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HUMAN VAGINAL EPITHELIAL IMMUNITY
AND INFLUENCES OF
HORMONAL CONTRACEPTIVE USAGE

by

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Cover illustration: Human vaginal epithelium from a DMPA user with a high density of intraepithelial leukocytes, shown by anti-CD45 MoAb in immunoperoxidase staining. Original magnification, ×100.
To My Family, with Love
ABSTRACT

The vagina is the port of entry for sexually transmitted diseases in women. Its epithelium constitutes the luminal border, thus comprising an important defence barrier. The objective of this work was to investigate the mechanisms of importance in the immune defence of the vaginal epithelium of healthy, fertile women, and possible menstrual cycle changes. Effects of hormonal contraceptive usage on oestrogen receptor (ER) and progesterone receptor (PR) expression were studied. The contribution of epithelial cell to the immune defence was estimated by assaying their expression of antimicrobial defensins and the epithelial thickness.

Vaginal biopsies and serum samples were collected during the follicular and luteal phases in regularly menstruating women (controls) and in users of combined oral contraceptives (COCs), levonorgestrel implants (LNGs), or depot-medroxyprogesterone acetate injections (DMPAs). Fifteen healthy women (aged 20–34 years) were enrolled in each group. Morphometry was performed on vaginal tissue stained with haematoxylin/eosin and by immunohistochemistry using monoclonal antibodies against immune cell markers, PR, and ER. Expression of mRNA for human α-defensins HD-5 and HD-6, and human β-defensins (HBD) 1 to 4 were determined by real-time qRT-PCR and in situ hybridization.

In controls, the epithelium was 261 ± 16 µm thick and harboured 241 ± 35 leukocytes (CD45+) per mm². T lymphocytes (CD3+) dominated. Both αβ T cells and γδ T cells were present with an approximate 4-fold dominance of αβ T cells. Cytotoxic T cells (CD8+) were more frequent than T helper cells (CD4:CD8 ratio: 0.7 ± 0.1). Macrophages (CD68+) constituted the second-largest population, followed by Langerhans cells (CD1a+). B cells, natural killer cells, monocytes and granulocytes were generally absent. No differences were found between the follicular and luteal phase. All four β-defensins analysed for were detected in vaginal epithelium and most samples expressed at least two. HBD-2 and HBD-3 were most frequent. HBD-3 and HBD-4 expressing cells were localized in the parabasal and intermediate cell layers. α-defensins were not detected.

The epithelium was significantly thicker (333 ± 9 µm) in COC, LNG, and DMPA users than in controls, and commonly showed hyperplasia. In DMPA and LNG users the frequency of intraepithelial leukocytes (CD45+) was increased, explained by increased frequencies of both αβ and γδ T cells. In DMPA users there was also a selective increase in CD8+ T cells. PR expression was significantly reduced in DMPA users compared with controls, COC and LNG users. COC and particularly DMPA users often had undetectable levels of serum E2.

In conclusion, both adaptive immunity, i.e. intraepithelial T cells, and innate defence mechanisms, i.e. intraepithelial macrophages and β-defensins, are believed to contribute to the immune defence in the human female lower genital tract. These parameters did not change during the menstrual cycle but hormonal contraceptive usage, especially DMPA, affected the quality of the epithelium. The use of DMPA and LNG was correlated with the accumulation of T cells within the epithelium. The effects of these changes on the risk of contracting infections are yet to be determined.

Key words: human vaginal epithelium, hormonal contraceptives, epithelial thickness, intraepithelial immune cells, steroid receptors, steroid hormones, defensins
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ABBREVIATIONS

ADCC  antibody-dependent cell-mediated cytotoxicity
Ag    antigen
ANOVA analysis of variance
APC   antigen-presenting cell
CCR   chemokine receptor
CD    cluster of differentiation
COC   combined oral contraceptive
CTL   cytotoxic T lymphocyte
DMPA  depot-medroxyprogesterone acetate injections
E₂    17-β-oestradiol
ER    oestrogen receptor
FSH   follicle-stimulating hormone
HBD   human β-defensin
hCG   human chorionic gonadotropin
HD    human α-defensin
HIV   human immunodeficiency virus
IFN   interferon
Ig    immunoglobulin
IL    interleukin
IUD   intrauterine device
LH    luteinizing hormone
LNG   levonorgestrel implants
MoAb  monoclonal antibody
MHC   major histocompatibility complex
mRNA  messenger ribonucleic acid
NK cell natural killer cell
PAMP  pathogen-associated molecular pattern
PR    progesterone receptor
qRT-PCR quantitative reverse-transcriptase polymerase-chain reaction
SIV   simian immunodeficiency virus
STD   sexually transmitted disease
TcR   T cell receptor
Th    T helper
tNF   tumour necrosis factor
This thesis is based on the following papers and manuscripts, which will be referred to in the text by their Roman numerals (I–IV).


II  **Ildgruben A**, Bäckström T, Sjöberg I, Hammarström M-L. Increased Frequency of both αβ and γδ-T Lymphocytes in Human Vaginal Epithelium of Progestin-Only Contraceptive Users (manuscript).


IV  **Ildgruben A**, Fahlgren A, Bäckström T, Hammarström M-L. Prominent Expression of β-defensins 2 and 3 in Vaginal Epithelial Cells of Healthy Fertile Women. (submitted)

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INTRODUCTION

The vaginal epithelium is one of the critical interfaces with the external environment. It must tolerate the presence of commensal bacteria and antigen (Ag) exposure from allogeneic sperm and yet be able to distinguish these from the persistent abundance of commensal microorganisms in the perineal area. In addition the vagina is a critical portal of entry for potentially pathogenic microorganisms causing sexually transmitted diseases (STDs) in women (UNAIDS 1999, Witkin 1993). The vaginal epithelium constitutes a first line of defence against STDs through the physical epithelial barrier and the epithelial immune components present. The description of these local immune protection components, their regulation under the physiological hormonal variations during the menstrual cycle, and the effects of exogenous steroid hormones (e.g. hormonal contraceptives) are essential for future functional studies on the effect of hormonal contraceptives on local vaginal immune response.

Two decades have passed since the worldwide acquired immunodeficiency syndrome (AIDS) epidemic began and efforts to control the growing human immunodeficiency virus (HIV) transmission rate have had limited success, particularly in the developing world (Fauci 1999, Piot 2001). Studies in rhesus macaques have shown that subcutaneous administration of progesterone implants resulted in increased vaginal transmission of simian immunodeficiency virus (SIV) and vaginal epithelial thinning (Marx 1996). Furthermore administration of depot-medroxyprogesterone acetate injections (DMPA) to mice was shown to increase herpes simplex virus transmission (Parr 1994). An association between DMPA or combined oral contraceptive (COC) usage and contraction of STDs is supported by the results from epidemiologic studies (Baeten 2001, Martin 1998, Nagachinta 1997, Ungchusak 1996), although causality and confounders could be multifactorial.

Hundreds of millions of women use hormonal contraceptives worldwide, including COCs and long-term, gestagen-only methods (e.g. DMPA and subdermal levonorgestrel implants [LNG]; Baird 1999). Given their widespread use by women, any modification of the risk of acquisition of STDs due to hormonal contraceptives, including HIV could be of significance for STD-associated morbidity and mortality, and HIV epidemic spread. To better understanding the impact of steroid hormones on the composition of immune mechanisms operating on the cellular level in the human vagina, more direct investigations are required, and this study was therefore initiated.

THE HUMAN VAGINA

The word vagina is derived from the Latin for “sheath” and is described in literature from the 1950s and 60s as “a female organ of copulation” and “a tube
between the vulva and uterine cervix” (Netter 1965). The vagina has similarities to the heart in that it is both an entrance and an exit. The heart is an entrance for deoxygenated blood and lymph and an entrance and exit for reoxygenated blood, although in separate chambers, while the vagina has a common entrance and exit (bidirectional). The products transported via this “tube” can be considered as normal/physiological or abnormal/pathological, and all products are regulated in the interface between the immune and endocrine systems in a sophisticated way. They also differ considerably in size, from microscopic, invisible small microorganisms (commensal flora and pathogens) and single sperm, to larger macroscopic constellations such as menstrual bleeding, discharge/secretion, miscarriages/abortions, amnion fluid, and the obvious appearance of newborn babies. Additionally, in humans visible traces of any product are registered and arouse emotional, occasionally strong, reflections of any consequences that may rise from their presence, or absence. The red and obvious sign of vaginal bleeding can be associated with a wide range of emotions: contentment (normal regular menstrual cycles), discomfort (e.g. sanitary problems, anaemia, dysmenorrhoea), disappointment (miscarriage or menstrual bleeding, i.e. non conception), relief (menstrual bleeding, i.e. not pregnant), or anxiety (e.g. pregnancy-related bleeding, cancer, infections, heavy menorrhagia or postmenopausal bleeding). The signs of vaginal discharge are also associated with thoughts ranging from normal physiological discharge and ovulatory secretions (chance/risk of conception) to pathologic discharge due to infections, e.g. STDs, and the source of pathogens involved and the correlated risk to infertility.

THE FEMALE REPRODUCTIVE SYSTEM
The vagina is a dynamic fibromuscular canal connecting the external genital tract, commonly referred to as the vulva, and the internal genital tract. A schematic illustration of the anatomical features of the human vagina and its relation to neighbouring organs in the pelvis is shown in frontal section in Figure 1. The anterior and posterior vaginal walls are normally opposed, except at the proximal end, where they forms a cuff around the protruding external cervix of the uterus, forming anterior, posterior and lateral pouches termed fornices. The vaginal wall consists of three layers: I) the surrounding superficial strong adventitia composed of fibrocollagenous elastic fibres, bloodvessels, lymphvessels and nerves, II) the smooth muscular layer, and III) the mucosa, facing the vaginal lumen. The latter will be dealt with in a separate section. The configuration of the vaginal walls, characterized by longitudinal columns and transversal rugae, and its property of elasticity, especially under the influence of steroid hormones during pregnancy, permits the reversible distension necessary during childbirth.

The anterior neighbour of the vagina is the bladder, and more distally the urethra is adherent to the anterior wall. The rectum is the posterior neighbour to the vagina, proximally separated from the posterior fornix of the vagina by the rectouterine pouch (the pouch of Douglas) related to the peritoneum. In attempts to induce illegal abortions, instruments may penetrate the posterior fornix rather than enter
the uterine cervix and may give rise to severe haemorrhage and peritonitis, complications still causing women’s deaths.

Blood is supplied to the vagina by several anastomosing branches, mainly from the internal iliac artery, which divide into the uterine vessels, the vaginal artery, the middle rectal artery (providing blood supply to the mid-vagina), the inferior vesical artery, and the internal pudendal artery which also receives blood from the femoral region via the external pudendal artery supplying the lower vagina. A rich venous plexus also surrounds the vagina which empties into the internal iliac veins (Robboy 1997).

The lymphatic drainage of the vagina follows its embryologic development; the upper two thirds drain into the external and internal iliac nodes in the pelvis and to some extent directly into the sacral lymph node area, while the lower third drains into the inguinal region (Robboy 1997).

In healthy women, the vaginal canal contributes to the continuous connection between the external genitalia and the abdominal cavity, via the uterine cervix, cavity and tubes (Stevens 2005). This link is one of the essential anatomical features for fruitful fertilization. Following successful ovulation, the ovum is picked up by the fimbriae of the uterine (Fallopian) tubes (Speroff 1999b). Healthy sperm deposited in the vagina will penetrate the cervical mucus of ovulation and hence in the direction of one of the uterine tubes, where fertilization can occur in the ampullae. Mitotic divisions of the fertilized ovum then starts and the morula moves to the endometrium in the uterine cavity where it adheres to, and becomes implanted in the hormone influenced endometrium.

THE VAGINAL EPITHELIUM

During embryo development the vagina is derived from two mesodermal sources; the upper part of the vagina arises from the müllerian ducts, while the lower part arises from the urogenital sinus. During foetal development the müllerian ducts fuse and extend caudally, and develop into the uterovaginal canal (primordial of uterine corpus, cervix and vagina), the lining of which is an immature columnar epithelium (Lawrence, 1992) while the urogenital sinus differentiates into the squamous epithelium, proliferating from below, and completely replaces the columnar glandular müllerian mucosa onto the squamocolumnar junction of the cervix (Ulfelder 1976). The transition from müllerian to squamous epithelium occurs at about the time (10th gestational week) when nuclear oestrogen receptors (ERs) appear in the vaginal stroma (Taguchi 1986). The sinus (squamous) epithelium begins to mature, thickening and accumulating glycogen at the 16th week, when the maternal oestrogen and hence foetal oestrogen levels increase and the epithelial cells in the vagina also express ERs. By the 18th to 20th gestational week, the foetal vaginal development is complete and the oestrogenic effects persist until the neonatal period, under the influence of maternal hormones.
Histologically, the vaginal mucosa consists of a stratified squamous epithelium with several layers, typical of surfaces that are subject to frictional stress, such as skin, the oral cavity and oesophagus (Ross 2003). In Figure 2 the morphology of the human vaginal mucosa is illustrated. The vaginal epithelium is made up of four layers (Robboy 1997): (i) a superficial layer of variable thickness, facing the vaginal lumen, the cells are polygonal when viewed from above and flattened when viewed in cross-section, (ii) an intermediate layer, also of variable thickness in which some glycogen may be present, (iii) a parabasal layer, usually consisting of two layers of small polygonal cells with relatively large nuclei, and (iv) a basal layer, consisting of a single layer of columnar-like cells, which together with the parabasal cells are active proliferative compartments with stem cells. The basal layer is supported by a basal membrane constituting the border with the underlying lamina propria, consisting of elastic and collagen fibres and a rich network of blood and lymph vessels. Papillae from the lamina propria project into the underside of the epithelium, often giving the basal membrane an uneven appearance. The epithelium of the vaginal mucosa is steroid-hormone sensitive, lacks glands and in humans and other primates, keratinohyalin granules may be present in the epithelial cells. Under normal conditions, keratinization does not occur and, therefore, even
the superficial cells facing the vaginal lumen still contain nuclei (Ross 2003). Vaginal epithelial cells proliferate and mature in response to ovarian or exogenous oestrogenic hormones and atrophy without oestrogenic stimulation. Hence, the cell layers vary during the various stages of the life cycle, i.e. birth, childhood, reproductive period and postmenopausal period (Steger 1978). Cells in the superficial and intermediate cell layers stimulated by oestrogen (E$_2$), contain abundant intracytoplasmatic glycogen and have pyknotic nuclei, detected on both tissue sections and in vaginal smears. Before puberty and after the menopause endogenous E$_2$ levels are low, the vaginal epithelial cells remain immature, parabasal and intermediate cells predominate in the smear, the epithelium is thin and glycogen-deficient, and the pH is elevated (Nilsson 1995).

