture supernatant was stored, cells lysed and mRNA extracted. Note different scale of x-axis compared to Figure 35. Data represents the mean and s.e.m of three experiments. 

(a) shows IL-8 mRNA expression. Each time point represents the fold change compared to the uninfected control sample (medium alone) at that time point. (b) shows IL-8 protein secretion into the culture supernatant as measured by ELISA. Letters indicate significant increases over control samples.

(a) a: P=0.0032, b: P=0.0092

(b) a: P=0.0440
6.3.2 Chemokine and cytokine mRNA up-regulated following infection with C. trachomatis

Pooled cDNA from the three experiments indicated that mRNA for MCP-1, IL-1α, TGFβ and COX-2 showed a trend to increased over 8 hours and then returned towards pre treatment levels (Figure 37 and 38). COX-2 mRNA expression was 11.4 fold above the control at 24 hours post infection (P=0.0002) (Figure 38a). In keeping with increased COX-2 mRNA expression, the trend of PGDH mRNA expression was observed to decrease and the PGES and PGFS to increase (Figure 38b). ELISAs were carried out for PGE₂ to determine the functional significance of the raised COX-2 mRNA expression, but levels had not increased significantly by 48 hours (data not shown).
Figure 37: mRNA expression of MCP-1, IL-1α and TGFβ shows a trend to increase up to 8 hours post infection with *C. trachomatis* serovar E (MOI 0.1). At the time points indicated cells were harvested and mRNA extracted. cDNA from three experiments was pooled and mRNA expression quantified. Data is presented as the fold change compared to the uninfected control sample (medium alone) at that time point using quantitative real time PCR.
Figure 38: Up-regulation of COX2 mRNA over 24 hours (a), with later increases in PGES mRNA and PGFS mRNA observed. The trend of PGDH mRNA expression is decreased over a similar time course (b). Cells were treated with *C. trachomatis* serovar E (MOI 0.1 or MOI 0.01) and then mRNA extracted at the time points indicated. Data for COX2 represents the mean and s.e.m (n=3). For PGDH, PGES and PGFS cDNA from three experiments was pooled and mRNA expression quantified. Data is presented as the fold change compared to the uninfected control sample (medium alone) at that time point using quantitative real time PCR.

Letters indicate significant increases over control samples.

(a) a: P=0.0212, b: P=0.0016, c: P=0.0002
For an innate response to be initiated TLRs are likely to play an important role. It was therefore decided to look at the expression of two of these, TLR9 and TLR4, and CD14 which complexes with TLR4. TLR9 mRNA expression was increased by 3.25 fold at 24 hours post infection with *C. trachomatis* (*P*=0.0275) (Figure 39). TLR4 expression did not change significantly over the 48 hours (Figure 39). In contrast the associated mRNA for CD14 clearly increased to a maximum 4 fold above the control at 4 hours (*P*<0.0001) (Figure 39). Flow cytometry was performed to assess CD14 expression on the HeLa cell surface. An activated monocyte cell line U937 showed 12% of cells being positive for CD14. Very low, if any, CD14 expression was observed on the HeLa cells and expression was not significantly changed 4 hours, 8 hours or 24 hours following chlamydial infection, with less than 1% of cells being CD14 positive in both the control and chlamydia infected HeLa cells (Figure 40).
Figure 39: Kinetics of TLR4, CD14 and TLR9 mRNA expression after infection with *C. trachomatis* serovar E (MOI 0.1). Each time point represents the fold change mRNA compared to the uninfected control sample (medium alone) at that time point. Data represents the mean and s.e.m (n=3), with the exception of data for TLR9 and TLR4 at 2 hours where n=2 and therefore no s.e.m shown.

Letters indicate significant increases over control samples.

a: P<0.0001, b: P=0.0275
Figure 40: CD14 expression as assessed by flow cytometry. Activated U937 monocytes express CD14 while HeLa cells have very low/absent expression, which is not increased after infection with \textit{C. trachomatis}.

(a) Unstained activated U937 cells (b) Activated U937 cells stained with an antibody for CD14, 12% of cells positive for CD14. (c and d) HeLa cells were stained with an antibody for CD14 4 hours, 8 hours and 24 hours post infection with \textit{C. trachomatis}. This shows the results at 8 hours post infection which were representative of samples at the other time points. (c) Uninfected HeLa cells (d) HeLa cells infected with \textit{C. trachomatis}.

Data is presented as forward scatter versus FL3, FL3 being used as the fluorochrome is PC5.
6.3.4 Downstream events following TLR ligation: up-regulation of Myd88, MAL and IκBα.

TLRs have a Toll/IL-1 receptor (TIR) domain and require an adaptor of signal transduction. Myd88 and Myd88-adaptor-like (MAL) are proteins with a TIR domain, which act as signal transducers. The mRNA for both Myd88 and MAL were up-regulated 1.75 fold 8 hours after infection with C. trachomatis (Figure 41a). This was not a significant rise over the control for MyD88, but was significantly higher than the control for MAL (P=0.05). Levels at 8 hours were significantly higher than levels at 2, 4 and 48 hours post infection for both MyD88 and MAL (P<0.03).

NF-κB has been implicated as a transcription factor following TLR ligation. IκBα expression was examined as a marker of NF-κB activation. By 8 hours this had increased 2 fold (P=0.002) and was increased by 3.8 fold at 24 hours (P<0.0001), suggesting NF-κB is activated following chlamydial infection. This increase in mRNA expression was not seen after LPS stimulation (Figure 41b).
Figure 41: (a) Up-regulation of Myd88 and MAL mRNA following treatment treated with C. trachomatis serovar E (MOI 0.1). Data represents the mean and s.e.m (n=3).
(b) Up-regulation of IkBα mRNA following treatment treated with C. trachomatis serovar E (MOI 0.1), but not after treatment with LPS (1000ng/ml). Data represents the mean and s.e.m (n=3), with the exception of samples at 2hours where no s.e.m is shown as n=2. Letters indicate significant increases over control samples.
(a) a: P=0.05 (b) a: P=0.002, b: P<0.0001, c: P<0.0001
mRNA expression of MHC class II (HLA-DR) and its transcriptional activator, CIITA, were examined to define whether the infected HeLa cells could be potential targets for recognition by activated CD4 T cells. The mRNA expression of CIITA increased 3.3 fold at 48 hours (P<0.0001) (Figure 42) but no parallel increase in HLA-DR mRNA expression was observed.
Figure 42: CIITA mRNA expression is up regulated after infection with *C. trachomatis* serovar E (MOI 0.1). However HLA-DR mRNA expression does not change substantially over the time course observed. Each time point represents the fold change mRNA compared to the uninfected control sample (medium alone) at that time point. Data represents the mean and s.e.m (n=3), with the exception of data at 2 hours where n=2 and therefore no s.e.m is shown.

Letters indicate significant increases over control samples.

a: P=0.0002, b: P<0.0001
6.3.6 Antimicrobial expression following C. trachomatis infection; up-regulation of SLPI

As expression of receptors involved in initiation of innate immune responses were modified by chlamydial infection, the expression of the natural antimicrobials HBD1, HBD2, HBD3 and SLPI were examined.

SLPI expression was initially down regulated, but at 24 hours was 2.6 fold above the control (P=0.0002) (Figure 43a). Stimulation with LPS induced a similar but non significant change in expression although the magnitude of the response was lower (Figure 43a). This increase in mRNA expression following chlamydial infection was associated with an increase in SLPI protein, as determined by ELISA (Figure 43b). While all supernatant samples had less than 0.05ng/ml of SLPI initially, after 48 hours uninfected control supernatant samples had a mean of 0.88ng/ml and chlamydia (MOI 0.1) treated samples had mean levels of 1.57ng/ml of SLPI in the cell supernatant.

Levels of HBD1 and 2 in Hela cells were almost undetectable and could not be induced by chlamydial infection (data not shown). HeLa cells expressed HBD3, but expression was not modified by chlamydial infection (data not shown).
**Figure 43:** Up-regulation of SLPI mRNA and protein expression after infection with *C. trachomatis*. Cells were exposed to treatments, *C. trachomatis* serovar E (MOI 0.1) or LPS (1000ng/ml), for the indicated periods of time. (a) SLPI mRNA expression. Each time point represents the fold change compared to the uninfected control sample (medium alone) at that time point. Data represents the mean and s.e.m (n=3), with the exception of samples at 2 hours where no s.e.m is shown as n=2. (b) SLPI protein secretion into the culture supernatant. Data represents the mean and s.e.m (n=3). Letters indicate significant increases over control samples.

(a) a: P=0.0002, b: P=0.0003

(b) a: P<0.0001
6.4 Discussion

Epithelial cells at mucosal surfaces are common targets for many intracellular pathogens, acting either as the principal host cell for replication or as a route of entry for dissemination to other sites of the body. *C. trachomatis* infects the epithelium of the genital tract and is responsible for over 90 million new cases of human sexually transmitted infections (STIs) worldwide each year (Beagley and Timms 2000). The design of a safe, effective vaccine has been hampered by the fact that *C. trachomatis* can cause a persistent infection with resulting pathology, mediated by the host inflammatory immune response (Beatty et al. 1994; Darville et al. 1997). It is therefore important to define the innate response following primary infection of epithelial cells, with a view to understanding how this could limit bacterial growth directly and also how it could shape the acquired immune response.

Secretion of IL-8 as a consequence of infection of HeLa cells with *C. trachomatis* has been shown previously (Rasmussen et al. 1997). This study was designed to demonstrate whether infection was required for IL-8 induction, or if exposure to dead organisms or the products of dead cells would induce the same effects. Figures 35 and 36 show that although infection induces the greatest increase in IL-8 production, it is not an absolute requirement. Heat killed organism and cell lysate both elicited a response, albeit reduced, when compared to the effect of the live organism. Techniques commonly used to evaluate *C. trachomatis* measure multiplicity of infection and therefore live organisms, the contribution of dead organisms and their components therefore remain unknown. We show that these components do contribute to responses observed to a small extent. Heat killed chlamydia will have intact heat stable components such as LPS which may be initiating effects. *S. minnesota* R595 is representative of and has been shown to be more potent than *C. trachomatis* LPS (Ingalls et al. 1995). LPS alone had the least effect on IL-8 expression suggesting any role that it does play in initiating the response to *C. trachomatis* is minimal. Other components of *C. trachomatis*, such as CpG motifs or heat shock proteins that may mediate this response were not examined
and could be the subject of further studies. Interestingly the cell lysate, which was devoid of chlamydia - acting as a control for cellular components which would be produced in the natural course of the lytic life cycle of C. trachomatis, also caused an increase in IL-8 expression. Cell damage and resulting debris may act as “danger” signals (Gallucci and Matzinger 2001; Matzinger 1994), alerting the body to potential pathology and may play a part in propagating the inflammatory response in the genitourinary tract. Sauter et al (Sauter et al. 2000) present data showing selective activation of dendritic cells when exposed to necrotic cells, but not when exposed to apoptotic cells, with the differentiation between processes putatively carried out via PRR. This data suggests that exposure of epithelial cells to cell products can also provide an activating signal, although the nature of recognition remains to be defined.

Toll-like receptors are a family of PRRs, to date ten TLRs have been described. Ligands have been described for 5 of these TLRs; TLR2 (bacterial lipoproteins and peptidoglycans), TLR3 (double-stranded RNA), TLR4 (lipopolysaccharide (LPS) and HSP60), TLR5 (flagellin), and TLR9 (unmethylated CpG DNA motifs) (Underhill and Ozinsky 2002). The most relevant of these to chlamydial detection appear to be TLR4 and TLR9. The exact mechanisms of recognition have not been elucidated for all the TLRs and may be via direct binding or via co-ligands such as CD14 and MD2, as in the case of TLR4 (da Silva Correia et al. 2001; Shimazu et al. 1999; Wright et al. 1990). In the present study mRNA expression of TLR4 was not greatly increased, while its co-receptor CD14 mRNA was up-regulated within 4 hours of infection. This supports data from Heinemann et al 1996 (Heinemann et al. 1996) who showed that monocytes infected with C. pneumonia up-regulated CD14 surface expression 8 hours post infection. The fact that TLR4 mRNA expression was not induced, correlates with the data presented here showing that LPS does not stimulate the maximal response in terms of IL-8 secretion (see Figure 36). Previous work has been unable to detect TLR2 or TLR4 mRNA in HeLa cells infected for 12 to 48 hours with C. trachomatis serovar E (Dessus-Babus et al. 2002). Fichorova et al (Fichorova et al. 2002) report a lack of TLR4 mRNA expression in immortalised human endocervical epithelial cells. They
suggest that CD14 can act as a co-receptor for non-TLR4 ligands, enhancing the sensitivity of genital tract epithelial cells to bacteria. Alternatively it has been suggested that TLR4 and CD14 are not solely responsible for LPS recognition and a number of other molecules (depending on the cell type), such as CD11b/CD18, CD66, HSP70 and HSP90 might be involved (Triantafilou and Triantafilou 2002). In other systems such as the gut it has been suggested that TLR4 down regulation may be a protective mechanism where cells are continually exposed to LPS and a constant inflammatory response would be deleterious (Abreu et al. 2001). Chlamydial DNA unmethylated CpG motifs, which are recognised by TLR9, are possible candidate PAMPs. This study demonstrated that mRNA expression of TLR9 increased 4 hours following inoculation with C. trachomatis, and was maximal at 24 hours. Prior to this study, TLR9 expression had not been described in cervical epithelial cells.

Downstream signalling events after ligand binding to the TLR follow those seen after activation of the IL-1R, culminating in the nuclear translocation of NF-κB (Cario et al. 2000; Kopp and Medzhitov 1999; Muzio et al. 1998). The data presented here shows small increases in the signal transduction proteins MyD88 and MAL, which are known to activate NF-κB (Fitzgerald et al. 2001). NF-κB is a transcription factor involved in inflammatory and immune responses (Baldwin 1996). IκBα is an endogenous inhibitor of NF-κB, whose expression is increased by activation of NF-κB, resulting in a negative feedback loop (Sun et al. 1993). These experiments show an increase in the expression of IκBα suggesting activation of the NF-κB pathway. Many of the cytokines, chemokines and antimicrobials examined in these experiments have NF-κB binding sites in their gene sequences including IL-8 (Mukaida et al. 1991), COX-2 (Schmedtje et al. 1997) and HBD2 (Diamond et al. 2000). NF-κB may therefore play a crucial part in the initiation of the cascade of results observed.

Infection of epithelial cells initiates a cascade response, which activates the innate immune response. The MHC class II transactivator (CIITA) is the major regulator of expression of MHC class II genes and therefore plays a fundamental role in the
regulation of the adaptive immune response. It is interesting to observe that the adaptive immune response appears to be up-regulated, with an increase in CIITA mRNA expression, but without MHC II mRNA up-regulation. This may be because it is too early in the cycle of events to observe these effects. This model system is limited in that there are no other complementary cells present. In vivo, natural killer cells, which infiltrate the area following infection, would be a source of IFN-γ. IFN-γ is known to play two roles in chlamydial infection, firstly it can regulate chlamydial growth (Beatty et al. 1993), arresting growth at the EB stage and secondly IFN-γ is an inducer of MHC class II. Alternatively C. trachomatis may be affecting host genes and down regulating MHC class II expression. Zhong et al (Zhong et al. 2001; Zhong et al. 1999) have demonstrated that C. trachomatis can down regulated MHC II through the degradation of upstream factor-1 (USF-1, a transcription factor required for IFN-γ induction of CIITA). This may be a mechanism whereby C. trachomatis attempts to evade recognition by activated T cells, leading to low grade persistent infection.

Infection with C. trachomatis induced mRNA expression of both intracellular (IL-1α, COX 2) and soluble (IL-8, MCP-1, TGFβ) mediators of the innate immune response. IL-8 and MCP-1 have chemotactic properties for both monocytes and NK cells, and may therefore provide a link between the innate and adaptive immune responses. Intracellular gene products such as PGE₂, following the increased expression of COX-2, may act in an autocrine or paracrine fashion to further modify the immune response (Yu and Chadee 1998). PGE₂ has been demonstrated to up-regulate CD14 expression in mouse macrophages and may thus maintain or amplify the immune response (Iwahashi et al. 2000). It is interesting to note that the mRNA expression of all of these mediators shows a trend to decrease by 48 hours post infection, after an initial trend of up-regulation. It was thought that this might be due to secretion of anti-inflammatory cytokines such as TGFβ but mRNA expression of this cytokine also decreased after 24 hours. Dessus-Babus et al (Dessus-Babus et al. 2002) suggest that IL-11 may exert anti-inflammatory activity, showing that HeLa cells secrete increased levels of IL-11 following infection with the disseminating serovar L2 strain of C. trachomatis compared
to levels seen with the non disseminating serovar E strain. Other markers such as those involved in the adaptive immune response detailed above did not decrease suggesting that the cells continue to be capable of mRNA synthesis. The housekeeping genes 18S and β actin were also observed to remain constant over time (see Figure 34). Whether this observed down regulation of mRNA expression is due to the inherent instability of mRNA or whether there is some as yet undefined anti-inflammatory mechanism C. trachomatis has evolved to dampen down the acute inflammatory response, thereby improve the chances of establishing a persistent infection, is uncertain.