A vaginal smear mirrors the oestrogenic state of the superficial desquamated cells from the epithelial surface, while the majority of leukocytes in the smear may originate from other sources (e.g. the uterine cervix and cavity). The changes seen in vaginal smears during the menstrual cycle should not be confused with the ovulation-induced changes in the vaginal epithelial tissue. After the pre-ovulatory oestrogenic peak the epithelial thickness increases, followed by normalized thickness in the mid-luteal phase, resembling the thickness of the follicular phase epithelium (Robboy 1997). In addition, the microbial environment in the vagina, including the commensal microflora and other invading microorganisms, can be detected in the smear. In cervical smears, epithelial cells from the cervix are analyzed for atypical cells, a procedure used in screening for precancerous stages of cervical cancer.

Figure 2
Tissue section of vaginal epithelium, haematoxylin/eosin staining. Original magnification, ×100. Ep = Epithelium; LP = Lamina Propria
The Menstrual Cycle and Steroid Hormones

In humans the first menstrual cycle starts between 9 and 17 years of age (menarche) and continues, more or less regularly, during the fertile period of life, until the last menstrual period (menopause). The median age at natural menopause is approximately 51 years (McKinlay 1996). The average length of the menstrual cycle is 28 days, although the inter-individual variation in the length of a normal menstrual cycle is 21–35 days, and the duration of the menstrual bleeding period is 3–7 days (Munster 1992). Menstrual bleeding is regulated by steroid hormones produced by the ovaries.

The reproductive cycle depends on the cyclic interaction between hypothalamic gonadotropin-releasing hormone (GnRH), the pituitary gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH), and the ovarian sex steroid hormones oestradiol and progesterone (Beckmann 2005). Control of the normal female reproductive cycle is based on this “hypothalamic-pituitary-ovarian axis” and its positive and negative feedback loops, where ovulation is followed by menstrual bleeding in a recurrent, predictable sequence. Referring to the ovarian status, the menstrual cycle can be divided into three phases: I) the menstrual and follicular phase, II) ovulation, and III) the luteal phase. A schematic illustration of the menstrual cycle and the serum concentrations of pituitary and ovarian steroid hormones are shown in Figure 3.

![Figure 3](image_url)

Figure 3

The human menstrual cycle. The biopsy sampling days in the follicular phase (c.d. 8–13) and the luteal phase (c.d. 20–25) in the 28-day cycle are indicated on the c.d.-axis. E₂ = oestradiol; FSH = follicle-stimulating hormone; LH = luteinizing hormone; c.d. = cycle day.
The pulsatile secretion of GnRH from the hypothalamus controls the delicate balance between the pituitary FSH and LH secretions, which is pivotal for the regulation of reproductive physiology in vertebrates. The ovarian response to FSH is increased E₂ production. E₂ is produced by granulosa cells of the maturing oocytes in the ovarian follicles. The follicle with the greatest number of granulosa cells, FSH receptors, and the highest E₂ and inhibin production becomes the dominant follicle from which ovulation occurs. The theca cells, surrounding the granulosa cells in the developing ovarian follicles, produce androgens from cholesterol, which serve as precursors for E₂ production by aromatization in the neighbouring granulosa cells. Increasing E₂ concentrations cause negative feedback to the pituitary gland and inhibit FSH secretion. Inhibin production is a further mechanism by which FSH levels are reduced below the threshold at which only the dominant follicle can respond, ensuring atresia of the remaining ovarian follicles. As the dominant follicle secretes increasing levels of E₂, there is positive feedback to the pituitary gland to secrete LH. By day 13 of an average 28-day cycle LH surge occurs, which triggers ovulation, causing the oocyte to be expelled from the dominant follicle. LH induces conversion of the dominant follicle into a corpus luteum which begins production of progesterone and E₂ to prepare the endometrium for implantation of a fertilized oocyte. Ovulation can be confirmed by measurement of the progesterone concentration in serum in the luteal phase (Campbell 2000, Israel 1997, Ludwig 2000, Makarainen 1998).

Unless pregnancy occurs, the corpus luteum has a fixed lifespan, thus the duration of the luteal phase is fairly constant, being around 14 days in the ovulating cycles of most women, irrespective of the length of the follicular phase of the reproductive cycle. Progesterone causes negative feedback on both FSH and LH, and hence both pituitary hormones are reduced to low levels during the luteal phase of the cycle and initiation of new follicular growth is inhibited for the duration of the luteal phase. If pregnancy does not occur, the corpus luteum undergoes regression, the progesterone level falls rapidly, menstruation begins, and the cycle is repeated.

The most obvious manifestation of the normal menstrual cycle is the presence of regular menstrual bleeding caused by cytokine-induced programmed cell death (apoptosis) through shedding of the endometrium in response to progesterone withdrawal, in the absence of conception. The first day of menstrual bleeding is considered day 1 of the menstrual cycle and marks the beginning of the follicular phase of the cycle where the plasma concentrations of E₂, progesterone and LH reach their lowest point. As the progesterone level falls at the end of the luteal phase, FSH begins to rise in preparation of the next reproductive cycle by stimulating the maturation of another group of ovarian follicles. E₂ is a mitogenic hormone, stimulating cell growth in the endometrium, and with increasing production during the follicular phase, the endometrial stroma thickens and reaches maximal thickness at the time of ovulation. After ovulation the hormonal balance changes from oestrogenic dominance to progestational dominance. Progesterone is not a mitogen, but causes differentiation of progesterone receptor- (PR) containing
tissues. Progesterone converts the proliferative endometrium in the follicular phase into a secretory endometrium in the luteal phase.

Under the influence of increasing E₂ in the follicular phase, the endocervical glands secrete large quantities of thin, clear, watery, endocervical mucus, facilitating sperm capture, storage and transport. This mucus production is maximal at the time of ovulation, but with the shift to progesterone dominance in the luteal phase, the endocervical mucus become thick, opaque and tenacious (Beckmann 2005).

If the oocyte is fertilized, secretion of human chorionic gonadotropin (hCG) from the syncytiotrophoblasts increases rapidly at the time of implantation of the embryo in the endometrium, beginning approximately one week after fertilization (Speroff 1999a). Thus, using conventional sensitive pregnancy tests, hCG can be detected one week prior to the expected but missed menstrual bleeding. hCG binds to the LH receptors in the corpus luteum, sustaining a corpus luteum gravitans that produces E₂ and progesterone for the next two month of the pregnancy. Thereafter, the steroid hormones are produced by the placenta. In menopausal women, the serum concentration of E₂ is significantly reduced (Rohr 1999) and the menstrual cycle changes in progesterone concentrations cease.

E₂ is crucial for normal female maturation during puberty including growth and differentiation of female genital organs, including the vagina and mammary glands. As described above, the ovarian steroid hormones are essential for efficient female reproduction. Circulating E₂ and progesterone in serum are mainly bound to plasma proteins (albumin, sex-hormone-binding globulin) but a free, unconjugated fraction can also be detected. Steroid hormones mediate their activity by interaction and activation of specific receptor proteins, the steroid receptors.

STEROID RECEPTORS
Steroid receptors develop already in utero in females and ER is first detected in the lower genital tract before the 16th gestational week (Taguchi 1986). Steroid receptors are members of a superfamily of genes expressed in target tissue, comprising nuclear receptors for diverse ligands. PR and ER are members of a subgroup of nuclear receptors regulating a number of physiological and morphological processes in response to binding of their ligands, i.e. progesterone and E₂. The ovarian steroid hormones are small lipophilic signals that enter the target cells by diffusion through the cell membrane.

Nuclear receptors are ligand-activated transcriptions factors, mediating the expression of target genes by binding to specific response elements. These single-chained receptors consists of a hypervariable N-terminal domain contributing to the transactivation function, a highly conserved DNA binding domain responsible for specific DNA binding and dimerization, and a C-terminal domain involved in ligand-binding nuclear localization and ligand-dependent transactivation (Whitfield 1999).
The transcription of gene segments to messenger ribonucleic acid (mRNA) sequences in the nucleus and the subsequent translation of mRNA into specific proteins in the target tissues results in a variety of different effects that are mediated by the so-called genomic pathway (within hours). An alternative non-genomic pathway, involving the interaction of the steroid hormone with membrane recognition sites may explain the rapid effects (within seconds) in the brain and reproductive system.

The PR exists in two isoforms, A and B, expressed from a single gene on chromosome 11. PR A is a shorter molecule (94 kDa), lacking 164 amino acids from the N-terminal region of the PR B form (116 kDa), but is otherwise identical to the PR B subtype. Hence antibodies recognising solely the PR A isoform can not be produced.

Two subtypes of ER are known, expressed by two chromosomes, 6 and 14. The presence of ERα, discovered in 1958 (Green 1986, Greene 1986), and the more recently detected ERβ, (Kuiper 1996, Mosselman 1996) may explain some of the different effects of steroid hormones in different tissues due to their tissue-specific and hormonal-state-dependent distribution and expression.

PR and ER have been demonstrated in human steroid-sensitive tissues, at the mRNA and / or protein levels in a wide range of organs using molecular biology and immunohistochemistry techniques (Amso 1994, Enmark 1997, Eva 2003, Kuiper 1998, Mokrzycki 1997). In the human endometrium PR and ER expression generally increases during the proliferative phase and decreases during the secretory phase of the menstrual cycle (Mote 1999, Noe 1999, Punyadeera 2003, Snijders 1992). Notably, in the outer portion of the uterine wall, as well as in the endometrium of postmenopausal women not on hormone replacement therapy, ER and PR expression does not exhibit a cyclic pattern, but are expressed corresponding to the peak expression levels in the late follicular phase (Noe 1999). As in the endometrium, ER expression in normal human breast tissue is down-regulated in the luteal phase when the progesterone concentration in serum is at its peak level, or under the influence of exogenous gestagens. In contrast, PR expression in normal human breast tissue remains at a high level throughout the menstrual cycle, but is down-regulated by exogenous gestagens (Soderqvist 1993, Soderqvist 1998). Although the endometrial epithelium is often referred to as the “standard” of steroid-hormone-sensitive tissue, it is a uniquely dynamic tissue, undergoing monthly cycles of proliferation and subsequent secretory activity, in order to create an environment suited for a histoincompatible foetus and extrapolation to other steroid-hormone-sensitive tissues is inadvisable. Consequently, steroid receptor expression is dependent on both the tissue and the hormonal status.

ER and PR mRNA has been demonstrated in vaginal tissue extracts (Chen 1999, Gebhart JB, 2001), and PR and ER expression has been shown reported in vaginal

The consequences of worldwide excessive use of steroid hormones, including hormonal contraceptives, on PR and ER expression in human reproductive tissue, have been poorly investigated. In endometrial biopsies from women using LNG-releasing intrauterine devices (IUDs) both ER and PR were lower after than before insertion (Pengdi 1999). Little is known about the effect of exogenous administration of steroid hormones on steroid receptor expression in the vagina. This is one of the aims of this study and will be discussed in detail.

**HORMONAL CONTRACEPTIVES**

Attempts to control fertility have probably been made throughout history. When synthetic hormonal contraceptives became commercially available in the 1960s the ability of women to control their fertility changed dramatically. The ideal method of contraception must be highly effective, safe, inexpensive, available, easy to use and reversible. Such a method does not exist, and among the methods available today none is 100% reliable. The Pearl index is defined as the number of pregnancies per 100 women-years of exposure (regular vaginal intercourse), thus including both method and user failure. The Pearl index is about 70–90 when no contraceptive method is used.

**Combined oral contraceptives**

COCs became available in the 1960s and consist of a combination of synthetically produced oestrogens (e.g. ethinyl-oestradiol) and progestins (i.e. gestagens). The first pills contained gestagen doses around 10 times higher and oestrogen doses 5 times higher than in currently used pills. Most modern COCs contain ethinyl-oestradiol, exclusively used in COCs, at a dose of 20–50 µg per pill. The addition of an ethyl group to the C-17 in 17-β-oestradiol greatly enhanced the oral bioactivity of this steroid hormone (Goldzieher 1980). The gestagens in most oral contraceptives belong to the group of 19-nortestosterone derivatives (LNG, norethisterone, lynestrenol, desogestrel and norgestimat). In the present studies, commonly used, low-dose COCs, containing 30 µg ethinyl-oestradiol and 150 mg LNG per pill were studied (Neovletta® and Follimin®). These are given over periods of 21 days, followed by a tablet-free interval of 7 days, causing a sudden drop in plasma steroid levels and subsequently predictable withdrawal bleeding. The mechanism of action is inhibition of ovulation by reduction of pituitary gonadotropin (FSH, LH) secretion, caused by serum levels of ethinyl-oestradiol in the range of negative feedback. Additionally, decidual reactions in the endometrium inhibiting implantation, should ovulation have occurred, and thickening of the cervical mucus which impairs sperm penetration, also contribute to the contraceptive effects.

Apart from binding to the PR, the gestagens derived from 19-nortestosterone also bind to the androgen receptor; LNG having the strongest affinity. The possible
androgenicity of the gestagen in COC is, however, strongly balanced by the oestrogen, by induction of the hepatic carrier protein, sex-hormone-binding globulin, which binds gestagens and androgens (El Makh-zangy 1979). The effectiveness of COCs is very high, although in actual practice the failure rate varies, with a Pearl index between 0.2 and 3, as a result of varying compliance and other factors reducing the efficacy, i.e. disturbed absorption and drug interaction.

**Gestagen-only methods**

Gestagen-only contraceptive methods were developed in an attempt to avoid side effects and risks associated with oestrogen usage, noted in certain women. The effects of gestagen-only formulations depend on gestagen type, dose and route of administration.

**High-dose gestagens**

Depot-medroxyprogesterone acetate injections (DMPA), given intramuscularly at doses of 150 mg every third month (Depo-Provera®) is considered to be a high-dose compound with high efficacy. The gestagen medroxyprogesterone acetate (MPA) belongs to the group of 17-α-hydroxyprogesterone derivatives that differ from 19-nortestosterone derivatives in their lower anti-oestrogenic and androgenic effects. Following a single injection, serum concentrations of MPA vary between individual women (Smit 2004) but a plateau generally is maintained for about 3 months, after which there is a gradual decline over 6 months (Kaiser DG, 1974, Mathrubutham 1981, Mishell 1996, Ortiz 1977). DMPA exerts its contraceptive effect predominantly by inhibiting ovulation. The median time to return of ovulatory cycles following one single injection of DMPA is 30 weeks and by the end of 1 year the cumulative rate of return to ovulation is 95% (Jain 2004). DMPA also affects the endometrium, which becomes thin and atrophic (Khoo 1971, Lee 1968). A major advantage of DMPA is the very low pregnancy rate, as disturbed absorption, missed pills and drug interactions are not involved. The Pearl index of the method is low, 0.25.

**Low-dose gestagens**

Gestagens at a low dose can be administered by different routes. In the present study LNG-releasing implants (Norplant®, Leiras) were studied. The subdermal placement of the implants in the upper arm allows for continuous release of LNG from 6 rods, providing contraceptive effectiveness for at least five years. Norplant affects cervical secretion and the endometrium, and anovulation is induced in 80% of the users during the first year, with increased frequency of ovulatory cycles as the drug release from the implants slowly declines (Faundes 1991, Segal 1991). The Pearl index is low, 0.5.

Other gestagen-only methods include mini-pills (lynestrenol, norethisterone, norgestrel and desogestrel), levonorgestrel-releasing IUDs (Levonova®) and the more recently available etonogestrel-containing implants (Implanon®, 1 single rod with a 3-year active period).
All the cells of an individual contribute in a specific way to maintaining a healthy condition and survival, and the result is the sum of the cells and their features and a cell orchestration adapted to its purpose. There is an ongoing battle to maintain host integrity, although mainly imperceptible, against both exogenous agents and endogenously produced components. The battle against potentially pathogenic microorganisms and other toxic agents in the environment will be induced only when they come into direct contact with the organism. The location, dose and time necessary to induce readily visible traces of the war (so-called acute inflammation; *tumour, dolour, calor, rubor*) are dependent on the pathogenicity of the invader in relation to the ability of the host to defend itself against the specific agent. In addition, endogenously produced agents (e.g. products from the intestinal, hepatic and renal metabolism, cell turn-over debris including apoptosis, and tumour cells) keep our defence mechanisms busy with “local housekeeping”.

The immune system constitutes an interactive network in the host, made up of different types of immune cells and humoral factors (e.g. antibodies and cytokines). Immune cells are present at high amounts in blood, lymph and, lymphoid organs, but are also distributed although less densely throughout most bodily tissues. To establish an infection the pathogen must first overcome the physical surface barriers, i.e. skin and mucous-lined body cavities, and this is facilitated by physical lesions providing more vulnerable sites of invasion. Any pathogen that breaches this barrier encounters the next levels of defence: the innate and acquired (adaptive) immune responses, which usually collaborate to eliminate pathogens. Innate immunity provides initial limitation of the invading microorganisms by responding directly (within hours) at the mucosal surface, allowing time for the subsequent activation of adaptive immunity, which takes several days, but generates a memory against recurrent infections with the same pathogen. In response to certain components from microorganisms, antigens (Ags), immune reactions cover a broad spectrum of effects aimed directly at the pathogen, and indirectly by activating secondary components of the immune response (Delves 2000). In addition, the epithelial cells in the surface barriers also contribute significantly to local immune reactions, e.g. by the production of antimicrobial peptides and cytokines (Fahey 2005, Wira 2005).

Reactions provoked by harmless exogenous components (i.e. allergy) or immune reactions against endogenously produced normal cells (i.e. autoimmune diseases) are the result of defective Ag recognition, rendering an over-reactive immune defence.

Innate immunity is the first-line host defence which serves to limit infection during initial exposure or re-exposure to microorganisms. In a phylogenetic perspective there are similarities between innate pathogen recognition in insects and mammals,
indicating a common ancestry of these defences (Hoffmann 1999). In mammals innate immunity also promotes the subsequent specific adaptive immune responses induced when the surface receptors of B and T lymphocytes specifically bind to the Ag.

The innate immune responses developed earlier in evolution than acquired responses, and include all immune defence reactions that lack immunologic memory and thus remain unchanged despite repeated challenge. The innate responses use phagocytes (neutrophils, monocytes and macrophages), cells releasing inflammatory mediators (basophils, mast cells and eosinophils), and natural killer (NK) cells. The humoral components of innate immunity include the complement system, acute-phase proteins, cytokines and antimicrobial peptides/proteins such as lysozyme and defensins.

Innate immune recognition relies on a limited number of germline-encoded receptors, which have evolved to recognize conserved products from microbial pathogens, pathogen-associated molecular patterns (PAMPs), but not those of the host, allowing the immune system to distinguish between infectious nonself and non-infectious self (Medzhitov 2000). Examples are lipopolysaccharide, lipoteichoic acid and mannan on Gram-negative bacteria, Gram-positive bacteria, and yeast cell walls, respectively, recognized by receptors (e.g. Toll-like receptors) on phagocytes. Thus, a central feature of innate reactions is recruitment and activation of phagocytes at the site of infection to eradicate pathogens. Macrophages release cytokines in response to activation by an infection. Hereby stimulating the production of granulocytes in the bone marrow, causing characteristic leukocytosis when they are released into the circulation and travel to the site of infection with the aid of pro-inflammatory mediators, adhesion molecules (facilitating transport through endothelia), and attract by chemokines. The recruited neutrophils phagocyte the organisms and killing of the pathogens is achieved by intracellular production of toxic substances. Pathogen ingestion and killing by neutrophils is much more effective if the pathogens are first opsonized with specific antibodies (upon repeated exposure) or certain components of the complement system.

Eosinophils have receptors for the Fc-portion of antibodies of the IgE class. IgE antibodies are commonly produced by the adaptive immune system in response to parasitic infections. Eosinophils release cytotoxic granules into IgE-coated parasites. Eosinophils are also a pathologic participant in allergic reactions (Parkin 2001).

Mast cells and basophils have similar functions but there is little evidence that blood basophils develop into tissue mast cells. Both cell types have high-affinity receptors for IgE (FceR), which become coated with IgE antibodies. In atopic allergies, allergen binding to the IgE cross links the FceR, which triggers the cell to secrete inflammatory mediators, for example histamines, prostaglandins, serotonin and leukotrienes.

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NK cells have lymphocyte morphology but do not bear a specific receptor for Ag. Instead they bear receptors for the Fc-portion of antibodies of the IgG class (FcR) which bind antibody-coated cells, leading to antibody-dependent cell-mediated cytotoxicity (ADCC). NK cells also have a multitude of surface receptors called NK receptors. Some of these are stimulatory and induce cytotoxicity without prior immunization, and some are inhibitory and prevent the NK cell from exerting its killing function. The inhibitory receptor reacts with major histocompatibility complex (MHC) class I molecules. Normal host cells express MHC class I molecules, while tumour cells and certain viruses cause down-regulation of MHC class I molecules. In this case there will be no interaction with the inhibitory receptor and the NK cell is programmed to lyse the target.

*Antigen-presenting cells* (APCs) constitute a heterogeneous group of dendritic cells including Langerhans cells, activated/differentiated monocytes, macrophages and B cells that are specialized in processing extracellular Ags. They present Ags to the lymphocytes and collaborate with them in the response to Ags. APCs express MHC class II molecules which present processed extracellular Ags to T helper cells, thereby initiating adaptive immune responses.

The *complement system* is a group of serum proteins that respond to presence of certain foreign substances or antibody-Ag complexes by a cascade of events, resembling the coagulation cascade, leading to the formation of transmembrane pores and cell lysis. Components formed after complement activation also increase vascular permeability, allowing antibody penetration, induce inflammation, recruit phagocytes and induce specific immune responses by stimulating APCs (Parkin 2001).

Epithelial surfaces not only serve as physical barriers against infection, but can also contribute to the innate defence by the release of effector molecules, e.g. antimicrobial peptides, at the surface of the epithelium thereby inhibiting invading microbes.

The *defensins* are the most prominent family of antimicrobial peptides in humans (Lehrer 2002, Lehrer 2004). All well-characterized defensins are encoded by a cluster of genes residing on chromosome 8p22–p23 (Linzmeier 1999, Jia 2001, Schutte 2002, Garcia 2001a). Defensin mRNA is translated into an inactive prepropeptide, which is activated by post-translational cleavage into a 3–5 kD peptide with three intra-chain disulphide bonds. Defensins can be divided into two structural subclasses, α- and β-defensins, on the basis of the position of their disulphide bonds. Hitherto, the expression of six α-defensins has been demonstrated in man. Four of these, human neutrophil peptides (HNP) 1 to 4, are mainly expressed in neutrophils, while human defensins (HDs) 5 and 6 are constitutively expressed in epithelial cells, namely Paneth cells in the crypts of Lieberkühn in the small intestine under normal conditions (Ghosh 2002, Ouellette 2004). Bowel inflammation in patients with ulcerative colitis and celiac disease...
leads to increased levels of HD-5 and HD-6 due to the development of metaplastic Paneth cells in the colon and small intestine, respectively (Cunliffe 2001, Fahlgren 2003, Forsberg 2004). HD-5 was also reported to be expressed in the female reproductive tract (Quayle 1998, Svinarich 1997). Human β-defensins (HBDs) 1 to 4 are preferentially expressed in epithelia.

**HBD-1** was first cloned from kidney and vagina tissue (Bensch 1995), but has later been demonstrated in a large variety of tissues (Valore 1998, Zhao 1996). It appears to be constitutively expressed and intestinal epithelial cell expression is not increased by exposure to bacteria or pro-inflammatory cytokines such as IL-1β, tumour necrosis factor-α (TNF-α) and IFN-γ in vitro or by inflammation in vivo (Fahlgren 2003, Forsberg 2004, O’Neil 1999). **HBD-2** was first isolated from psoriatic scale extracts and was later shown to be locally induced by bacteria and pro-inflammatory cytokines in skin, the lungs, stomach and intestines (Fahlgren 2003, Harder 1997, Liu 1998, Wehkamp 2002). **HBD-3** was initially cloned and isolated from epidermal keratinocytes from patients with psoriasis, and was reported to be induced by IFN-γ, TNF-α and bacteria in lung and intestinal epithelial cells (Fahlgren 2004, Garcia 2001a, Harder 2001, Jia 2001). **HBD-4** was first demonstrated in cDNA from lung tissue (Garcia 2001b) and can be up-regulated by Gram-negative and Gram-positive bacteria in respiratory cells, and by IFN-γ and TNF-α in intestinal cells (Fahlgren 2004, Garcia 2001a). HBD-3 and HBD-4 were also shown to be expressed in the endometrium (King 2003).

Defensins as a group manifest antimicrobial activity against a broad spectrum of microbes, including Gram-positive and Gram-negative bacteria, fungi and enveloped viruses (Ganz 2003, Lehrer 2004). However, different defensins are selective in their activity. HBD-1 is active against Gram-negative bacteria (Harder 1997, Valore 1998,), while HBD-2, HBD-3 and HBD-4 are also active against Gram-positive bacteria and yeast (Ganz 2001). Furthermore, HBD-1 and HBD-2 are sensitive to salt whereas HBD-3 is not. Defensins display activities that implicate them as regulators of inflammation and they may provide a link between innate and adaptive immune responses. Chemoattractant activity toward dendritic cells, memory T cells and mast cells has been reported for HBD-2 and HBD-3 (Chertov 1996, Chertov 2000, Durr 2002, Niyonsaba 2002, Yang 1999) and both HBD-3 and HBD-4 are chemotactic to monocytes (Garcia 2001a, Garcia 2001b, Harder 2001).

**Cytokines** are small peptide messengers with a variety of functions, produced and secreted mainly by immune cells to alter their own behaviour or that of other cells by binding to specific chemokine surface receptors, thereby sending intracellular signals resulting in activation, division, apoptosis or movement. Chemokines are cytokines with chemoattractant activity. Interleukins (ILs) are produced by and mainly effect leukocytes. Today, several ILs with different functions are known. Upon phagocytosis, the macrophages become activated and release IL-6 into the blood, thereby inducing production of acute phase proteins (e.g. C-reactive protein
by the liver. Interferons (IFNs) interfere with viral replication. IFN-α and -β are produced by any virus-infected cell while IFN-γ is only produced by Th 1 helper cells and NK cells. IFN-γ activates macrophage and neutrophil intracellular killing, stimulates NK cell function and enhances Ag presentation by increasing MHC class II expression on APCs. Cytokines are also important messengers in adaptive immunity (Mackay 2001).

ADAPTIVE IMMUNITY
The acquired (adaptive) immunity consists of Ag-specific responses mediated through two classes of specialized lymphocytes, B and T cells, derived from the bone marrow and thymus, respectively, where they have undergone antigen receptor gene rearrangement, before exposure to Ag, which leads to millions of T cell receptors (TcRs) and antibody specificities (Arstila 1999), sufficient to cover a range of pathogens likely to be encountered in life. These naïve lymphocytes populate the secondary lymphoid tissues of the lymph nodes, spleen and mucosa-associated lymphoid tissue, where a microenvironment for immune responses to be generated is provided. The lymphoid organs communicate with the tissues using lymphatics and blood vessels, and are able to produce chemotactic cytokines, so-called chemokines, to attract and maintain B and T cells. They also contain APCs that present extracellularly processed Ags. Antigen receptors are clonally distributed and both the TcR and the antibody, which is transmembrane-anchored in the B cell membrane, the B cell receptor, have binding sites for specific Ag. An acquired immune response is activated when the surface receptors of Ag-specific lymphocytes bind to the Ag, resulting in clonal expansion and induction of cell-mediated and humoral immune responses. B cells activated by Ag differentiate into plasma cells secreting antibodies, a process that is generally dependent on help from T helper cells. The Ag-specific antibodies (immunoglobulin [Ig]) secreted by plasma cells are responsible for elimination of extracellular microorganisms (humoral immune responses). Igs not only function in isolation but in several ways trigger other components of the immune system to provide defence against the invader. Igs can neutralize toxins, prevent adhesion of pathogens on mucosal surfaces, activate the complement system, opsonize microbes for phagocytosis and induce ADCC. T lymphocytes can also participate in cell-mediated immune responses, either by secreting cytokines as IFN-γ which activate macrophages into more forceful phagocytes and increased expression of MHC class II molecules giving them the capacity of fully fledged APCs, or by inducing differentiation of pre-cytotoxic T lymphocytes to cytotoxic effector cells (CTLs) which specifically kill virus-infected cells. Immune cells resident in body surfaces play a critical role in Ag recognition and local surveillance.

Ags presented by APCs activate T lymphocytes, recognized by the expression of the TcR in association with the cluster of differentiation 3 (CD3) molecules on their surface, and they can eradicate intracellular pathogens by activating macrophages and by killing virus-infected cells. T cells also help B cells to
differentiate to antibody-secreting plasma cells. In addition, memory cells develop so that the individual is protected from repeated infection by the same pathogen or at least reduce infection by faster and enhanced response (Klonowski 2005). There are two types of effector αβ T cells: T helper cells bearing CD4, and cytotoxic T cells (CTL) bearing CD8 molecules on their surface (Parklin 2001).

αβT cells recognize processed Ag presented by MHC molecules. CD8+ T cells recognize Ag presented by MHC class I molecules expressed by most normal cells. T helper (CD4+) cells recognize Ag by MHC class II molecules expressed by APCs. T helper cells are subdivided functionally by their cytokine products. Th1 cells produce IL-2 and interferon (IFN)-γ which stimulate cell-mediated responses by activation of CTLs and macrophages. Th2 cells produce IL-4, 5, 6 and 13 which favour humoral immunity by stimulation of B cells, leading to Ig production. CTLs lyse abnormal cells recognized by their expression of a foreign antigen on MHC class I by activation of apoptosis using perforins and granzymes or by Fas/Fas ligand interaction (Delves 2000b).