SLPI, a protease inhibitor that has antimicrobial activity, was up-regulated in this study and may play a role in limiting chlamydial invasion of epithelial cells. In addition to antimicrobial effects, SLPI also inhibits the NF-κB signal transduction pathway (Jin et al. 1997; Lentsch et al. 1999) and may be limiting the inflammatory response to C. trachomatis in an attempt to protect the host while at the same time potentiating the pathology caused by infection. Due to their low ionic strength α and β defensins are likely to exert their effects intracellularly in phagocytic vacuoles, or extracellularly at the skin surface and at mucosal surfaces (Yang et al. 2002). Natural antimicrobials are thought to exert their effects through the disruption of microbial membrane integrity (Risso 2000). In vitro β defensin 2 has been up-regulated following stimulation with LPS and bacterial lipoprotein. This has been dependent on CD14 and TLR2 expression respectively (Becker et al. 2000; Birchler et al. 2001). Furthermore, human defensin and porcine protegrin have been shown to protect McCoy cells from infection by C. trachomatis, by damaging the membrane and allowing leakage of cellular contents (Yasin et al. 1996). The antimicrobials may therefore play an important part in resisting infection. The expression of the β defensins in HeLa cells was low, and was not up-regulated by infection. Islam et al (Islam et al. 2001) have demonstrated that mRNA expression of some antibacterial peptides, including HBD1 can be down regulated in enteric Shigella infections. It may be that chlamydia is employing a similar strategy of down regulation and hence self-preservation.
Recognition of pathogens and resultant sequelae at mucosal surfaces can occur through a variety of processes. This data suggests that LPS is not a predominant ligand in the activation of epithelial cells as a primary defence mechanism to *C. trachomatis*. Other components such as bacterial DNA or cellular debris released as a consequence of the lytic cycle of *C. trachomatis* may play a role. Figure 44 summarises the effects observed in this study following *in vitro* infection of a cervical epithelial cell line with *C. trachomatis*. A better understanding of the early immune responses to *C. trachomatis* provides insight into the pathogenesis of infection and allows consideration and development of rational control strategies.
Figure 44: A summary of the effects observed in this study following in vitro infection of a cervical epithelial cell line (HeLa) with *C. trachomatis*.

Components of *C. trachomatis*, such as LPS, HSP60 or CpG DNA, will interact with Toll-like receptors. Co-factors such as CD14 and MD2 will be recruited if required. A signal will be transduced intracellularly via adaptor proteins including MyD88 and MAL with a cascade of events following culminating in the activation of NFκB with phosphorylation and subsequent degradation of IκBα. NFκB translocates to the nucleus where a series of genes are activated.

This study shows an increase of mRNA for TLR9 and CD14, which are part of the recognition pathway. MyD88 and MAL mRNA are up-regulated and are part of the signal transduction pathway. IκBα is an inhibitor of NFκB and IκBα mRNA is up-regulated as part of the negative feedback response following activation of NFκB. Finally mediators of the immune responses, cytokines, chemokines and natural antimicrobials are up-regulated. Following an initial up-regulation of gene expression, decreased mRNA expression of most mediators examined was observed.
Extracellular

- LPS
- HSP60
- CpG DNA

Intracellular

- MD-2
- TLR4
- CD14

MyD88/MAL^ Map kinases
-TrAF6

NFkB

Pro-inflammatory cytokines, IL-1
Chemokines, IL-8, MCP-1
Prostaglandins, COX2 - immunomodulation
Natural Antimicrobials, SLPI
Adaptive immune responses, CIITA -
?HLA class II and antigen presentation
Chapter 7

General Discussion
This chapter summarises the results obtained in this thesis. Detailed discussion accompanied each chapter and therefore the wider context of this work is discussed. Finally, suggestions for future study are presented.

7.1 Synopsis of Results

The aim of this thesis has been to characterise and investigate the control of natural antimicrobial peptides in the female reproductive tract. Work has concentrated on uterine tissue: endometrium, first trimester decidua, a cervical cell line; and briefly vaginal and salivary secretions. In the course of study the role of the pattern recognition receptor family, the Toll-like receptors (TLRs) as an integral part in the recognition of and initiation of a response to pathogens was realised and this role was investigated further. A schematic diagram of possible interactions between these components of the innate immune system is shown in Figure 45.

The presence of mRNA for the natural antimicrobial peptides, HBD1, HBD2, SLPI and granulysin in the endometrium has been described and immunohistochemical studies carried out to localise SLPI protein expression. Expression of all the natural antimicrobials displayed cyclical variation. HBD1 was maximal in the mid secretory phase, granulysin in the late secretory phase, HBD2 in the menstrual phase and SLPI in the late secretory and menstrual phases. Similarly the presence of mRNA for HBD1, SLPI and granulysin but not HBD2 within first trimester decidua was described. None of the antimicrobials under investigation were expressed in trophoblast tissue. Exogenous sex steroid hormones in the form of the combined oral contraceptive pill and the levonorgestrel intrauterine system significantly decreased mRNA expression of HBD1 and 2 and granulysin within endometrial tissue. mRNA levels of SLPI were not significantly altered by the combined oral contraceptive pill or the levonorgestrel intrauterine system. There was no significant effect of chlamydial infection on natural antimicrobial mRNA expression in first trimester decidual samples. Preliminary work was undertaken to investigate the possibility of using salivary measurements of natural
antimicrobials as non-invasive surrogate markers of natural antimicrobial secretion in the genital tract, but no correlations between levels of SLPI in saliva and vaginal secretions were found.

*In vitro* studies investigated the expression of the natural antimicrobials, SLPI, HBD1 and HBD2, the Toll-like receptors, TLR4 and TLR9 and the co-receptor CD14 in primary endometrial epithelial cells and two endometrial epithelial cell lines (MFE and Hes). The role of bacterial (Gram negative - lipopolysaccharide and Gram positive – lipoteichoic acid) and viral (polyinosinic-polycytidylic acid) mimics of infection and some inflammatory mediators, including prostaglandins, IFNγ, and combinations of IL-1β and TNFα in the regulation of natural antimicrobials and TLRs in human endometrial epithelial cells was determined. LPS and poly I:C increased the mRNA expression of HBD1 while decreasing the mRNA expression of SLPI. LTA had no significant effect on the expression of the natural antimicrobials. The response to treatment with LPS was augmented in the presence of human serum, which may be providing required co-factors such as CD14 or undefined cytokines. The prostaglandins, PGE₂ and PGF₂α, did not alter natural antimicrobial, TLR4, TLR9 or CD14 expression in primary endometrial epithelial cells. However PGE₂ and IFNγ increased TLR4 mRNA expression in the endometrial epithelial cell line Hes and TNFα and IL-1β increased TLR4 mRNA expression in the endometrial epithelial cell line MFE. LPS increased CD14 expression in Hes cells, while decreasing CD14 expression in MFE cells. TLR9 expression was not altered by any of these treatments.

Having examined the effects of mimics of infection, work was undertaken to characterise early events following infection of a cervical epithelial cell line (HeLa) with *C. trachomatis* in vitro. Both live organisms and heat-killed organisms initiated IL-8 and COX-2 production suggesting that invasion of host cells and subsequent multiplication of the organism are not essential to induce an inflammatory response. LPS appeared to play a minor role. Interestingly, products of lysed cells, which would be produced during the natural lytic cycle of the organism also induced IL-8 production.
HeLa cells expressed mRNA encoding TLR4 and TLR9, and CD14. TLR9 and CD14 mRNA expression were up-regulated by *C. trachomatis* infection, no effect was seen on TLR4 mRNA expression. mRNA for the Class II transactivator was increased, while MHC class II expression was unchanged up to 48 hours post infection. The natural antimicrobial SLPI was up-regulated while HBD1 and HBD2 were not expressed by HeLa cells. HBD3 mRNA expression was unaffected by *C. trachomatis* infection.

Expression profiles of the natural antimicrobials and Toll-like receptors suggest that they contribute to the innate immune armoury of the female reproductive tract. Further studies are required to take these concepts and convert them into clinically applicable treatments within reproductive health care.
Figure 45: The role of the Toll-like receptors and the natural antimicrobials in response to infection. The mucosal epithelial surface of the female reproductive tract is exposed to commensal and pathogenic organisms. Pattern recognition receptors such as the Toll-like receptors putatively differentiate between these organisms and initiate a cascade of intracellular signals, resulting in effector mechanisms that can be both beneficial and detrimental. The natural antimicrobials are an effector mechanism of the innate immune response. Natural antimicrobials may exert a direct antimicrobial action and/or via their chemotactic activity provide a link to the adaptive immune response. The response can be further modulated by internal and external factors such as the sex steroid hormones and inflammatory mediators.
Commensals
Pattern recognition receptor:
e.g. Toll-like receptors
Mucosal surface epithelial cells

Response
Non specific, innate response
- Natural antimicrobials
- Cytokines/chemokines
Specific, acquired response
- Leukocytes

Modifiers of response
- Endogenous
- Exogenous
- Local inflammatory mediators

Beneficial and/or detrimental

Pathogens

Pattern recognition receptors:
e.g. Toll-like receptors
7.2 Discussion: The wider context

Research work is undertaken to further knowledge and understanding of the environment. Questions are answered and further questions arise. Ultimately within medical research it is hoped that the understanding gained may lead to benefits for others, through the design of medical interventions. The studies in this thesis increase our understanding of a component of the innate immune events within the female reproductive tract. As the use of synthetic antibiotics are more widely and commonly used the resistance of organisms to the antibiotics that we have is becoming of greater concern (Nollette 2000). The discovery of natural antimicrobial substances has opened up new areas of possibility for the treatment of infections. These treatments could be utilised both in the non-pregnant and pregnant state to prevent or treat sexually transmitted infections. The potential maternal and fetal sequelae of these infections might therefore be attenuated.

Within the female reproductive tract it has been envisaged that the natural antimicrobials may make up an important component of topical microbicides. An ideal microbicide should be broad spectrum, inexpensive, easy to use, safe to use and easy to store. Initial interest focused on existing spermicides, which reportedly have microbicidal activity, such as nonoxynol-9 (N-9) and benzalkonium chloride (Belec et al. 2000), both detergent like substances. The safety of these compounds, particularly N-9, has been called into question. N-9 can inactivate many sexually transmitted pathogens including *C. trachomatis* and *N. gonorrhoea* (Cook and Rosenberg 1998; Patton et al. 1992), however, it has been demonstrated to cause vaginal irritation, inflammation, immune cell infiltration and changes in the natural vaginal flora. N-9 may therefore enhance the infectivity of some microbes, particularly HIV (Fichorova et al. 2001). Investigators are therefore exploring new potential microbicides such as sulphated polysaccharides (Pauwels and De Clercq 1996; Pearce-Pratt and Phillips 1996) and natural antimicrobials.
Natural antimicrobials under investigation include the insect derived cecropins and the vertebrate derived magainins and protegrins. The cecropins have been shown to have anti-chlamydial activity in vitro (Ballweber et al. 2002). A synthetic 22 amino acid analogue of the magainin peptide from the African clawed frog has undergone testing as an antifungal, but has not got FDA approval. Dr R. Lehrer (California, USA) is undertaking work developing protegrins for clinical treatment, and a pharmaceutical company, Intrabiotics, is developing a protegrin derived antimicrobial. The limitation of the defensins as antimicrobials is that they are pH and salt sensitive (Bals et al. 1998; Goldman et al. 1997; Singh et al. 1998). Defensins are also inactivated by serum (Yasin et al. 1996). Yu et al (Yu et al. 2000) have therefore been designed salt insensitive α defensins, and these compounds still have wide antimicrobial activity. Theta defensins have been described in rhesus monkeys. The theta defensins are cyclical peptides formed by the ligation of two α defensins, which results in a peptide with greater antimicrobial activity (Tang et al. 1999). The natural antimicrobials, although small peptides, are larger than other agents used therapeutically. This and their positive overall charge makes the penetration of these substances into tissues slow. Smaller ring peptides, which penetrate tissues more effectively have therefore been developed (Fernandez-Lopez et al. 2001). These limitations will need to be further addressed before these compounds could be used within the female reproductive tract. The expense of large-scale production of these peptides has also been a limitation, although synthesis of neutrophil defensins has been achieved (Raj et al. 2000).

Another approach to use of natural antimicrobials would be to develop compounds that induce local production of natural antimicrobials. A synthetic salicylic acid-like compound has been described to induce antimicrobial peptides in plants (Schroder and Harder 1999). Isoleucine, an essential amino acid, induces HBD2 expression and is a candidate for further investigation (Fehlbaum et al. 2000). It has been suggested that the amino acid may work by binding to a Toll-like receptor. A number of inflammatory cytokines, such as IFNγ, IL-1β and IL-1β + TNFα, induce antimicrobial expression within human endometrial epithelial cells (King et al. 2002). The limitation of these
molecules is the cascade of other inflammatory events that they initiate which may cause detrimental effects if not controlled. The studies presented in this thesis examine the role of mimics of infection such as LPS as potential inducers of natural antimicrobial activity. Once again the limitation of these compounds is the other inflammatory events that are potentially stimulated.

Mimics of infection, LPS and LTA, are examples of pathogen associated molecular patterns (PAMPs) that act as ligands for pattern recognition receptors (PRR), particularly the TLRs. Signalling events after ligation of the receptors initiate gene expression. The TLR pathway could therefore be a target for manipulation and modulation. Shock due to Gram-negative bacterial sepsis is a consequence of an acute inflammatory response to LPS. A stabilised endotoxin antagonist has been developed which can block the symptoms of experimentally induced septic shock (Bunnell et al. 2000; Christ et al. 1995). This antagonist blocks TLR4-mediated transgene activation in a dose-dependent manner (Chow et al. 1999). Others have also examined the potential of agonists and antagonist to TLR4 as a mechanism to influence the inflammatory processes. C. trachomatis LPS is a weak inducer of an inflammatory response (Ingalls et al. 1995). Sweet et al (Sweet et al. 2001) therefore re-engineered the LPS containing Lipid A of C. trachomatis, expressed it in an E. coli mutant and determined the residues responsible for the poor induction of inflammatory response. They suggest a possible future strategy would be to delete and replace this area with the complimentary region from E. coli, which may result in a more effective immune response to C. trachomatis.

Epithelial cells release antimicrobial peptides in response to PAMPs such as LPS and LTA (Becker et al. 2000; Birchler et al. 2001; Diamond et al. 1996) and these responses are putatively TLR dependent. TLRs can also induce differential gene expression, resulting in the release of distinct cytokine patterns which may then direct the adaptive immune response (O’Neill 2002). Further research into this area is required, but it could be envisaged that once microbial stimulators of specific cytokine responses are known,
with resultant beneficial or detrimental immune responses, these responses could be modified to the host's benefit.

The application of studies defining components of the innate immune system are not limited to the site at which they have been defined, such as within the female reproductive tract, but can be used to help understand immune responses at other mucosal surfaces. Treatments developed for use at one site might well be applicable for use at other sites or in other situations. It is hoped that the research described in this thesis adds to our understanding and will provide the basis for further studies and possibly, with development, future clinical interventions.
7.3 Suggestions for future studies

1) The contraceptives studied, COCP and LNG-IUS, significantly decreased mRNA expression of natural antimicrobials. There are wide ranges of effective contraceptives available at present. Future contraceptives need to either provide improved side effect profiles, or additional benefits to the user. A contraceptive designed with an optimal antimicrobial profile would have such additional benefits. There are two ways in which to address this problem; firstly when a new contraceptive is designed, trials should assess the effects of the method on components of the immune system including the natural antimicrobials. Studies developing new contraceptives are ongoing and are incorporating this approach into the assessment of new contraceptives. The other approach would be to take existing available contraceptives and modify them to enhance their antimicrobial activity.

2) Preliminary results looking for correlations between antimicrobial secretions at different mucosal surfaces were inconclusive. Techniques to determine the levels of antimicrobials other than SLPI, particularly the β defensins, need to be optimised. Studies could then be extended to examine expression of the natural antimicrobials in situations of acute infection. The role of the natural antimicrobials through pregnancy could also be assessed in longitudinal studies. Antimicrobial levels at different mucosal sites could be determined at different stages through pregnancy and the pregnancy outcomes evaluated, possible markers for adverse pregnancy outcomes may be defined.

3) The research on Toll-like receptors is in its infancy and future studies need to continue to define the roles of these receptors in the female reproductive tract more clearly. Down stream events following receptor ligation need to be assessed and the therapeutic potential of these receptors investigated.


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### Appendix I: Source General Materials

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<th>Tissue Collection and Culture</th>
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<td>Pipelle suction curette</td>
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<td>Epithelial growth medium</td>
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**RNA Extraction**

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**RT-PCR**

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Appendix II: Recipes for solutions

All chemicals listed were from Sigma and all dilutions were in distilled water unless otherwise stated.