Successful immune response eliminates the challenging Ags and generates memory. Then the activity is down-regulated and returns to near-basal level. Within both CD4+ and CD8+ T cells there are subgroups of T cells that act down-regulatory by secretion of cytokines. Th1 cells secrete IFN-γ which inhibits Th2 cells, and the secretion of IL-10 from Th2 cells inhibits Th1 cells. In the intestinal mucosa distinct subsets of regulatory T cells are detected and defined by their phenotype and their secretion of down-regulatory cytokine, e.g. IL-10 and TGF-β1. Most CD4 regulatory T cells (CD4+ CD25+) interact with dendritic cells and CD8+ CD28− cells may recognize Ags presented by epithelial cells (Allez 2004). They have an important role in self-tolerance and may have an important role also in the tolerance of commensal flora.

T lymphocytes can be divided into two major groups based on their TcR type: αβ T cells and γδ T cells. αβ T cells have been demonstrated in epithelia at several mucosal sites, e.g. small and large intestine, uvula, as well as nasopharyngeal and pharyngeal tonsils (Jarry 1990: Lundqvist 1995; Olofsson 2000; Olofsson 1998; Olofsson 1996). γδ T cells are detected within the epithelium at most mucosal surfaces in man (Groh 1998; Lundqvist 1995; Lundqvist 1993; Moretta 1991; Olofsson 1998; Olofsson 1996; Olofsson 2000). TcRs consist of either α/β or γ/δ heterodimers, each with a constant and a variable domain like antibodies, but unlike antibodies TcRs are produced and recognize Ags as transmembrane-anchored surface molecules in both naïve T cells and effector cells. TcR are associated with the CD3 complex of molecules on the surface of T cells. CD3 transmits signals into the cell when the TcR binds Ag. Ag binding to the TcR activates transcription of various genes, including those encoding cytokines that stimulate and regulate the recruitment of immune cells and proliferation of additional T cells.
The TcR-αβ cells recognize Ags as processed peptides in complex with MHC molecules, while most γδT cells recognize Ag by MHC-like molecules (non-classic MHC), such as CD1 molecules presenting certain Ags or unprocessed Ags with conserved molecular patterns. Other γδT cells recognize Ag directly, as antibodies do (Allison 2001). αβ and γδT cells differ in their abilities to stop bacterial invasion. It has been postulated that γδT cells contribute to the first line of defence in a chronological sense, whereas αβT cells clear bacteria at a later stage (Chien 1996). Experiments with TcR gene-knock-out mice suggest that γδT cells also play a key role in the surveillance of epithelia (Girardi 2001; Hayday 2003).

In vitro Ag priming of γδT cells with alkylamine Ags results in a memory response to these Ags. Such priming also results in a direct non-memory response to whole bacteria and to lipopolysaccharides, characterized by γδT cell effector functions like IL-12-dependent secretion of IFN-γ and also by γδT cell proliferation. This unique combination of innate immune response and immunologic memory shows that γδT cells can function as a bridge between innate and acquired immunity (Kamath 2003). γδT cells are also able to secrete cytokines (e.g. MIP-1α, MIP-β and RANTES; Hayday 2000, Hayday 2003, Lehner 2000). Thus, αβ and γδT cells recognize Ags in a fundamentally different way.

In humans, major subsets of γδT cells are recognized on the basis of their TcR Vδ-gene utilization. While Vδ2 T cells dominate in the peripheral blood (Esin 1996), Vδ1 cells are preferentially localized in the mucosal tissues (De Rosa 2001, Kabelitz 2005) and respond to stress-induced expression of the MHC class I related chains A and B, independent of antigen presentation (Groh 1998, Wu 2002).

**MUCOSAL IMMUNOLOGY**

The mucosal surfaces of the body and their cellular composition and secretory products provide a mechanical and physiological interface between the external environment and the systemic immune system. Mucosal immunity involves non-specific components common to most mucosal surfaces and constituents restricted to certain mucosal sites. The secretory products of mucosal surfaces, including locally produced, naturally occurring enzymes, mucus and Igs have a wide range of appearances, i.e. tears, saliva, mucus and milk, and respiratory-, gastrointestinal-, urinary- and genital secretions. Certain constituents are common to most but not all secretions (e.g. lysozyme, lactoferrin, proteolytic enzymes and mucins), i.e. protection against bacteria and mechanical protection against epithelial invasion (Menge 1993).

Among the mucosal surfaces, the female reproductive tract has evolved to meet the dual challenge of providing a receptive environment for allogeneic sperm and embryo implantation while providing continuous protection against potential pathogens. Although the endometrial surface has been studied much more extensively than the mucosal surface in the vaginal epithelium, the mucosal
immunology of the female genital tract is dependent both on the tissue, and the hormonal status and has to be investigated accordingly.

The mucosal-associated lymphoid system has been well defined in the gastrointestinal, respiratory and nasal tracts, including the inductive sites of aggregated lymphoid tissues, such as Peyer’s patches in the gut, and effector sites of the respective mucosal tissues. However, a corresponding genital-associated lymphatic tissue has not been clearly identified. The specific humoral defence in mucosal secretions is provided by the Igs. In most mucosal secretions Igs are produced locally by plasma cells in lamina propria and IgA is the dominating Ig isotype (Mestecky 1991), whereas cervico-vaginal secretions contain more IgG than IgA (Bouvet 1994, Mestecky 1999, Mestecky 2005). The predominance of plgA and the ratio of IgA1 to IgA2 in cervical secretions indicate that much of the IgA originates from local production, mainly from Ig-secreting cells in the uterine endocervix, not from plasma (Russell 2002). The vaginal lamina propria contains dispersed IgA- and J-chain-positive plasma cells, and both IgA- and IgG-containing vaginal epithelial cells are found on the luminal surface and dispersed in the squamous epithelium. However, the epithelium does not stain for the polyIg-receptor, suggesting that vaginal epithelial cells can acquire locally produced and plasma-derived antibodies by an alternative route (Russell 2002). The mechanisms involved in the appearance of IgG in cervico-vaginal secretions are unknown. However, hysterectomy greatly reduces Ig levels in the vagina, suggesting the tissues in the upper genital tract, lined with columnar epithelium, as the main sources of Ig in cervico-vaginal secretions (Miller 2003a).

In the adaptive arm of cell-mediated immunity T lymphocytes with potential cytolytic capacity have been demonstrated in vaginal epithelium of healthy women, suggesting their importance as effectors against intracellular pathogens in the front-line defence also of the lower genital tract (Patton 2000; White 1997a, White 1997b). Tracing the migration of T and B cells in experiments on primates revealed that the internal and external iliac lymph nodes may function as an inductive site from which these cells migrate to the effector site in the lamina propria of the cervico-vaginal mucosa and rectum (Mitchell, 1998), suggesting a putative genito-rectal-associated lymphoid tissue (GERALT; Lehrer 2003). The importance of T lymphocytes in the defence of the vagina was also suggested by murine studies in which CD4+ cells and CD8+ cells, as well as γδT cells, were shown to respond to intravaginal challenge with common viral and bacterial agents of STDs (Gillgrass 2003, Johansson 2001, Nishimura 2004, Parr 1998, Parr 2000).

One important component of mucosal immunity is the site-specific commensal flora. *Lactobacilli* are normal commensals of the vaginal microflora and beneficial for vaginal health because they compete with exogenous microbes for nutrients. The protective role in inhibiting colonization of potentially harmful microorganisms is facilitated by their fermentation of glycogen released from the desquamated cells into form lactic acid, which maintains an acid environment in the vagina (pH 4.0–4.5) and production of hydrogen peroxide.
During and following vaginal intercourse, the vaginal mucosa is directly exposed to the penile mucosa and semen deposition. As it is the first site of contact with potential pathogens transferred from the counterpart, the vaginal mucosal immunity is particularly important in this context. The ability to prevent infections depends on both innate and adaptive immune mechanisms.

**IMMUNITY AND SEX STEROID HORMONES**

Steroid hormones may be important in regulating the immune system (Grossman 1984). There is growing evidence of the effect of sex steroid hormones on immune reactivity in the female genital tract (Beagley 2003, Franklin 1999, Lü 2002, Parr 1994. Polanczyk 2003, Rakaszi 2002, Smith 2000, Thongngarm 2003). The human endometrium undergoes local cycle changes also with regard to immune functions, and over the years several studies have indicated a immuno-regulatory role of steroid hormones (Flynn 2000, Laird 2003, Mettler 1996). The role of sex steroid hormones in regulating the immunity may be especially pronounced in the genital tract. Steroid hormone variations during the menstrual cycle are known to affect cervical Ig and cytokine secretion patterns in rhesus macaques and humans (Lü 2002, Kutteh 1998, Franklin 1999). This fluctuation in Ig levels occurs despite the fact that immune cell populations in the cervico-vaginal mucosa remain constant under the influence of endogenous steroid hormone concentrations during the menstrual cycle (Ma 2001, Patton 2000). In a mouse model the immunomodulating effect of oestrogen has been demonstrated since it can stimulate antibody response but inhibit T-cell-mediated inflammation (Joseffson 1992). In postmenopausal women, treatment with oestrogens prevents recurrent urinary tract infections (Privette 1988, Raz 1993). Recently, it was shown that oestrogen drives the expansion of regulatory T cells (CD4+CD25+ cells) in the mouse spleen in an ERα-dependent manner (Polanczyk 2004). In COC users the IgA content in cervical mucus is increased (Franklin 1999). The immunosuppressive role of gestagens on immune regulation has been shown from results in mouse models where MPA increase susceptibility to infection by herpes simplex virus-2 much more than progesterone and, unlike progesterone, to significantly decrease immune response to the Ag due to decrease in the humoral and the cellular immune responses (Gillgrass 2003, Kaushic 2003, Parr 1994, Parr 2003). Administration of DMPA to mice was shown to increase susceptibility to the herpes simplex virus (Gillgrass 2003, Kaushic 2003, Parr 1994). Studies in SIV/rhesus macaque models has been demonstrated that exogenous oestrogen administration decreases SIV transmission (Smith 2000), while exogenous gestagen administration increases SIV transmission after intravaginal inoculation of SIV. It has been suggested that in macaques, gestagens are factors that causes loss of genital tract epithelial integrity (Miller 20035). In a study, human papilloma virus type 16 viral transcription was induced by progesterone- mediated induction through hormone response elements in the regulatory region of the viral genome (Mittal 1993). It has been suggested that the immune suppression manifested by MPA is mediated by its glucocorticoid rather than progestogenic actions (Kurebayashi 2003, Koubovec 2004). Within the T cells
there are subtypes that act down-regulatory by secretion of cytokines (Allez 2004). Immuno-regulation also involves interactions of the immune system with both the endocrine and nervous systems, with cross-talk between these systems involving hormones, neurotransmitters and cytokines (Delves 2000\textsuperscript{9}). Also the lymphoid cells bear steroid and insulin-like growth factor receptors on their surface and can respond to changes in hormone concentration (Parkin 2001).

The role of steroid hormones on the transmission and progression of STDs in women must be further investigated, since studies in rhesus macaques have shown that subcutaneous progesterone administration by implants resulted in vaginal epithelial thinning and increased vaginal SIV transmission (Marx 1996). In a human epidemiologic study, DMPA usage was found to be associated with a higher virus set point during the early stages of HIV infection (Lavreys 2004). This raises questions regarding the effect of sex steroid hormones commonly used in hormonal contraceptives on local immune protection against potentially pathogenic microorganisms in the vagina, the route of entry for STDs in women. Thus, the direct consequences of changes in the concentrations of steroid hormones, as well as the indirect effect of hormonal contraceptive use on immune regulation in the vaginal mucosa must be further investigated.

STD–HIV

The female reproductive tract includes the vulva, vagina, cervix, uterus and ovaries, all at risk of infection, e.g. STDs or virus induced tumour development. STDs, including HIV are a major health problem worldwide, and a leading cause of morbidity and mortality in both adults and newborns (WHO 2004). There are many agents of varying virulence threatening the genital tract mucosal surfaces. STDs can be caused by viruses (e.g. human papilloma virus, herpes simplex virus, hepatitis virus, HIV), bacteria (e.g. Chlamydia trachomatis, Neisseria gonorrhoeae, Treponema pallidum), parasites (e.g. Trichomonas vaginalis) and yeast (e.g. candida species). Following intravaginal deposition of pathogens, the infection may travel via the cervix into the uterine cavity and Fallopian tubes and develop into a serious infection; i.e. pelvic inflammatory disease. Due to irreversible post-inflammatory scarring of the tubes (as well as in the deferent ducts in men) the fertilized egg can be trapped and develop in the narrow Fallopian tube, instead of completing its normal journey to the uterus. Such ectopic placental development can lead to heavy, life-threatening intra-abdominal bleeding due to rupture of the tube, a condition requiring urgent surgical intervention. In cases of severe inflammation, complete obstruction of the tubes occurs constituting a common cause of infertility. In pregnant women STDs can cause miscarriage or premature rupture of membranes and preterm labour. The molecular pathways through which the immune responses to pathogens are activated differ with different sources of Ags. For example, responses to Neisseria gonorrhoeae and Treponema pallidum are mediated via TLR2 (Lien 1999, Massari 2003). In contrast, endotoxin from Chlamydia trachomatis activates cells via TLR4 (Prebeck 2003).
The cell-mediated immune responses to viral infections are integrated in a complex system of responses regulated in an intricate manner, and are often difficult to modify. Analysis of diseases where the immune response fails to clear up the infection has contributed to the understanding of some of the host defence mechanisms involved, which in the longer term may help to provide new methods for controlling or preventing viral infectious diseases, including AIDS.

Heterosexual intercourse is the most common vector of HIV transmission to and from women, and therefore a better understanding of mucosal cervico-vaginal immune mechanisms and the effects of hormonal contraceptives is critical. Individuals infected with HIV develop symptoms as a result of progressive disruption of cell-mediated immune responses and this mechanism is described here as an example of a viral STD and its impact on immune responses.

HIV is a lentivirus of the retrovirus family. The major HIV surface glycoprotein (Gp 120) serves as the primary attachment for HIV particles to the preferred host cells by binding of the viral glycoprotein to a CD4 receptor molecule on the host cell surface. Susceptible host cells include T lymphocytes, monocytes, macrophages, follicular dendritic cells and microglial cells. After attachment to the host cell surface, the virion enters the host cell membrane via binding to the co-receptor chemokine receptor (CCR) 5, internalizes and uncoats its single-stranded RNA. The exposure of viral reverse transcriptase products to cytoplasmatic nucleotides activates the production of complete double stranded DNA copies from the viral RNA. The DNA copy, compiled with viral integrase and reverse transcriptase, is transported to the cell nucleus where the viral DNA is attached to the host cell DNA by the viral integrase. Upon host cell activation, transcription of the integrated HIV DNA is initiated, resulting in the production of genomic RNA for incorporation into new virions and mRNA, the translation of which generates several regulatory proteins facilitating viral replication, assembly and release. Subsequently, new complete HIV virions are released from the host cell surface.