1. Blocking/protecting solution

In 1 litre:
- 20g 2% polyvinylpyrrolidone
- 5g bovine serum albumin
- 1ml preservatives Boehringer Mannheim
- 1.9g EDTA (5 mmol/L)
- 6.1g Tris (50 mmol/L)

2. Complete medium (cRPMI)

500ml RPMI 1640 supplemented with:
- 10% FBS
- 20μg/ml gentamycin
- 100 IU/ml penicillin
- 100μg/ml streptomycin
- 2 mM L-glutamine

3. Chlamydia Transport Medium (Mordun Research Institute scientific services)

373g sucrose
2.6g KH₂PO₄
6.2g K₂HPO₄
3.6g L-glutamic acid

made up to 5L in deionised water, supplemented with:
- 20ml of 0.4% phenol red solution
- 500ml FBS Gibco
- 25ml gentacin Gibco
- 0.5g streptomycin Gibco
- 25ml 10 000U/ml Nystatin suspension
4. Epithelial growth medium
500ml cRPMI supplemented with
- 25ng/ml epidermal growth factor
- 1ng/ml vascular endothelial growth factor
- 5ng/ml basic fibroblast growth factor
- $10^{-8}$ mol/l oestradiol

5. ELISA buffer
In 1 litre:
- 9g NaCl (150mmol/L)
- 12.1g Tris (100mmol/L)
- 300μl phenol red solution (0.00015%)
- 0.7g EDTA (2mmol/L)
- 150mg 2-methylisothiazolone (1mmol)
- 150mg bromonitrodioxane (1mmol)
- 2g BSA

pH 7.2

6. SLPI ELISA buffer, as ELISA buffer above with additional:
- 0.05% Tween-20
- 0.01% hexadecyltrimethylammonium bromide

7. ELISA substrate
1ml tetramethyl benzidine : 1ml urea-hydrogen peroxidase : 10ml sodium acetate

5g/l urea-hydrogen peroxidase (0.5% in 50mM sodium acetate, pH6)
3g/l tetramethyl benzidine in dimethylformamide
in 100mmol/l sodium acetate, pH6

100mM sodium acetate
In 1 litre: 13.6g sodium trihydrate
1ml preservatives
pH 6
8. ELISA wash buffer
In 1 litre: 9g NaCl (150mmol/L)
1.21g Tris (5mmol/L)
0.5ml Tween-20 (0.025%)
\[\text{pH 7-7.5}\]

9. Methyloximating solution
10mg/ml methoxyamine hydrochloride
10% ethanol
1M sodium acetate
\[\text{pH 5.6}\]

10. Neutral buffered formalin (NBF)
In 1 litre: 6.5g Na$_2$HPO$_4$ BDH
4.5g NaH$_2$PO$_4$.2H$_2$O BDH
100ml 40% formaldehyde
900ml distilled water

11. Phosphate buffered saline (PBS)
In 1 litre: 5 PBS tablets
\[\text{pH 7.4 – 7.6}\]

12. PBS + Tween
In 1 litre: 5 PBS tablets
8g NaCl
100μl Tween-20
\[\text{pH 7.4 – 7.6}\]

13. Preservatives
200mg/ml 2-methylisothiazolone
200mg/ml bromonitrodioxane
in dimethylformamide (DMF) / dimethylsulphoxide (DMSO) 1:1
diluted 1:1000 to use
14. **0.1M Sodium citrate buffer**

In 1 litre: 29.4g Tri-sodium citrate BDH
0.1g sodium azide
pH 6
Diluted 1:10 to use

15. **TE Buffer**

10mmol/l Tris pH8.0
1mmol/l EDTA in DEPC water

16. **Tris buffer**

In 1 litre: 121.1g Trizma base
pH 7.2
Appendix III: HBD2

Immunohistochemistry method

5μm paraffin sections were dewaxed in histoclear and rehydrated in descending grades of alcohol. After washing, an antigen retrieval step was performed. Sections were pressure cooked for 5 minutes in 0.01M sodium citrate (pH6), left to cool for 20 minutes and then washed in PBS for 10 minutes. Frozen sections were fixed in NBF for 10 minutes and then washed in PBS for 10 minutes. All sections where then treated with 3% hydrogen peroxide in distilled water for 10 minutes to block non-specific endogenous peroxidase activity. Sections were then washed for 10 minutes in PBS. Additionally in one run, to reduce non-specific staining due to endogenous biotin, an avidin-biotin blocking step was carried out. Sections were incubated in avidin for 15 minutes at room temperature, then washed in PBS, followed by an incubation with biotin for 15 minutes at room temperature. Depending on the primary anti-HBD2 antibody subsequently used, a non-immune block of diluted horse or goat serum was then applied for 20 minutes in a humidified chamber at room temperature. In an attempt to further reduce non-specific staining, on one occasion a rabbit serum non-immune block with 5% bovine serum albumin (BSA) was used in place of the horse serum. The non-immune block was then carefully removed and the primary anti-HBD2 antibody applied. The two antibodies tested were:

i) HBD2 goat polyclonal diluted 1:50, 1:100, 1:150 and 1:200 in horse serum incubated for 1 hour at 37°C

ii) HBD2 rabbit polyclonal diluted 1:100 in goat serum incubated overnight at 4°C

The primary antibody was substituted with an equimolar concentration of goat or rabbit immunoglobulin (IgG) respectively in negative control sections.

After the primary incubation, sections were washed for 10 minutes between each stage with PBS + Tween. Antibody binding was detected by the application of biotinylated horse anti-goat IgG or goat anti-rabbit IgG as appropriate, followed by an avidin-biotin-peroxidase complex (Elite ABC), both for 60 minutes at room temperature. The protocol was then completed as for SLPI (section 2.5.1).
Appendix IV: Conference Proceedings

D.C. Fleming, G. Entrican and R.W. Kelly: Mediators of the Response to *Chlamydia trachomatis* in Epithelial cells. Poster: *Society for Gynecologic Investigation, Los Angeles, USA, 2002*

D.C. Fleming, G. Entrican and R.W. Kelly: Innate immunity and cytokine response to *Chlamydia trachomatis*. Poster: *British Society of Immunology, UK, 2001*


Appendix V: Publications


Regulation of natural antibiotic expression by inflammatory mediators and mimics of infection in human endometrial epithelial cells

Anne E. King1,3, Diana C. Fleming1, Hilary O. D. Critchley2 and Rodney W. Kelly1

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The natural antibiotic molecules, β-defensins 1 and 2 (HBD1/2) and secretory leukocyte protease inhibitor (SLPI), have an important role in mucosal defence and are present in the uterus. This study details their regulation in primary endometrial epithelial cells and in two endometrial cell lines (MFE/HES). Cells were treated with proinflammatory molecules and mimics of infection [lipopolysaccharide (LPS) and lipoteichoic acid (LTA)]. mRNA for HBD1, HBD2 and SLPI was detected in primary endometrial epithelial cells using real-time quantitative PCR. HBD1 mRNA was present at very low levels preventing conclusive study of its regulation. However, HBD2 mRNA expression was increased by interferon-γ, interleukin (IL)-1β alone and IL-1β + tumour necrosis factor (TNF)-α. SLPI mRNA was not affected by proinflammatory mediators, although protein levels fell in the presence of IL-1β + TNFα. LPS had little effect on antimicrobial expression. However, there was a trend towards increased expression with LTA treatment for 4–8 h. Antimicrobial expression in endometrial cell lines was similar to that in primary cells, although SLPI was increased by IL-1β + TNFα treatment. These results suggest that in endometrium some natural antibiotics (e.g. SLPI) may be constitutively expressed providing a basal level of protection, while others (e.g. HBD2) are inducible allowing maximal antimicrobial activity during infection. Natural antimicrobials will have an important role in endometrium in protecting against infection.

Key words: defensins/endometrium/infection/inflammatory mediators/SLPI

Introduction

Successful human implantation and pregnancy are reliant on the prevention of genital tract infections that can compromise both fertility and the fetus itself. The molecules of the innate immune system, present at mucosal surfaces, are likely to be crucial in the limitation of such infections.

Natural antimicrobial molecules are small, cationic peptides often present at epithelial surfaces (Huttner and Bevins, 1999; Hancock and Diamond, 2000). These molecules are a crucial component of the innate immune system offering a broad range of antibacterial, antiviral and antifungal protection. In the human there are two major groups: the α and β defensins. The α-defensins 1–4 [human neutrophil peptide (HNP) 1–4] are present in neutrophils while α-defensins 5 and 6 [human defensin (HD) 5/6] are located in the Paneth cells of the gut and at other epithelial surfaces. The β-defensins [human beta defensin (HBD) 1–4] are located at epithelial surfaces and are likely to play a major role in mucosal defence. Similarly, secretory leukocyte protease inhibitor (SLPI), a neutrophil elastase inhibitor with antimicrobial activity, is present at mucosal sites and may have an anti-infective role (Franken et al., 1989; Tomee et al., 1998).