Following primary HIV infection, an acute decrease in circulating CD4⁺ and CD8⁺ cells occurs with rapid viral replication and dissemination throughout the lymph nodes and monocyte/macrophage cells (Pantaleo 1991). The clinical signs are acute viral syndrome characterized by fever, pharyngitis, myalgia and tender adenopathy (Cooper 1985). Within a couple of months IgM antibodies appear, followed by the development of IgG antibodies, specifically anti-Gp 120 antibodies, that persist for life. With rising HIV antibody titres the circulating viral load falls (Daar 1991), the number of circulating CD4⁺ and CD8⁺ cells normalizes and infected individuals enter an asymptomatic period.

During the asymptomatic period, HIV is effectively trapped in the lymph nodes, primarily in follicular dendritic cells. In response to viral replication, activated CD4 cells are recruited to the lymph nodes and become infected by contact with these follicular dendritic cells. Subsequently, the HIV infection leads to defective
CD4 proliferative responses to recall Ags, e.g. tetanus and Candida albicans. In addition, the CD4 receptor of uninfected T cells can bind free Gp 120 molecules released from neighbouring infected cells. Thus, uninfected cells coated with HIV Ags may be attacked by CTLs. The hallmark of HIV infection is slow, progressive depletion of circulating CD4+ T lymphocytes. In later stages most of the follicular dendritic cells within the nodes are overloaded and then destroyed, and unable to trap the virus, or other pathogens, resulting in opportunistic infections and the development of the clinical conditions that define AIDS.

In infected macrophages and monocytes viral replication occurs slowly with the accumulation of HIV in the cytoplasm but without Ag expression of viral Ag on the surface. This allows the virus to escape immune-recognition by HIV specific T cells, and with the migration of macrophages and monocytes the infection disseminates throughout the body. HIV infection also causes dysfunction of macrophages and monocytes including cytokine production, ADCC and chemotaxis, which enhances T cell dysfunction.

Does the recruitment of specific activated lymphocyte populations to vaginal mucosa surfaces play a role in the relative efficiency of HIV infection in women? Are there potentially protective mucosal immune responses in highly exposed but uninfected women? Interestingly, some subsets of women highly exposed to HIV continue to be seronegative. This is, to some extent, correlated with viral epitope-specific immune responses, but when present these responses rapidly decline following interruption of pathogen exposure leading to the acquisition of HIV (Laurence 2003). Absence of HIV infection following exposure to HIV is also associated, in a minority of exposed but uninfected women, with mutation of the CCR5 co-receptor (Kostrikis 1998, Liu 1996, Paxton 1995) or with genetic factors including particular HLA subtypes (Buchacz 1998, Luscher 2001, MacDonald 2000). Additionally, innate immunity could be involved in the generation of exposed but seronegative status. Lytic activity from NK cells, as well as interferon-γ and TNF-α production, was shown to be significantly elevated in Vietnamese HIV-exposed but uninfected intravenous drug users (Truong 2003). Robust constitutive production of α-defensin was detected in HIV-exposed uninfected individuals; peptides that may contribute to the potentially protective immune response that characterizes exposed but uninfected individuals (Trabattoni 2004). The understanding of the immune mechanisms involved in HIV transmission is crucial in the development of an effective treatment or vaccine against HIV (Locher 2005).
AIMS OF THE STUDY

The vaginal mucosa constitutes an important interior barrier in the lower genital tract in two respects; its squamous epithelium provides an effective physical barrier, and its composition of epithelial cells and intraepithelial immune cells constitutes an efficient barrier to the underlying lamina propria against potentially pathogenic agents in the vaginal lumen. These physical and biochemical barriers are under regulation by steroid hormones, and the question of possible effects of hormonal contraceptives on these protective barriers was raised. This study was performed to investigate the effects of hormonal contraceptives on the immune components in the vaginal epithelium. The aims were as follows:

- To study the vaginal epithelial thickness in healthy, fertile women and to determine any menstrual cycle changes (Paper I).
- To study the effect of long-term hormonal contraceptive use on vaginal epithelial thickness (Paper I).
- To study the composition and frequency of intraepithelial immune cells in the vaginal epithelium of healthy, fertile women and to determine any menstrual cycle changes (Papers I and II).
- To study the effect of long-term hormonal contraceptive use on the composition and frequency of intraepithelial immune cells in the vaginal epithelium (Papers I and II).
- To determine the effect of long-term hormonal contraceptive use on vaginal tissue sensitivity expressed as the frequency of steroid receptors in vaginal epithelial cells (Paper III).
- To investigate the production of important antimicrobial peptides, e.g. defensins, in vaginal epithelial cells of healthy, fertile women (Paper IV).
SUBJECTS AND METHODS

SUBJECTS (PAPERS I–III)
The studies presented in Papers I–III describes the composition and frequency of intraepithelial immune cell types and steroid receptors expressing cells resident in the human vaginal epithelium of healthy, fertile women in the follicular and luteal phase of the menstrual cycle, and in users of hormonal contraceptives, using a panel of monoclonal antibodies (MoAbs) in immunohistochemistry.

A total of 60 healthy women volunteers recruited via an advertisement were included in a single-centre study at the Department of Obstetrics and Gynaecology, University Hospital of Umeå, Sweden. Healthy and informed women, aged 20–34 years, were eligible for inclusion in either the control group \((n = 15)\), or in one of the three hormonal contraceptive user groups studied: combined oral contraceptives (COCs, \(n = 15)\), levonorgestrel subdermal implants (LNG, \(n = 15)\), or depot-medroxyprogesterone acetate injections (DMPA, \(n = 15)\). Women reporting a history of regular monthly menstrual cycles \((25–33\) days), presently using condoms as their only contraceptive method since at least \(8\) weeks, and without hormonal medication for at least \(8\) weeks constituted the control group \((n = 15)\).

The hormonal contraceptive user groups constituted of current users of COCs (NeoVletta\textsuperscript{TM}, Schering Nordiska or Follimin\textsuperscript{TM}, Wyeth Lederle; \(30 \mu g\) ethinyl-oestradiol and \(150 \mu g\) LNG), LNG subdermal implants (Norplant\textsuperscript{TM}, Schering Nordiska, 6 rods containing \(36 mg\), with \(5\) years’ lifetime), or DMPA injections (Depo-Provera\textsuperscript{TM}, Pfizer Pharmacia, \(150 mg\) intramuscularly, every third month) as their only contraceptive method for the past year and not more than \(5\) years.

Exclusion criteria were: (i) significant macroscopic vaginal pathology, (ii) current pelvic inflammatory disease, (iii) vaginal bleeding of unknown aetiology, (iv) pregnancy, (v) known or suspected bleeding disorders, (vi) known or suspected malignant disease, (vii) treatment with investigative drugs other than the contraceptive steroids under study during the previous \(8\) weeks, (viii) any systemic or vaginal medications other than the contraceptive during the last \(2\) weeks before sampling, or (ix) use of an IUD. Current use of barrier methods (mechanical or chemical) in addition to the reported hormonal method was an exclusion criterion.

Volunteers were recruited sequentially, informed and interviewed by telephone. Eligible subjects were selected for age homogeneity between the groups, and appointments at the clinic were made. Subjects attended the clinic twice, once in the follicular phase \((days 8–13)\) and once the luteal phase \((days 20–25 after first day of the last menstrual period)\) of a normal menstrual cycle (control group), and at the same intervals for the hormonal contraceptive user groups. At the first visit, subjects were interviewed concerning socio-demographic and medical history. Weight and height were measured for calculation of body mass index (BMI; \(kg/m^2\)). On both visits a gynaecological examination was performed for evaluation
of any clinical signs of exclusion criteria and subsequent tissue sampling. As a precaution against mechanical or immunological effects on the vaginal mucosa due to condoms or semen, intercourse, as well as use of tampons, was not allowed the last week before biopsy sampling. Blood samples were drawn at both visits for analysis of E₂ and progesterone concentrations in serum. The criterion for ovulation was set at a serum progesterone concentration of ≥16 nmol/L in the luteal phase (Israel 1997, Makarainen 1998, Ludwig 2000). Urine samples were collected at both visits and analysed for exclusion of pregnancy with a one-step, dip-stick test (OriPreg, detection limit 50 IU hCG/mL). Data were collected on coded, standardized forms.

There were no significant differences between the four study groups concerning age homogeneity and BMI. None of the women had detectable levels of hCG in urine on either of the two visits. Six women were smokers; they were all hormonal contraceptive users.

In the control group, all women had regular menstrual cycles and 11 women had verified ovulatory cycles. In two subjects the progesterone levels did not indicate ovulation, and for one subject the value of progesterone in serum in the luteal phase was missing. In one control subject, both biopsies were collected in the follicular phase of two successive cycles. In all the remaining subjects the criteria for inclusion were fulfilled. All individuals in the COC user group had regular bleeding, and samples were collected on days 7–13 and 17–27 after the first day of the last bleeding period. In the LNG user group six women had regular bleeding (regular monthly menstrual cycles of 25–33 days), of whom only one had an ovulatory cycle, as determined by the method used in this study; seven subjects had oligomenorrhoea and two had amenorrhoea. All DMPA users were amenorrhoic. The subjects in the COC, LNG, and DMPA user groups had used their current hormonal contraceptive for an average of 2.8 (range 1–5), 2.1 (1–4), and 1.9 (1–4) years, respectively.

The socio-demographic characteristics of the study subjects in Papers I–III are summarized in Table I. As expected, the frequency of women with no previous pregnancy was highest in the COC user group (11 of 15) and lowest in the DMPA user group (2 of 15), while the frequency of parous women was highest in the DMPA user group (12 of 15) and lowest in the COC user group (3 of 15). One subject in the DMPA user group had a compulsory school certificate only (9 years), while the remaining subjects had graduated from senior high school, and of these 61% had a university degree.

No adverse events occurred, with three minor exceptions, these women underwent short-term therapy within the two weeks prior to biopsy sampling, as follows. One COC user had taken anti-Candida treatment 9 days before biopsy sampling and became symptom free, clinically and subjectively. One LNG user, with regular bleeding for 1 year during LNG implant treatment, had recently been prescribed gestagens because of irregular bleeding and had been treated until 10 days before
sampling. Another LNG user had used antihistamine treatment for a few days before sampling. There were no obvious differences between the results obtained from the two biopsies in any of these three women.

**SUBJECTS (PAPER IV)**

The study presented in Paper IV describes the presence and expression levels of $\alpha$- and $\beta$-defensin mRNA in the human vaginal epithelium of healthy premenopausal women (median age 40 years, range 27–48 years). Eleven women were included in a single-centre study at the Department of Obstetrics and Gynaecology, University Hospital of Umeå, Sweden. Five of the women used no hormonal contraceptive or IUD, while 3 had an IUD of copper, and 3 had an IUD containing 52 mg LNG (IUD/LNG; Levonova®, Schering Nordiska).

**ETHICAL CONSIDERATIONS**

In these studies only women who consented participated after having received comprehensive information about the aims of the study, the content of the interview (Papers I–III), and information about the vaginal biopsies. In the cross-sectional studies (Papers I–III) women were assigned to the different study groups according to their current contraceptive method, which were chosen prior to and independently of this study, and in the descriptive study (Paper IV) women were included on the basis of fertile age.

The procedure of potential contention used in this study is the vaginal biopsy sampling which was performed in all participating women. Contrary to common belief, vaginal biopsy sampling is usually not perceived as painful, but may in some cases be described as uncomfortable with a brief dull pelvic ache of short duration. Bleeding from the site of biopsy sampling is commonly minimal and stops spontaneously within a few minutes, and complications resulting from bleeding are rarely seen. The epithelial defect created by the biopsy usually heals macroscopically within a few days.

Awareness of the reason for the study, i.e. the hypothesis of increased risk of acquiring STDs among hormonal contraceptive users, could lead to concern among these women which might lead to discontinuation of contraception and unplanned pregnancies. Consequently, it must be made clear that: the risk of contracting a STD is primarily dependent on sexual behaviour and not on the hormonal contraceptive per se. The studies were approved by the Scientific and Ethical Review Group of the Human Reproduction Programme at the World Health Organization (Papers I–III) and by the local Research Ethics Committee of the Medical Faculty, Umeå University, Sweden (Papers I–IV).
TISSUE SAMPLING AND PREPARATION (PAPERS I–III)

Four biopsies were taken from each included woman, two in the follicular and two in the luteal phase of the normal menstrual cycle in the control group, and with the same interval in the hormonal contraceptive user groups. The order of sampling (i.e. first biopsy in follicular or luteal phase) was randomized to avoid errors due to possible effects of biopsy sampling on the vaginal environment. A biopsy forceps (Hartmann, Stille, Germany) were used to obtain two full-thickness biopsies of vaginal mucosa, approximately 4 × 8 mm, from the lateral vaginal fornices (Figure 1) at positions “4:30” and “10:30” on the first visit and at positions “1:30” and “7:30” on the second visit. No local anaesthesia of any form was applied. A ferric sulphate paste was applied at the site of biopsy as needed. In most subjects the bleeding was negligible.

On each occasion, one of the biopsies was immediately formalin fixed by immersion in 10% buffered formalin for 12–24 h at room temperature, dehydrated, oriented, and embedded in paraffin. The second biopsy was immediately placed in ice-cold tissue culture medium RPMI1640 supplemented with 3.5% sucrose, human serum albumin, antibiotics, and fungizone, then oriented and embedded in O.C.T.™ (Tissue-Tek®) compound, snap frozen by immersion in isopentane pre-cooled in liquid nitrogen and stored at −70°C until use. Tissue samples were coded and analysed in a blinded manner.

IMMUNOHISTOCHEMISTRY

For the investigation of intraepithelial immune cells and T cell receptors, serial sections of frozen vaginal biopsies were cut at 5-µm thickness perpendicular to the epithelial surface and mounted on poly-L-lysine pre-treated microscopic slides, and processed for immunoperoxidase staining. Sections were air-dried, fixed in acetone at −20°C, and then processed at room temperature.

STAINING FOR IMMUNE CELL TYPES

The frequency of intraepithelial immune cells (CD45+, CD3+, CD4+, CD8+, CD68+, CD1α+, CD14+, CD15+, CD57+ cells and B cells) were determined by immunohistochemistry on fixed cryostat sections. Following washing in phosphate-buffered saline (PBS), endogenous peroxidase activity was blocked by incubation in PBS containing 0.003% hydrogen peroxide and 1 mmol/L sodium azide. Unspecific binding sites were blocked by incubation with bovine serum albumin (BSA). Thereafter, the sections were incubated with the relevant MoAbs for 60 minutes, followed by incubation with horseradish peroxidase-conjugated F(ab′)2 fragments of sheep antimouse Ig. Sections were developed with 0.05% 3,3′-diaminobenzidine tetrahydrochloride and hydrogen peroxide in Tris-buffer and counterstained with Mayer’s haematoxylin. Negative and positive controls were included in every staining procedure. Sections incubated with the isotype- and
concentration-matched MoAb specific for *Aspergillus niger* glucose oxidase served as negative controls. Sections incubated with the anti-CD45 MoAb served as positive controls. A limited number of vaginal samples were taken from volunteers outside the controlled study. Sections of these samples were stained in parallel on each occasion as additional positive controls. The MoAbs used and their main specificities are listed in Table II. B cells were analysed with a mixture of the anti-CD19, anti-CD20 and anti-CD22 MoAbs to obtain an obvious staining of positive cells. The optimal concentration for each MoAb was determined by titration in immunohistochemistry on tissue sections of frozen vaginal mucosa and palatine tonsils.

**STAINING FOR T CELL RECEPTOR TYPES**

The frequency of TcR-αβ and TcR-γδ expressing cells was determined by immunohistochemistry on cryostat sections, fixed and air-dried, followed by rehydration in Tris-buffered saline (TBS). Endogenous peroxidase activity was blocked by incubation in TBS containing 0.003% H2O2 and 1 mM NaN3. Unspecific binding sites were blocked by pre-incubation with BSA and foetal calf serum in TBS. Thereafter, the sections were incubated with specific MoAbs directed against T cells (anti-CD3), T lymphocytes expressing TcR-αβ (a mixture of two anti-TcR-αβ MoAbs) or TcR-γδ (a mixture of three anti-TcR-γδ MoAbs), respectively, for 60 minutes followed by incubation with DAKO EnVision+™ (goat anti-mouse Ig conjugated to peroxidase polymer) for 30 minutes. Sections were developed with the DAKO® Liquid DAB+ Substrate-Chromogen System according to the manufacturer’s instructions. To increase the sensitivity, immunohistochemical analysis was performed using this enhancement technique utilizing peroxidase-labelled polymers consisting of a dextran backbone bound to a large number of horseradish peroxidase molecules. Goat anti-mouse Igs (secondary antibodies) were coupled to the dextran backbone, forming peroxidase-rich conjugates with the primary mouse anti-human MoAb. The advantage of this enhancement technique is the possibility of achieving obvious DAB chromogen signals from marker-positive cells, despite using more diluted primary antibodies, and thus the major problem associated with background staining from anti-TcR antibodies on human vaginal tissue was satisfactorily overcome. Finally, sections were counterstained with Mayer’s haematoxylin. The MoAbs used and their main specificities are listed in Table II.

Each specific MoAb was optimized to give a sufficient signal from tissue sections of frozen vaginal mucosa and tissue with known TcR-αβ and TcR-γδ expression. Negative and positive controls were included in every staining procedure. Sections incubated with isotype- and concentration-matched MoAbs specific for *Aspergillus niger* glucose oxidase served as negative controls. Sections of a limited number of vaginal samples, taken from volunteers outside the controlled study, were stained in parallel on each occasion as positive controls.
STAINING FOR STEROID RECEPTORS

Determination of PR and ER expression by immunohistochemistry was done on serial sections of formalin-fixed, paraffin-embedded biopsies cut at 5-µm thickness perpendicular to the epithelial surface and mounted on poly-L-lysine-pretreated microscopic slides and then de-paraffinized in xylene and re-hydrated in consecutive baths containing 100, 95 and 70% ethanol and distilled water. For Ag retrieval the sections were boiled in citrate buffer in a microwave oven and thereafter processed in a semi-automatic staining instrument (Ventana Inc.) for Ag visualization using the Ventana Medical Systems Basic DAB Detection Kit according to the manufacturer’s manual. Briefly, slides were placed in wash solutions until being loaded into the instrument. Hydrogen peroxide, sodium azide inhibitor solution, was applied to quench endogenous peroxidase activity, followed by incubation with specific MoAbs directed against PR (isoforms A and B) and ERα (Bivitt 1997, Mote 2001). Each specific MoAb was optimized to provide a satisfactory signal from tissue with known ER and PR expression. The MoAbs used and their specificities are listed in Table II.

Staining for PR was enhanced by using the Ventana Medical Amplification Kit, providing a rabbit anti-mouse IgG heavy- and light-chain antibody, binding to the primary anti-PR antibody, followed by washing and application of mouse anti-rabbit IgG. Biotinylated anti-mouse antibody was applied to all sections followed by streptavidin horseradish peroxidase conjugate, forming streptavidin-biotin-horseradish peroxidase complexes, visualized by precipitates formed by diaminobenzidine substrate solution and enhanced by copper sulphate solution. Sections were counterstained with haematoxylin and eosin. Negative controls were run according to the manufacturer’s recommendations. In each run tissue with known steroid receptor expression served as positive controls, observed as nuclear staining in epithelial cells. The set up of the immunohistochemical staining procedure for detection of steroid receptor expression in vaginal epithelial tissue was optimized and judged by two observers (B. Bozoky, Department of Pathology, Umeå University Hospital and the author).

MORPHOMETRY

Morphometry analysis was performed in a blind manner with coded slides by one observer (the author.), using an integrating, cooled colour 3CCD camera (Hamamatsu C5810 camera) on a standard light microscope combined with a computer image analysis system (LeicaQWin) utilizing interactive, computerized morphometry programs developed for the determination of vaginal epithelial thickness, frequency of positively stained intraepithelial immune cell markers and the percentage of steroid-receptor-expressing cells in vaginal epithelium in immunohistochemically stained tissue sections, as described in the following sections.
**EPITHELIAL THICKNESS AND MORPHOLOGY**

Vaginal epithelial thickness was determined on serial sections of formalin-fixed, paraffin-embedded biopsies cut at 5-µm thickness perpendicular to the epithelial surface, mounted on poly-L-lysine pre-treated microscopic slides, and stained with haematoxylin and eosin. The average epithelial thickness of each vaginal tissue sample was determined from measurements of five randomly chosen microscopic fields visualizing a full-thickness epithelial segment in ×100 magnification. In each microscopic field, the mean direction of the basal membrane of the epithelium was oriented vertically on the microscope screen; thus, the vertical length of each epithelial segment in each microscopic field corresponded to the constant height of the screen (600 µm in the actual magnification). The epithelial segment in each microscopic field was outlined using the cursor and the enclosed area was calculated by computerized morphometry. The average epithelial thickness in each microscopic field was calculated by dividing the estimated epithelial area by the constant vertical length (600 µm) of each epithelial segment (Figure 4).

**INTRAEPITHELIAL IMMUNE CELL TYPES AND TcR UTILIZATION**

An interactive, computerized immunohistomorphometry program was developed to determine the frequencies of positively stained intraepithelial immune cells in immunohistochemically stained vaginal tissue sections. A microscopic field (×400 magnification) was selected and transferred to the computer screen, and the

Figure 4

![Image of cellular thickness measurement](attachment:image.png)

Average epithelial thickness = 136200 / 600 = 227 µm
The epithelial section to be studied was computed by outlining the perimeter with the cursor; the enclosed area was then calculated by morphometry. Thereafter, positively stained cells within the enclosed area were labelled manually, and the number of labelled cells was recorded by the computer program. The full height of the epithelium, from the basal lamina to the apical part facing the vaginal lumen, was analyzed by analyzing microscopic fields contiguously situated to each other. Positive cells were counted in a total of twenty microscopic fields in each slide, corresponding to a total epithelial area of collectively 0.3–0.5 mm², and expressed as the number of positively stained cells/mm² of epithelium.

Langerhans cells were identified on the basis of anti-CD1a staining and morphological structure with a stained cell body. Solitary anti-CD1a-positive dendrites with no visible connection to a stained cell body were not included. The immune cells commonly exhibited an irregular distribution regarding the distance to the basal lamina, and the vaginal epithelium was also significantly thicker in hormonal contraceptive users than in controls. Consequently, the relation between immune cell frequency and epithelial thickness exhibited different linear regression coefficients in the four groups studied. To allow comparison of intraepithelial immune cells in the various groups, immune cell frequency was investigated as a function of vaginal epithelial thickness in each sample. The values were normalized by multiplying the estimated frequency of marker positive intraepithelial cells/mm² of vaginal epithelium by the average epithelial thickness, expressed as the number of positive cells along a linear length of the epithelial tissue (number of positive cells/mm).

**Steroid Receptors**

To determine the percentage of steroid-receptor-expressing cells in vaginal epithelium in immunohistochemically stained tissue sections, an interactive, computerized immunohistomorphometry program was developed according to Weibel stereological principles (Weibel 1979). Each sample was evaluated regarding ER and PR expression (×400 magnification). Cells were considered positive only if nuclear staining was present, and no grading of staining intensity was performed. A microscopic field was selected and transferred to the computer screen, and a Weibel graticule was digitally superimposed on the image (Figure 5). Positively stained cell nuclei crossed by the graticule’s coarse points were labelled manually with the cursor and recorded in the computerized morphometry program. All remnant areas crossed by the graticule coarse points within the area of the displayed graticule (i.e. stromal tissue and areas outside the tissue sections) were labelled manually with the cursor and deselected. The percentage of labelled cells within the epithelium was then calculated by the program. The total epithelial area was analysed continuously, both horizontally and vertically by analysing adjacent microscopic fields. An average of nine to ten microscopic fields was evaluated in each section, designed to cover $\geq 1000$ epithelial cells.
Morphometry of ER expressing cells according to Weibel in ×400 magnification. The 121 coarse points graticula is superimposed on the computer screen. All coarse points crossing remnant areas (i.e. lamina propria and areas outside the tissue section) are deselected (red dots). The remaining coarse points crossing an ER expressing nucleus are selected (green rings). In the microscopic field exemplified here the number of coarse points within the epithelium are 121 – 26 = 95, and the percentage of ER expressing cells are thus 19 / 95 = 20%. Notable, the microscopic field exemplified here has a basal location whereas the next microscopic field to be analysed (located adjacent to the right in this example) has an apical localisation where the number of ER expressing cells are less dense. Therefore, the average frequency of ER expressing cells are calculated from analyses of several microscopic fields comprising the full thickness of the epithelium.

Ep = Epithelium; LP = Lamina Propria
BLOOD SAMPLING AND HORMONAL ANALYSES

At both visits, serum samples were taken for determination of concentrations of E₂ (detection limit 72 pmol/L; 2.6% cross-reactivity with ethinyl-E₂) and progesterone (detection limit 0.6 nmol/L; 0.03% cross-reactivity with MPA). These were analysed at the Department of Clinical Chemistry, Umeå University Hospital, using a competitive immunoassay (Immulite 1). For serum E₂, the inter-assay coefficient of variation (CV) was 7.2% and 8.6%, and the intra-assay CV was 8.5% and 6.5% for the 104 and 531 pmol/L quality control (QC) standards, respectively. For serum progesterone the inter-assay CV was 9.2% and 3.6% and the intra-assay CV was 15.8% and 7.3% for the 2.3 and 53.9 nmol/L QC standards, respectively.

ANALYSIS OF DEFENSIN GENE EXPRESSION AT THE mRNA LEVEL (PAPER IV)

TISSUE SAMPLING AND PROCESSING

From each of the eleven subjects included, three to four biopsies of vaginal mucosa, approximately 4×8 mm, were taken from the lateral vaginal fornices of each woman using a biopsy forceps. Each biopsy was placed in ice-cold transport medium RPMI-1640, supplemented with 12.5 mM HEPES buffer, human serum albumin, antibiotics, and amphotericin B, and processed within 15 minutes of sampling. The median total wet weight of the tissue samples taken from each subject for isolation of epithelial cells was 60 mg (range 20–155 mg). In four subjects, one biopsy was immediately snap frozen by immersion in liquid nitrogen and stored at –80°C until analysis by in situ hybridization.

CELL ISOLATION

Biopsies were washed in sterile Tris-buffered Hank’s salt solution (TH) containing antibiotics, cut into small pieces and suspended in RPMI 1640 containing collagenase Type IV, CaCl₂, HEPES, heparin and antibiotics in heat-inactivated human AB+ serum (HNS), incubated at 37°C with vigorous shaking for 60 minutes, and thereafter pressed through a stainless steel sieve. Cells in the resulting single-cell suspension were washed with TH containing 20% HNS and suspended in 67% Percoll (Pharmacia), overlaid with a gradient of 44% and 20% Percoll and centrifuged for 30 minutes at 900 g. Epithelial cells were recovered at the interface between 44% and 20% Percoll, washed and counted. The average cell recovery was 3.2×10³ epithelial cells per mg tissue. All cells had polygonal vaginal epithelial cell morphology and no lymphocyte-like cells were seen.

Four samples were purified further to ensure that the epithelial cell preparations were free from contaminating leukocytes. Cells binding to paramagnetic beads coated with anti-CD45 MoAbs (Dynabeads M-450) added at a bead-to-cell ratio of 20:1 were depleted, as described by Fahlgren (2003). This procedure reduced the
cell recovery by approximately 50%. However, most of the cells adhering to the anti-CD45 MoAb coated beads had epithelial morphology, while only sporadic cells had leukocyte morphology, suggesting high unspecific losses of epithelial cells. No leukocytes were present in the unbound fraction as determined by analysis for CD45 mRNA by RT-PCR, as described by Lundqvist (1994). Isolated cells were washed in RNase-free PBS and kept at –80°C until RNA extraction.

**mRNA EXTRACTION AND REAL-TIME qRT-PCR**

Total RNA was extracted using RNeasy® Mini Kit (Qiagen) according to the manufacturer’s instructions and dissolved in RNase-free water containing 1000 U rRNasin (Promega)/ml and stored at –80°C until analysis. Expression levels of HD-5, HD-6, HBD-1, HBD-2, HBD-3, and HBD-4 mRNAs were determined using real-time quantitative reverse-transcriptase polymerase-chain reaction (qRT-PCR) assays. The assays have been previously defined at the laboratory and utilize TaqMan EZ® technology (Applied Biosystems) in combination with specific RNA copy standards (Fahlgren 2003, Fahlgren 2004). The assays give linear correlations between log concentration of standard RNA and PCR cycles over a range of at least 5 and have a detection range from 25 - 100 up to at least 10^5 RNA copies. Samples were analysed in triplicate and expressed as copies of mRNA/µl using the external copy standard. The concentration of 18S rRNA was determined in each sample by using real-time qRT-PCR and an external standard of total RNA extracted from *in vitro* stimulated human peripheral blood lymphocytes. One 18S rRNA unit was defined as the signal obtained from 1 µg of this RNA, and the results are expressed as defensin mRNA copies per unit of 18S housekeeping gene rRNA (Bas 2004). Because of the high sequence homology between the end of exon 1 and the intron of HBD-3 there is a risk of amplification of contaminating nuclear DNA in the HBD-3 qRT-PCR assay (Fahlgren 2004). Possible DNA signals were assessed by quantitative PCR for each RNA sample using the HBD-3 primers, probe and PCR profile without the RT step and using AmpliTaqGOLD DNA polymerase (Applied Biosystems) instead of *Thermus thermophilus* polymerase. None of the samples had a detectable DNA signal and were consequently analysed for HBD-3 mRNA content without DNase treatment.