Several natural antimicrobials have been detected in the human endometrium. HBD1, HD5 and SLPI have been detected in endometrial epithelium, with both HD5 and SLPI showing maximal expression in the secretory phase of the menstrual cycle (Quayle et al., 1998; Valore et al., 1998; King et al., 2000). SLPI is also present in first trimester and term decidua (Denison et al., 1999; King et al., 2000). HBD2 and 4 have been detected in the uterus (Bals et al., 1998; Garcia et al., 2001), while HBD1 and 3 have been located in placenta and placental membranes respectively (Zhao et al., 1996; Jia et al., 2001). The presence of these molecules in endometrium at the time of implantation and during pregnancy suggests a role in the protection of the fetus from infection.

Several of the natural antibiotics are regulated by inflammatory molecules such as interleukin (IL)-1, tumour necrosis factor (TNF)-α and lipopolysaccharide (LPS) in systems such as lung, gut and gingiva (Sallenave et al., 1994; Diamond et al., 1996; Jin et al., 1998; O’Neil et al., 1999; Harder et al.,
A.E. King et al.

2000; Krisanaprakornkit et al., 2000). Conserved motifs on pathogens, pathogen-associated molecular patterns (PAMPs), such as LPS can be used to mimic infection. The control of natural antimicrobial expression in endometrium is unclear, although the IL-1 system is thought to be active in the glandular epithelium (Simon et al., 1993). This study investigates the role of inflammatory molecules and bacterial products associated with infection in the regulation of natural antibiotics in human endometrial epithelial cells.

Materials and methods

Tissue collection

Endometrial biopsies were collected from women undergoing gynaecological procedures for benign conditions. All women reported regular menstrual cycles (25–35 days) and had not received any form of hormonal treatment in the 3 months preceding biopsy. Biopsies were dated according to the patient’s last menstrual period (LMP). Histological dating according to published criteria (Noyes et al., 1950) and circulating sex steroid concentrations were consistent with the date of LMP. Written informed consent was obtained from all patients prior to biopsy collection and ethical approval was received from the Lothian Research Ethics Committee.

Tissue samples were collected in RPMI 1640 medium (Sigma, Poole, Dorset, UK) and were subsequently divided into two portions. Endometrium was: (i) fixed in 10% neutral buffered formalin overnight at 4°C, stored in 70% ethanol and then wax embedded; and (ii) separated into glandular and stromal compartments for cell culture.

Separation of endometrial biopsies into glandular and stromal compartments

This method for separation of glandular and stromal compartments of endometrium was adapted from that of Osteen et al. (Osteen et al., 1989). Several modifications were made and the details of the method used in this study are described below. Endometrial biopsies were washed twice in phosphate-buffered saline (PBS; Sigma), sliced into small fragments, immersed in collagenase/DNAase (1 and 0.1 mg/ml; both Sigma) and incubated for 80 min at 37°C. After incubation, RPMI 1640 medium was added and tissue was broken up using a syringe. This yielded single cells and larger, glandular fragments. This suspension was centrifuged (450 g, 3 min) and then cells/fragments were resuspended in fresh medium and allowed to separate by density sedimentation. After 5 min the supernatant (stromal compartment) was removed leaving 2 ml of medium. Fresh medium was added and the density sedimentation was repeated. The remaining 2 ml of medium contained glandular fragments that were centrifuged as above. Medium was discarded and the epithelial fragments were incubated with collagenase/DNAase for 2 h at 37°C. After incubation, medium was added and the cell suspension was centrifuged (see above). Medium was removed and the epithelial cell pellet was resuspended in 50% Matrigel (BD Biosciences, Bedford, MA, USA).

Cell culture

Primary endometrial epithelial cells

Primary endometrial epithelial cells were grown in Matrigel (see Figure 1 showing cells immunostained for SLPI) in RPMI 1640 medium supplemented with 10% fetal calf serum (Mycoplex; PAA Laboratories, Teddington, UK), penicillin (50 μg/ml; Sigma), streptomycin (50 μg/ml; Sigma), gentamycin (5 μg/ml; Sigma), epidermal growth factor (25 ng/ml; Peprotech Ltd, London, UK), vascular endothelial growth factor (1 ng/ml; Peprotech), basic fibroblast growth factor (5 ng/ml; Peprotech) and estradiol (10^-7 mol/l). These growth factors were included in the culture medium as there is evidence that endometrial epithelial cells express their receptors and hence they are likely to be involved in modulation of cell growth (Li et al., 1994; Zhang et al., 1995; Sangha et al., 1997; Meduri et al., 2000). Cells were grown to near confluence (7–10 days) in 12-well plates (Nunc, Gibco, Paisley, UK) and then treated with inflammatory mediators or mimics of infection. The inflammatory mediators investigated were as follows: IL-1β (1 ng/ml; Peprotech), TNFa (2 ng/ml; Peprotech), IL-1β + TNFa, interferon (IFN)-γ (10 ng/ml; Peprotech), IL-6 (5 ng/ml; Peprotech) and phorbol-1-myristate, 13-acetate (PMA, 10^-7 mol/l; Calbiochem, Nottingham, UK). These molecules were investigated because they have been reported to influence natural antibiotic expression in other systems (Sallenave et al., 1994; Harder et al., 2000; Krisanaprakornkit et al., 2000). Dose dependence and kinetics of the response of endometrial epithelial cells to IL-1β and TNFa were determined in the HES endometrial epithelial cell line by measuring SLPI mRNA expression. The response observed at 24 h was greater than at 4 h and concentrations 5-fold higher than those...
Immunohistochemistry

fixed in Matrigel was calculated of the above concentrations and samples (without ABI et) PE genomic DNA contamination. major probe (all 50 nmol/l; Biosource, Nivelles, Belgium) and probe (200 nmol/l; Biosource) for the natural antibiotic. Primers and probe (all at 200 nmol/l) for specific and reverse primers (300 nmol/l; Biosource, Nivelles, Belgium) and probe (200 nmol/l; Biosource) were specific amplicons in cDNA determined that at the time of treatment the purity of epithelial cells was >90%. The majority of contaminating cells were likely to be of stromal origin and morphologically resembled fibroblasts.

RT and real-time quantitative PCR

After treatment, cells were harvested in Tri-reagent (Sigma) and RNA was extracted as detailed in the manufacturer's protocol. Amounts of HBDS, HBD2 and SLPI mRNA were determined using real-time quantitative PCR. This method detects fluorescence release from a specific probe allowing measurement of PCR progress. The amount of specific amplicon was related to ribosomal 18S (constant relative to the amount of cDNA present) and subsequently to an internal control. Details of RT and quantitative PCR are described elsewhere (King et al., 2000). Briefly, RNA samples were reverse transcribed using random primers. PCR reaction mixtures were made containing Sure Start Taq DNA polymerase (0.025 IU/µl; Stratagene, Amsterdam, The Netherlands), dNTPs (all at 200 µmol/l) and specific forward and reverse primers (300 nmol/l; Biosource, Nivelles, Belgium) and probe (200 nmol/l; Biosource) for the natural antibiotic. Primers and probe (all at 50 nmol/l; PE Biosystems, Warrington, UK) for ribosomal 18S were also added. Samples were measured in triplicate and no template controls were included in all runs. PCR reactions were run on ABI Prism 7700. Contamination of cDNA samples with genomic DNA was measured by detection of β-actin signal in control RNA samples (without reverse transcriptase). All samples had a β-actin signal that was only detectable above a previously defined, arbitrary level of 27 cycles (King et al., 2001), indicating that there was no major genomic DNA contamination.

Primers and probes were designed using PRIMER express software (PE Biosystems; Table I). Primers and probes were validated at the above concentrations and linearity of response was confirmed using serial dilution of a standard pool of cDNA. Within assay variations of the PCR measurement of specific amplicons in cDNA were calculated from six replicates (Table I).

Immunohistochemistry

Matrigel was removed from primary epithelial cells and they were fixed in neutral buffered formalin for 10 min at room temperature and subsequently permeabilized with Triton X100 (0.05%; Sigma). Non-specific endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxidase (BDH Laboratory Supplies, Poole, UK) in distilled water for 10 min at room temperature. Cells then underwent a non-immune block using diluted normal horse serum (Vestacain 4002; Vector Laboratories, Peterborough, UK) for 20 min at room temperature. Cells were incubated overnight at 4°C with 200 µl mouse anti-SLPI antibody (1:200 in diluted horse serum; HyCult Biotechnology, Uden, The Netherlands). In negative control wells, cells were incubated with equimolar concentrations of mouse immunoglobulin (Ig; Sigma). Cells were then incubated with biotinylated horse anti-mouse IgG (Vector Laboratories), followed by an avidin–biotin peroxidase detection system (both for 60 min at room temperature; Elite ABC 6101; Vector Laboratories). Diaminobenzidine (DAB; Vector Laboratories) was used to identify positive staining (Figure 1). Cells were counterstained with Harris's haematoxylin (Pioneer Research Chemicals Ltd, Colchester, UK) and stored in ethanol.