**CLONING AND SEQUENCING**

Products from real-time qRT-PCR were cloned and sequenced as described by Fahlgren 2003. Briefly, RT-PCR products were purified by gel electrophoresis, extraction and ligation into dT-treated EcoRV pBluescript vectors. Competent *E. coli* XL1blueMRF’ were transformed with plasmids and grown on selective medium. Transformants were checked for the presence of insert of the expected size using agarose gel electrophoresis after restriction cleavage of plasmids. Two to three µg DNA was used for cycle sequencing using T7 or Rev primers and Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP. Cycle-sequenced products were analysed using ALFexpress equipment.
**In Situ Hybridization**

Digoxigenin (DIG)-labelled antisense and sense cRNA probes for HBD-3 mRNA (144 bases) and HBD-4 mRNA (260 bases) were synthesized using the primers utilized for preparation of the RNA copy standards (Fahlgren 2004). *In situ* hybridization was performed on 10-µm cryosections of vaginal biopsies and human colon mucosa (positive control), according to Bas (2003), with minor modifications. Briefly, tissue sections fixed in paraformaldehyde were hybridized with DIG-labelled cRNA probes. After RNase A treatment, the sections were incubated in sodium citrate/NaCl/formamide, followed by alkaline phosphatase-conjugated Fab fragments of sheep anti-DIG antibodies (Roche Diagnostics). Hybridized probe was visualized by incubation with nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, MgCl₂, and levamisole in Tris-HCl. The sections were thereafter rinsed in dimethylformamide, counterstained with methylgreen, and mounted in Canada balsam. The corresponding DIG-labelled sense cRNA probes served as negative controls.
Statistics

The statistical procedures are described in detail in the Papers (I–IV). Results for the follicular phase/first sample and the luteal phase/second sample, respectively, are given as means plus or minus one standard deviation (SD) for normally distributed data and as medians and interquartile ranges (IQRs) for data with a skewed distribution. The average results of continuous variables for each group studied, are based on individual values represented by the mean value of two samples or, if not available, by one single value from each subject, and the results are given as the mean ± standard error of the mean (SEM) for normally distributed data and as the median (IQR) for skewed data.

All data were entered into a database in Microsoft Excel 2000 (Microsoft, Redmond, WA, USA) for storage and subsequently exported to statistical SPSS package (SPSS 11.0, Chicago, IL) and GraphPad Prism 3.02 (GraphPad Software, San Diego, CA, USA) for statistical analysis and graphic representation. Comparisons between the follicular phase/first sample and the luteal phase/second sample within the study group were performed with the two-tailed Student’s paired t-test for normally distributed data and the two-tailed Wilcoxon signed rank test for nonparametric data. Normality was evaluated using the F test. Differences in frequencies of intraepithelial immune cells, epithelial thickness, age and BMI between the controls and hormonal contraceptive user groups were analysed using one-way analysis of variance (ANOVA), with the Dunnett post hoc test or the test for linearity (Paper I), or with the LSD post hoc test (Paper II). Kruskal-Wallis one-way ANOVA by rank was calculated when differences between the study groups regarding the follicular phase/first sample and the luteal phase/second sample, respectively, were analysed concerning frequencies of steroid receptor expressing cells, and serum concentrations of steroid hormones. Post hoc comparisons were made using the Mann-Whitney U test, in the follicular phase/first sample and luteal phase/second sample, respectively, with the application of a Bonferroni correction for downward adjustment of the α-level to compensate for multiple comparisons between the groups. Differences in socio-demographic parameters were analysed with the Fisher exact test. Statistical analysis of correlations was performed using bivariate analysis (Spearman’s rank correlation test). Differences with a P-value < 0.05 were considered statistically significant.
Table I. Socio-demographic characteristics of women studied in Papers I-III.

<table>
<thead>
<tr>
<th>Study group³</th>
<th>Controls</th>
<th>COC</th>
<th>LNG</th>
<th>DMPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of study subjects</td>
<td>11-15*</td>
<td>15</td>
<td>15</td>
<td>15</td>
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<tr>
<td>Age (years)⁵</td>
<td>27.5 ± 3.9</td>
<td>26.7 ± 3.4</td>
<td>28.1 ± 4.0</td>
<td>28.1 ± 3.7</td>
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<tr>
<td>BMI (kg/m²)⁵</td>
<td>24.7 ± 5.7</td>
<td>23.3 ± 3.6</td>
<td>23.3 ± 3.3</td>
<td>23.6 ± 3.6</td>
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<tr>
<td>hCG in urine &gt; 50 IU / mL</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Current partner</td>
<td>15</td>
<td>14</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Luteal phase S-progesterone ≥16 nmol/L</td>
<td>11</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Medication last 2 weeks</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Hormonal contraceptive use (years, median [range])</td>
<td>3 (1-5)</td>
<td>2 (1-4)</td>
<td>1.5 (1-4)</td>
<td></td>
</tr>
<tr>
<td>Non-smoker (last year)</td>
<td>15</td>
<td>13</td>
<td>14</td>
<td>12</td>
</tr>
</tbody>
</table>

* 15 subjects, whereof 11 had ovulatory cycles and in one subject both biopsies were collected in the follicular phase. ³ Combined oral contraceptive users (COC), levonorgestrel implant users (LNG), and depot-medroxyprogesterone acetate injection users (DMPA).

⁵ Mean ± 1SD.
### Table II. Monoclonal antibodies (MoAbs) used in the studies (Papers I-III)

<table>
<thead>
<tr>
<th>Marker</th>
<th>MoAb/clone</th>
<th>Main reactivity</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45</td>
<td>2B11+PD7/26</td>
<td>All leucocytes</td>
<td>I</td>
</tr>
<tr>
<td>CD3</td>
<td>UCHT1</td>
<td>T cells</td>
<td>I</td>
</tr>
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RESULTS AND DISCUSSION

VAGINAL EPITHELIAL THICKNESS AND MORPHOLOGY
(PAPER I)

Vaginal biopsies were taken on two occasions, prepared and paraffin embedded, and successfully analysed regarding epithelial thickness for all 60 participants.

In agreement with earlier reports, the histology of the vaginal epithelium in normally menstruating women showed the typical features of non-keratinized stratified squamous epithelium overlying a loose connective tissue (Figures 2 and 6; Robboy 1999; Ross 2003, Witkin 1993). In controls with verified ovulatory cycles ($n = 11$), the mean epithelial thickness in the follicular and luteal phase was $280 \pm 109 \mu m$ and $211 \pm 57 \mu m$ (mean ± SD), respectively. There were no statistically significant differences in epithelial thickness between the follicular and luteal phase samples ($P > 0.05$). Therefore, in subsequent comparative analyses of vaginal epithelial thickness between controls and hormonal contraceptive users, all individuals in the control group were included, and the two biopsies from each individual were considered as duplicates. The average epithelial thickness of $261 \pm 16 \mu m$ in controls ($n = 15$) agrees well with values reported in earlier studies (Bahamondes 2000, Mauck 1999, Witkin 1993).

A significant increase in vaginal epithelial thickness was found in all three groups of hormonal contraceptive users, compared with controls. This increase was most pronounced in COC and DMPA users, with mean thicknesses of $344 \pm 14$ (mean ± SEM) and $348 \pm 16 \mu m$, respectively. The increased epithelial thickness among the hormonal contraceptive users has commonly been associated with morphological changes due to a distended superficial portion of the epithelium constituting loose hyperplastic layers of cells (Figure 6). In this study condoms were used by controls but not by hormonal contraceptive users. Although intercourse was not allowed during the week prior to biopsy sampling the bias of this can be discussed. However, intercourse was not previously found to be associated with changes in the mean number of epithelial cell layers in the vaginal mucosa between users and nonusers of condoms, before or after intercourse (Eschenbach 2001).

No change in vaginal epithelial thickness was reported within 3 months after one DMPA injection (Mauck 1999). In another short-term study the epithelial thickness was found to be $1020 \pm 40$ (mean ± SE) before and $890 \pm 50 \mu m$ 6 months after initiation of DMPA injections every third month, which constituted a significant epithelial thinning (Miller 2000). Morphological changes were not reported but the initial epithelial thickness was remarkably high in their study subjects. In one previous study the effect of long-term use (2 to 3 years) of DMPA on vaginal epithelial thickness was investigated and no significant change was reported (Bahamondes 2000). The discrepancies between these and our results may have methodological explanations. To minimize manipulation of the tissue, we did not
use local anaesthetics and did not pin-mount the samples, while in the earlier studies cited above both procedures were used (Bahamondes 2000, Mauck 1999, Patton 2000, Miller 2000). Furthermore, in the present studies the average epithelial thickness was determined by morphometric analysis of a large surface area (at least 0.3 mm² epithelium), whereas the other groups measured the height of the epithelium at a small number of randomly chosen locations. In any event, the use of exogenous gestagens is not associated with a dramatic thinning of vaginal epithelium in humans, as was observed in rhesus macaques and speculated to be an important component in their increased susceptibility to vaginally inoculated SIV (Marx 1996, Hild-Petito 1998).

The increased epithelial thickness observed in hormonal contraceptive users in this study does not necessarily indicate an increase in the immune defence barrier. The morphological change towards distension and hyperplasia may even indicate the opposite, since the hyperplastic epidermal changes seen in dermatoses (e.g. psoriasis vulgaris and eczema) are associated with increased susceptibility to secondary infections (Grossman 1989, Bikowski 1999).

**Figure 6**

Vaginal epithelial thickness in a) a control, and b) a DMPA user. Note the distended peripheral region of epithelial hyperplasia in the DMPA user (arrow head). Arrows indicate basal membrane. Original magnification, ×100.
In postmenopausal women gestagens are given to reduce the risk of endometrial hyperplasia and the associated risk of endometrial cancer during oestrogen treatment (Creasy 1992). Likewise, atrophy in the endometrium induced by DMPA usage is one of the contraceptive mechanisms of DMPA (Mishell 1996). In contrast, in the breast tissue E2 replacement therapy combined with gestagens induces additional hyperplasia (Hofseth 1999). Use of COCs also induces hyperplasia in breast tissue (Isaksson 2001). In postmenopausal women the vaginal epithelial thickness was found to increase from 114 µm before, to 220 µm after, oestrogen treatment (Nilsson 1995). Obviously, the effects of steroid hormones on tissue morphology are different in different tissues. Regarding the vagina, the epithelial morphology is associated with steroid hormone levels; both endogenously produced E2 and progesterone, as well as different forms of exogenous steroid hormone treatment.

INTRAEPITHELIAL IMMUNE CELLS (PAPER I)

In all subjects ($n = 60$) the frequencies of CD45+, CD3+, CD4+, CD8+, CD68+, CD1a+, CD14+ CD15+, CD57+ and B cells were determined by immunohistomorphometry analyses. The MoAb panel covers total number of immune cells and all major subtypes. Immune cell analyses were performed separately on two biopsies from each woman, with the exception of one single biopsy from one subject in the DMPA user group.

The total number of vaginal intraepithelial immune cells was determined by indirect immunoperoxidase staining using anti-CD45 MoAbs. The CD45+ cells were of two morphological types: cells with round, lymphocyte-like morphology and cells with Langerhans dendritic cell morphology. Although CD45+ cells were present throughout the epithelium, the majority was located in the basal and parabasal cell layers, thus in the vicinity of the basal membrane and the underlying lamina propria including the connective tissue papillae. Langerhans cells were scattered throughout the epithelium, often with dendrites spanning several epithelial cell layers. In all groups studied T lymphocytes (CD3+ cells), both T helper (CD4+) and T cytotoxic (CD8+) cells, as well as tissue macrophages (CD68+ cells) were commonly seen. T lymphocytes have previously been demonstrated in vaginal epithelium of healthy women, suggesting that cellular immunity is also important in the front-line defence of the lower genital tract (Johansson 1999, Patton 2000; White 1997a, White 1997b).

In controls, immunohistomorphometry analyses of total leukocytes (CD45+ cells) and the panel of immune cell types were performed separately on biopsies from the follicular and luteal phase, respectively. Statistical analysis comparing the results for the 11 women with verified ovulation showed that there were no significant differences between the follicular and luteal phase with regard to frequency of any of the intraepithelial immune cell types analysed. For this reason, the values obtained from the two biopsies were used to calculate the mean frequency of each
immune cell type in each subject. The average value representing each parameter was based on the mean values from all 15 subjects in the control group.

The frequency of intraepithelial CD45⁺ cells was 241 ± 35 cells per mm² of vaginal epithelium, which is equivalent to 65 cells per millimetre linear length of the vaginal epithelial tissue. The majority consisted of T cells (CD3⁺ cells; 186 ± 34 cells per mm²), predominantly of T cytotoxic phenotype (CD8⁺ cells; 109 ± 19 cells per mm²), as usually most common in human mucosal epithelia (Lundqvist 1993, Lundqvist 1995, Olofsson 1998, Olofsson 2000), followed by T helper cells (CD4⁺ cells; 61 ± 18 cells per mm²). Tissue macrophages were also frequent (CD68⁺ cells; 40 ± 11 cells per mm²). Langerhans cells (CD1a⁺ cells) were detected in eight of the 15 women and constituted a minor population (14 ± 9 cells per mm²). Granulocytes (CD15⁺ cells) and NK cells (CD57⁺ cells) were detected at low densities (less than 1 cell per mm²) and only in occasional samples (n = 5 and 2, respectively). B lymphocytes (CD19⁺, CD20⁺, or CD22⁺ cells) and monocytes (CD14⁺ cells) were not detected in the vaginal epithelium of controls.

Subsequent comparative analysis of intraepithelial immune cell frequency in controls and hormonal contraceptive users was performed using the mean value from the determinations on two biopsies in each subject. Comparisons were made for each parameter between groups with 15 subjects in each group.

In LNG and DMPA users, the number of CD45⁺ cells was significantly greater, than in controls (P < 0.04 and < 0.001, respectively), whereof cytotoxic T cells (CD8⁺ cells) showed a statistically significant linear trend between controls and DMPA users (P < 0.04). Interestingly, there was a statistically significant difference in the CD4:CD8 ratio between DMPA and LNG users (P < 0.01). Taken together, these results suggest a selective expansion of lymphocyte subsets in LNG and DMPA users. Selective increase in the number of CD8⁺ cells in DMPA users is suggested. The role of intraepithelial CD8⁺ cells in immune protection of mucosa has yet to be determined, but surveillance by cytotoxicity against virus-infected cells and memory T cells control life-long immunity against a particular pathogen is possible (Klonowski 2005).

CD4⁺ T lymphocytes (T helper cells) constituted the second largest population of vaginal intraepithelial immune cells. In LNG users a selective expansion of CD4⁺ cell subtype is suggested, but their role in local defence is unclear. Other types of analysis are required to determine whether these are helper cells for antibody production, cell-mediated immune responses, or even regulatory T cells with suppressive effects. Their front-line position makes them potential targets for vaginal transmission of HIV infection.