SLPI ELISA

SLPI concentrations in culture supernatants were determined by ELISA. Throughout the ELISA all dilutions were made in ELISA buffer (150 mmol/l NaCl, 100 mmol/l Tris, 0.00015% phenol red solution, 2 mmol/l EDTA, 150 mg/l 2-methylisothiazolone, 150 mg/l bromonitrooxadine, 2 mg/ml bovine serum albumin, 0.085% Tween-20, 0.01% hexacycliltrimethylammonium bromide, pH 7.2). Assay plates (96-well; Nunc Maxi-Sorp) were coated overnight at 4°C with 200 µl/well of goat anti-SLPI (2 µg/ml; R&D Systems, Oxford, UK) and then blocked for 30 min with 200 µl/well of blocking/protecting solution (2% polyvinylpyrrolidone, 5 mg/ml bovine serum albumin, preservatives, 5 mmol/l EDTA, 50 mmol/l Tris). Plates were washed with wash buffer (150 mmol/l NaCl, 5 mmol/l Tris, 0.025% Tween-20, pH 7–7.5) and subsequently 200 µl of standard/sample were added to each well and incubated for 2 h at room temperature on a plate shaker. Two non-specific binding wells (200 µl buffer only) were included on each plate. Standards were added in duplicate and their concentration range was 0.025–25 ng/ml (recombinant SLPI; R&D Systems). Plates were washed and then incubated for 1 h with 200 µl/well of biotinylated mouse anti-SLPI (1:10000; Hycult) as above. After further washing, 200 µl/well of streptavidin peroxidase (1:4000; Roche, Lewes, UK) was added and incubation was for 20 min, as above. Plates were washed again and then 200 µl/well of substrate (0.4 g/l urea-hydrogen peroxidase, 0.16 g/l tetramethyl benzidine in 100 mmol/l sodium acetate; pH 6.0) was added. After 10 min, wells were quenched with 50 µl/well of 1M sulphuric acid. Plates were read in a plate reader at 450 nm. Intra- and inter-assay coefficients of variation were 12.80 and 14.76% respectively.

Statistical analysis

Significant difference was determined by analysis of variance (ANOVA; Statview 3.0). Fisher's protected least squares differences test was used to assign individual differences. P < 0.05 was considered significant.

No attempt was made to analyse experiments involving epithelial cells derived from proliferative and secretory endometrium separately. However, SLPI mRNA expression in epithelial cells originating from the proliferative and secretory phases was compared using the control samples. There was no significant difference between SLPI levels in cells derived from the proliferative phase and from those in the secretory phase. In vivo, SLPI is expressed only in secretory endometrium. This suggests that after 7–10 days in culture, the cells do not exhibit characteristics specific to their original menstrual cycle phase and so it is appropriate to treat them as one group for statistical analysis.
Results

Regulation of natural antibiotic expression in primary cultures of endometrial epithelial cells

The role of inflammatory mediators and mimics of infection in the regulation of HBD1, HBD2 and SLPI in primary endometrial epithelial cells was investigated.

HBD1

Primary cultures of endometrial epithelial cells expressed very low levels of HBD1 (data not shown). This low level of expression made investigation of HBD1 regulation inappropriate. Successful detection of HBD1 mRNA in whole endometrial biopsies served as a positive control.

HBD2

HBD2 mRNA expression was up-regulated by treatment with several inflammatory mediators. IL-1β alone, TNFα alone and IL-1β+TNFα increased HBD2 levels by 9-, 4- and 8-fold respectively (Figure 2a; P < 0.03). Treatment with IFNγ caused mRNA expression to increase by 9.5-fold (P < 0.01).

IL-6 and PMA treatment had no effect on HBD2 expression. LPS at several doses had no effect on HBD2 mRNA expression (data not shown). However, treatment with LPS (1000 ng/ml) for 8 h showed a decrease in HBD2 mRNA levels followed by an increase at 24 h (Figure 2b; P = 0.055). LTA treatment caused a trend towards increased expression of HBD2 at 4 h, with a subsequent significant decrease in expression at 24 h (Figure 2b; P < 0.02). No significant synergetic effect of treatment with LPS in combination with LTA or IL-1β was detected, although increased HBD2 expression was apparent in the presence of both LPS and IL-1β (Figure 2b; P < 0.04). However, this can be accounted for by the up-regulation of HBD2 mRNA in the presence of IL-1β alone (see above).

SLPI

SLPI mRNA expression was not affected by treatment with any of the inflammatory mediators tested (Figure 3a; not significant).

SLPI mRNA expression was down-regulated by treatment with LPS (1000 ng/ml) for 4 and 24 h (Figure 3b; P < 0.05). All other doses and incubation times had no effect on SLPI levels. LTA treatment resulted in a trend towards increased expression of SLPI at 4 h (Figure 3b; not significant). LPS, in combination with LTA or IL-1β, caused a down-regulation of SLPI mRNA expression after 24 h of treatment (Figure 3b; P < 0.01). This is consistent with the results for treatment with LPS alone and does not reflect an effect of LTA or IL-1β.

In addition, SLPI protein was localized to the cytoplasm of the primary epithelial cells using immunohistochemistry (Figure 1) and changes to SLPI protein concentrations in cell culture supernatants were determined by ELISA. In the presence of IL-1β+TNFα, SLPI concentrations were reduced (Figure 3c; P < 0.04), although treatment with the other inflammatory molecules did not affect SLPI levels. SLPI concentrations were reduced by treatment with LTA for 24 h (Figure 3d; P < 0.03). There was also a trend towards increased SLPI release in the presence of LPS+IL-1β.

Regulation of natural antibiotic expression in endometrial epithelial cell lines

Expression of HBD1, HBD2 and SLPI was investigated in the MFE and HES endometrial epithelial cell lines. Natural antibiotic expression in these cell lines was similar to that found in primary endometrial epithelial cells. HBD1 mRNA was expressed at very low levels in MFE cells and was undetectable in HES cells (data not shown). Similarly, HBD2 was undetectable in both cell lines under control conditions, although mRNA expression became detectable at very low levels in MFE cells treated with IL-1β+TNFα for 24 h (in two of three experiments; data not shown). SLPI mRNA was detected in both cell lines and was up-regulated by treatment with IL-1β+TNFα (Figure 4a; MFE, P < 0.01; Figure 4b; HES, P = 0.05). SLPI protein release by HES cells into culture supernatants was increased by treatment with TNFα both alone and in combination with IL-1β (Figure 4c; P < 0.01). MFE cells released very little SLPI into culture supernatants and so it was not possible to determine the regulation of protein release.

Discussion

This study details the presence and regulation of natural antibiotic molecules in endometrial epithelial cells. Human β-defensins 1 and 2 and SLPI were detected in primary cultures of endometrial epithelial cells. This is consistent with previous reports detailing the expression of HBD1 and SLPI in the endometrial epithelium and at other mucosal sites (Zhao et al., 1996; Valore et al., 1998; King et al., 2000). HBD2 has also been detected in the uterus (Bals et al., 1998), although this report did not investigate the site of expression within the organ. However, HBD2 is present at other epithelial surfaces (Harder et al., 2000; Krisanaprapornkit et al., 2000).

The regulation of natural antibiotic molecules has not been previously described in endometrial epithelial cells. Our in-vitro study, the first detailing natural antimicrobial regulation
in genital tract cells, shows that HBD2 expression is induced by the presence of inflammatory mediators such as IL-1β, TNFα, and IFNγ. This is consistent with previous reports stating that HBD2 is up-regulated by inflammatory molecules in gingival and respiratory epithelial cells and at sites of inflammation (Liu et al., 1998; Harder et al., 2000; Krisanaparakornkit et al., 2000). It should be noted that the actions of IL-1 are affected by IL-1 receptor antagonist and IL-1 receptor levels which vary throughout the menstrual cycle (Simon et al., 1993, 1995) and hence, levels of these molecules may influence natural antibiotic expression in vivo. The HBD2 promoter is reported to have several consensus sites for the proinflammatory transcription factors NFκB and AP-1, accounting for its induction by the above mediators (Harder et al., 2000). Additionally, several sites for the transcription factor NF-IL6 have been detected in the HBD2 promoter, although the cytokine IL-6 (which stimulates transcription via NF-IL6) was reported to have no effect on HBD2 expression in lung epithelial cells (Harder et al., 2000). Similarly, IL-6 did not alter HBD2 levels in endometrial epithelial cells. The protein kinase C stimulator, PMA, up-regulates HBD2 in gingival epithelial cells (Krisanaparakornkit et al., 2000), although it had no effect in our system, suggesting that differences in defensin regulation exist between epithelial cells from distinct sites.

HBD1 mRNA was found to be expressed at very low levels in both primary endometrial epithelial cells and in endometrial cell lines. As HBD1 has previously been detected in endometrial epithelium in vivo (Valore et al., 1998), our results suggest that a stromal mediator is needed to maintain expression of this defensin in the epithelium. Previous studies have detailed constitutive HBD1 expression in several epithelial cell types and have reported resistance to treatment with proinflammatory cytokines. Our investigation into the regulation of HBD1 expression in endometrium was limited by the low expression of the molecule.

SLPI is also reported to be up-regulated by inflammatory cytokines and LPS (Sallenave et al., 1994; Jin et al., 1998). However, in primary endometrial epithelial cells, SLPI mRNA expression was unaltered by treatment with proinflammatory molecules, while protein concentrations in culture medium fell after treatment with IL-1β+TNFα. In a previous study, we reported that SLPI expression in endometrium is increased during the secretory phase of the menstrual cycle, suggesting regulation by progesterone (King et al., 2000). This, along with our current findings, suggests that in endometrium SLPI may be controlled primarily by the local steroid environment, resulting in peak expression around the time of implantation. However, inflammatory pathways such as the IL-1 system are also involved at this time (Simon et al., 1994) and may be involved in the regulation of SLPI expression under certain circumstances. It should be noted that treatment of both the HES and MFE cell lines with a combination of IL-1β and TNFα resulted in increased SLPI expression. This may represent differences in regulation of SLPI between the primary cells and cell lines, e.g. there may be differences in IL-1 and TNF receptor numbers on the cell surfaces. Also, in the MFE cell line a synergistic action of IL-1β and TNFα was observed. This may be due to the presence of several sites on the SLPI promoter that can respond to these two cytokines, as has previously been reported for the rat nosII gene (Kuenmerle, 1998). For example, the SLPI promoter contains several AP-1 sites and is thought to be responsive to NFκB (Abe et al., 1991; Nguyen et al., 1999).

LPS had little effect on HBD2 expression when dose and

Figure 2. Regulation of HBD2 mRNA expression in primary endometrial epithelial cells. Sample numbers are shown below the x-axis. Note that the total number of treatment samples exceeds the number of controls. In most experiments, there were several different treatments relating to one control. Due to the distribution of results from primary cell cultures, data were logarithmically transformed prior to statistical analysis by ANOVA.

(a) Proinflammatory mediators. Cells were treated with IL-1β (1 ng/ml), TNFα (2 ng/ml), IL-1β+TNFα, IFNγ (10 ng/ml), PMA (10−7 mol/l) or IL-6 (5 ng/ml) for 24 h. a,b,c,d: P < 0.03. (b) Mimics of Gram-negative, Gram-positive, and dual infection. Cells were treated with LPS (1 μg/ml) or LTA (5 μg/ml) for 4, 8 or 24 h or with LPS in combination with LTA or IL-1β for 24 h. a: P = 0.055; b,c: P < 0.04.

Endometrial antimicrobials
Figure 3. Regulation of SLPI mRNA and protein expression in primary endometrial epithelial cells. Sample numbers are shown below the x-axis. Note that the total number of treatment samples exceeds the number of controls. In most experiments, there were several different treatments relating to one control. Treatment of cells was as described in Figure 2. Due to the distribution of results from primary cell cultures, data were logarithmically transformed prior to statistical analysis by ANOVA. (a) SLPI mRNA—proinflammatory mediators. (b) SLPI mRNA—mimics of Gram-negative, Gram-positive and dual infection. a,b,c,d,e: P < 0.05. (c) SLPI protein—proinflammatory mediators. a: P < 0.04. (d) SLPI protein—mimics of Gram-negative, Gram-positive and dual infection. a: P < 0.03.

Figure 4. Regulation of SLPI mRNA and protein expression in endometrial cell lines. Cells were treated with IL-1β (1 ng/ml), TNFα (2 ng/ml), IL-1β + TNFα, IFNγ (10 ng/ml) or PMA (10⁻⁷ mol/l) for 24 h. The results shown represent the mean of three experiments. (a) SLPI mRNA—MFE cell line. a,b,c: P < 0.01. (b) SLPI mRNA—HES cell line. a: P = 0.05. (c) SLPI protein—HES cell line. *Mean of two samples. a,b: P < 0.01.
time of treatment were varied. Synergistic actions between LPS and other mediators were not observed. Previous studies in other systems have reported contradictory results. Some have shown HBD2 to be up-regulated by LPS (McNamara et al., 1999; Becker et al., 2000). However, others have reported little effect of LPS, but have found increased HBD2 expression upon infection with pathogenic bacteria, suggesting that other components of the bacterial cell wall are involved in stimulating defensin expression (O’Neil et al., 1999; Harder et al., 2000). This also suggests that LPS actions may depend on the cell type investigated. Also, our study used E. coli LPS and it may be that endometrial epithelial cells would respond to LPS from genital tract pathogens (e.g. Chlamydia). SLPI has been reported to be an LPS inducible gene in macrophages (Jin et al., 1997). However, we found SLPI expression to be decreased after treatment for 24 h with 1 μg/ml of LPS. This may indicate that, as expected, innate immune responses to LPS occur over a short time period. Following initiation of the adaptive response, high levels of a leukocyte protease inhibitor may be inappropriate, hence the observed down-regulation. Similarly, at shorter time points LTA caused a trend towards increased expression of HBD2 and SLPI that was not observed after 24 h. Indeed, protein concentrations of SLPI fell after exposure to LTA for 24 h. Responses to PAMPs such as LPS and LTA are thought to be mediated by interactions with cellular or soluble CD14 and subsequent signalling via Toll-like receptors (Chow et al., 1999; Becker et al., 2000). It may be that the poor response to PAMPs by the endometrial epithelial cells was due to low levels of CD14 expression and inadequate soluble CD14 in bovine serum.

The main role for HBD1, HBD2, and SLPI in human endometrium is likely to be in the prevention of uterine infection. Each of these molecules has been shown to have antimicrobial activity, and hence they are likely to be important in the control of sexually transmitted infections. They may serve to limit the spread of disease from the lower to upper genital tract, where infection is associated with infertility (Cates and Wasserheit, 1991; Paavonen, 1993). Also, local defences are crucial to successful pregnancy. A total of 20% of preterm births are associated with uterine infection (Romero et al., 1989c) and increased amounts of HNP1–3 and another antimicrobial compound, lactoferrin, have been detected in these circumstances (Heine et al., 1998; Pacora et al., 2000). This suggests that these natural antimicrobials have a protective effect and HBDs and SLPI are likely to have a similar role.

Constitutively expressed natural antibiotics such as SLPI will provide a basal level of antimicrobial protection in the uterus. This will ensure antimicrobial protection at times when infection may jeopardize endometrial receptivity (e.g. implantation). IL-1β, TNFα and IFNγ are produced by the endometrium under physiological conditions, with TNFα produced by epithelial cells (Tabibzadeh et al., 1995). IL-1β by epithelial and isolated stromal cells (Tabibzadeh and Sun, 1992; Simon et al., 1993) and IFNγ by T cells and polymorphonuclear cells (Tabibzadeh, 1994; Yeaman et al., 1998). These molecules may contribute to regulation of the normal antibiotics during the normal menstrual cycle. In addition, up-regulation of these cytokines occurs during infection and the presence of both IL-1β and TNFα has been reported in amniotic fluid from patients undergoing preterm labour associated with infection (Romero et al., 1989a,b).

Although an antimicrobial role is likely to be the main function of HBDs and SLPI in the endometrium, it should be noted that these molecules have several other actions that may be important. HBD2 chemotaxates immature dendritic cells and memory T cells to sites of inflammation via the CCR6 chemokine receptor (Yang et al., 1999). This will result in activation of the adaptive immune system during infection. HBD2 has also been shown to activate rat mast cells resulting in histamine and prostaglandin D2 release (Niyonsaba et al., 2001). SLPI has generalized anti-inflammatory actions including inhibition of neutrophil elastase and catepsin G (Thompson and Ohlsson, 1986), down-regulation of matrix metalloproteinase production by monocytes (Zhang et al., 1997) and inhibition of NFκB (Lentsch et al., 1999). Activation of DNA synthesis in porcine endometrial epithelial cells has also been reported (Badinga et al., 1999).

In summary, the natural antimicrobials HBD1, HBD2 and SLPI have been studied in a primary endometrial epithelial cell culture model and in two endometrial epithelial cell lines. Each of these molecules is expressed in the endometrial epithelium and, as in other systems, they are differentially regulated, suggesting that they provide both constitutive and inducible antimicrobial protection.

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References


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