Tissue macrophages were the third most common cell type, probably acting as scavengers and a first line of defence against bacterial attacks and as Ag presenting cells. They may also release cytokines in response to activation by an infection.
Langerhans cells were detected in two to three women in each hormonal contraceptive user group at low densities, and no significant changes were found in the frequency of Langerhans cells between the groups. This is in agreement with previous studies on the effect of short-term (Mauck 1999) and long-term (Bahamondes 2000) use of DMPA. However, one study reported an increase in Langerhans cells following intravaginal administration of progesterone (Wieser 2001). The presence of Langerhans cells in vaginal epithelium has been reported in previous studies (Bahamondes 2000, Mauck 1999, Patton 2000, Hussain 1992). There are some notable differences between these studies and the present work, making comparisons difficult. In the studies described here the frequency of Langerhans cells was estimated by counting intraepithelial CD1a-positive distinct cell bodies, occasionally associated with dendrites. Two studies used CD1a as a marker (Hussain 1992, Patton 2000), in one, solitary dendrites were also counted (Patton 2000), and in the other, tissue from women undergoing hysterectomy was used (Hussain 1992), with the possible risk of influence of age (34–54 years) as well as of the underlying diseases necessitating surgery. Two studies used the polyclonal S100 marker (Bahamondes 2000, Mauck 1999), which might detect a cell population partly different from CD1a. Langerhans cells have been suggested as major targets for HIV infection (Miller 1998, Turville 2001) and that they become rapidly infected after intravaginal inoculation of SIV in rhesus macaques (Hu 2000). However, the more frequent CD4+ lymphocytes would be expected to be more likely targets in humans.

Monocytes (CD14+ cells) were detected solely in the DMPA user group (median: less than 1 CD14+ cell per mm; n = 4). Granulocytes (CD15+ cells) and natural killer (NK) cells (CD57+ cells) were detected in low quantities in two to three subjects in all three groups, with no significant differences between the groups (medians: 1 CD15+ cell per mm and less than 1 CD57+ cell per mm). B cells (CD19+, CD20+, or CD22+ cells) were detected at low densities in two subjects in each hormonal contraceptive user group (median: 2 B cells per mm).

In conclusion, significant differences were demonstrated in several aspects, although the study population was relatively small (15 subjects in each group). From among the three hormonal contraceptives studied, low-dose levonorgestrel-combined pills seem to affect the immune cell types in the vaginal epithelium least. The site of the biopsy sampling in our study is an area of the lower genital tract at increased risk of exposure to infectious agents, e.g. STDs. The present results suggest changes in the immune protection capacity at this site. To identify the effects of DMPA and LNG usage on the vaginal immune system and to determine whether the use of hormonal contraceptives increases the risk of contracting STD, additional in-depth studies must be conducted.
**T cell Receptors (Paper II)**

With the knowledge of the effects of progestin-only hormonal contraceptive use on the frequency and cellular composition of intraepithelial leukocytes, including CTLs, in vaginal epithelium (Paper III) questions arose regarding cellular adaptive immunity. The frequency of TcR subtypes utilized in vaginal intraepithelial T cells was addressed. Initial attempts to determine intraepithelial frequencies of TcR subtypes, γδT cells in particular, were severely hampered. The MoAb concentrations required in indirect immunoperoxidase or immunofluorescence techniques for optimal staining of TcR-γδ in other mucosal tissues such as decidua, gingiva, intestine and tonsils (Mincheva-Nilsson 1992, Lundqvist 1993, Lundqvist 1995, Olofsson 1996, Olofsson 1998) frequently gave a strong non-specific staining of vaginal epithelial tissue, precluding any quantitative assessment in these tissues. By utilizing an efficient enhancement system (goat anti-mouse immunoglobulins coupled to a dextran backbone with a large number of peroxidase molecules as secondary antibody) which alone did not give non-specific staining, more diluted MoAbs could be used. In this way, an accurate anti-TcR-γδ staining method was obtained from at least one biopsy of each subject. γδT cells with intraepithelial localization have previously been demonstrated in mouse vagina (Hayday 2003). This is the first demonstration of intraepithelial γδT cells in the human female genital tract.

Positive cells of both TcR-αβ and TcR-γδ types were detected in the vaginal epithelium of controls as well as steroid hormone contraceptive users, and they were preferentially situated in the basal and parabasal areas of the epithelium with co-localization to CD3+ cells. In accordance with our previous determinations utilizing indirect immunoperoxidase staining (Paper I) there was no significant difference in the frequencies of intraepithelial CD3+ cells between the follicular and luteal phase samples in controls. Furthermore, there were no obvious variations in the frequencies of either intraepithelial αβT cells or γδT cells during the menstrual cycle in controls. In control samples TcR-αβ+ cells (21 ± 3 positive cells/mm) dominated over TcR-γδ+ cells (5 ± 1 positive cells/mm) giving an average TcR-αβ+ : TcR-γδ+ ratio of 4:1. The frequency of CD3+ cells (46 ± 7 positive cells/mm) commonly exceeded the sum of TcR+ cells in each sample, indicating an underestimation of TcR+ cells.

In hormonal contraceptive users there were no significant differences in the frequencies of intraepithelial CD3+, TcR-αβ+ or TcR-γδ+ cells between the first and the second biopsy, as expected. Consequently, mean values from both biopsies in each subject were used in subsequent comparisons between the groups.

The frequency of CD3+ cells was significantly higher in LNG and DMPA users than in controls (P < 0.05 and < 0.01, respectively) while there was no significant difference between controls and COC users. Similarly, the frequencies of intraepithelial TcR-αβ+ cells were significantly higher in both LNG and DMPA.
users than in controls ($P < 0.05$ and $P < 0.001$, respectively), while there was still no significant difference between controls and COC users. DMPA users also had a significantly higher frequency of TcR-$\alpha\beta^+$ cells than COC users ($P < 0.01$). The dominance of TcR-$\alpha\beta^+$ cells over TcR-$\gamma\delta^-$ cells was also seen in the hormonal contraceptive user groups, and there was no significant difference between controls and hormonal contraceptive users with regard to this ratio.

The frequency of intraepithelial TcR-$\gamma\delta^-$ cells was also increased in both LNG and DMPA users, by approximately two-fold compared with controls, and reached an average of $\approx 13$ positive cells/mm ($P < 0.05$ in both groups). The frequency of intraepithelial TcR-$\gamma\delta^+$ cells in COC users was not significantly different from that in controls. The frequency of TcR-$\gamma\delta^-$ cells was significantly higher in LNG and DMPA users than in COC users ($P < 0.05$ and $P < 0.01$, respectively).

In all four groups studied, there was a significant correlation between the frequencies of CD3$^+$ cells and TcR-$\alpha\beta^+$ cells (controls, $rho=0.87$, $P < 0.001$; COC users, $rho=0.58$, $P < 0.05$; LNG users, $rho=0.79$, $P < 0.001$, and DMPA users, $rho=0.55$, $P < 0.05$), while a significant correlation between the frequencies of CD3$^+$ and TcR-$\gamma\delta^+$ cells was found only in the DMPA user group ($rho=0.64$, $P < 0.01$) in which the frequency of TcR-$\gamma\delta^+$ cells was also highest.

T lymphocytes (CD3$^+$ cells) constitute approximately 75% of the immune cells in the vaginal epithelium of healthy, regularly menstruating women (Paper I). In the present study it became evident that the majority of these are $\alpha\beta$T cells, although $\gamma\delta$T cells also constitute a significant population, on average $\approx 20\%$ of the TcR$^+$ cells. This is similar to the non-keratinized stratified squamous epithelium of the human uvula (Olofsson 2000), but in sharp contrast to the keratinized epithelium of gingiva, in which only $\gamma\delta$T cells have been detected (Lundqvist 1993). $\alpha\beta$T cells have been demonstrated in epithelia at several mucosal sites, e.g. the small and large intestine, uvula, as well as nasopharyngeal and pharyngeal tonsils (Jarry 1990, Lundqvist 1995, Olofsson 1996, Olofsson 1998, Olofsson 2000). $\gamma\delta$T cells have been detected in the epithelium at several mucosal surfaces in man (Groh 1998, Moretta 1991, Lundqvist 1995, Lundqvist 1993, Olofsson 1998, Olofsson 1996, Olofsson 2000). $\gamma\delta$T cells previously demonstrated in decidualized endometrium were not intraepithelial (Mincheva-Nilsson 1997) and the localization of $\gamma\delta$T cells previously detected in the cervix has not been determined (Christmas 1993).

A dominance of $\alpha\beta$T cells over $\gamma\delta$T cells prevails in the single-cell-layer epithelium of the human intestine, corresponding to the TcR-$\alpha\beta :$ TcR-$\gamma\delta$ ratio detected in the vaginal epithelium (Brandtzaeg 1989, Jarry 1990, Lundqvist 1995). This suggests that intraepithelial $\alpha\beta$T cells seem to be a property of non-keratinized epithelial surfaces where concomitant immune protection and immune tolerance is required. In the vagina the immune system has to tolerate semen and commensal flora, while food constituents and commensals have to be tolerated.
along the alimentary tract. A certain proportion of intraepithelial αβ T cells consists of cytotoxic T lymphocytes specific to virus-infected cells since CD3⁺CD8⁺ lymphocytes with cytolytic activity were isolated from vaginal mucosa (White 1997), and intestinal intraepithelial lymphocytes were shown to exert protective cytotoxicity during acute virus infection (Müller 2000). It is also possible that the vaginal epithelium is a site where virus-specific cytotoxic memory T cells accumulate, in accordance with what has been found in the small-intestinal epithelium in mice (Kim 1999, Klowinski 2005). The cytokine profile and cytolytic capabilities of small-intestinal intraepithelial αβ T cells in man suggest that they are involved in cell-mediated immune reactions and execute specific cytotoxicity (Lundqvist 1996, Melgar 2002).

γδ T cells comprise two functional groups which in humans can be distinguished by their δ-chain V-gene utilization. Systemically distributed γδ T cells utilize Vδ2 and are mainly reactive against microbial components via “pattern recognition”, while γδ T cells residing in mucosa utilize Vδ1 and can be cytotoxic to stressed epithelial cells (Allison 2001, Allison 2002, De Rosa 2001, Esin 1996, Groh 1998, Hayday 2001, Kabelitz 2005, Mauerer 1996, Sugita 2000, Wu 2002). γδ T cells may contribute to anti-viral responses in humans and probably play an important role in the surveillance of the vaginal epithelium. However, they could also participate in anti-viral responses since it was recently shown that intraepithelial Vδ1⁺ γδ T cells in mice help protect against intravaginal infection with herpes simplex virus type 2 by promoting Th1 responses (Nishimura 2004). In the present study a pan-TcR-γδ MoAb was used in combination with two Vδ1-specific MoAbs for the detection of γδ T cells. Therefore, it is not possible to say whether all γδ T cells detected utilize the expected Vδ1⁺ mucosa type or if γδ T cells utilizing Vδ2 are also present.

By utilizing an enhancement technique the frequency of intraepithelial T cells (CD3⁺ cells) detected in vaginal samples from LNG and DMPA users was significantly higher than in controls, and represented by a significant increase in both γδ T cells and αβ T cells. The significant increase in intraepithelial leukocytes (CD45⁺ cells) detected in gestagen-only users (Paper I) was thus explained. Interestingly, no such changes were seen in the COC user group, suggesting an influence on T-cell-mediated local immune defence in the vaginal epithelium of progestin-only contraceptive users.

**Steroid Receptors (Paper III)**

The distribution and quantification of steroid-receptor-expressing vaginal epithelial cells were analysed completely in all 118 samples collected on two occasions from 59 women. PR- and ER-expressing cells were visualized using PR- and ER-specific MoAbs in immunohistochemistry on sections of formalin-fixed tissue, in combination with a morphometry technique according to Weibel’s stereological technique. Steroid-receptor-expressing cells displayed an obvious brown nuclear
staining and presented epithelial cell morphology, whereas nuclei not expressing steroid receptors appeared blue due to haematoxylin/eosin counterstaining.

The control group samples were collected in the follicular and luteal phase in 14 women. However, comparisons between the follicular and luteal phase samples within the control group were initially performed on 11 controls with verified ovulatory cycles, as determined by the method used in this study. There were no significant differences between the follicular and luteal phase samples concerning frequency of PR- and-ER expressing epithelial cells. Following inclusion of three subjects with regular menstrual cycles, albeit without verified ovulatory cycles, there were still no significant differences in PR- and ER- expressing cells between the follicular and luteal phase samples. In the subsequent comparative analysis between controls and the three hormonal contraceptive user groups, results from the follicular and luteal phase samples from 14 controls were analysed.

PR-expressing cells were distributed predominantly in the basal cell layer, and occasionally within the parabasal and intermediate cell layers, commonly in the vicinity of the basal cells. PR-expressing cells were detected in the epithelium of controls, COC and LNG users, and the majority of samples had a PR-expression of less than 3%. In contrast, epithelial PR-expressing cells were undetectable in all but one of the 30 samples collected from the women in the DMPA user group. No significant difference was seen in PR-expression levels within the control group or within any of the hormonal contraceptive groups studied when comparing the follicular phase/first sample versus the luteal phase/second sample. PR-expressing cells in lamina propria were detected in all four study groups. PR-expressing cells in the stroma generally showed more obvious nuclear staining and were usually more frequent than PR-expressing cells in the epithelium.

For comparison of PR expression between controls and the hormonal contraceptive user groups a Kruskal-Wallis test was conducted showing that the groups differed from each other on both biopsy sampling occasions (follicular phase/first sample, \( P < 0.001 \), and luteal phase/second sample, \( P < 0.01 \)). In DMPA users, the frequency of PR-expressing cells was down-regulated and was significantly lower at both occasions than in controls as well as COC and LNG users, shown by using the Mann-Whitney \( U \) test for post hoc comparisons between pairs of means in the follicular and luteal phase, respectively (\( P < 0.017 \)). The significant down-regulation of PR expression in human vaginal epithelial cells observed in DMPA users could be explained by the decrease in E2 production induced by DMPA use, causing the women to be in a hypo-oestrogenic state similar to menopause, and having an indirect effect on PR expression. In premenopausal as well as in postmenopausal women receiving hormone replacement therapy, PR expression in the vaginal epithelium was found to be significantly higher than in postmenopausal women not receiving treatment (Blakeman 2000), and vaginal PR expression was shown to increase after vaginal administration of oestrogens (van Haafften 1997). However, apart from this hypothesis on the indirect effects on PR expression, DMPA may have its own down-regulating effect on PR expression in vaginal
epithelial cells. MPA administered to *in vitro* organ cultures from human hormone-sensitive tissues, e.g. proliferative endometrial cells and mammary gland cells has shown a decrease in PR expression in a dose-dependent way (Illouz 2003, Zhuang 2003). In the present study, both direct and indirect effects of DMPA on PR expression could explain the mechanism behind the significant down-regulation of PR expression in human vaginal epithelial cells observed in DMPA users.

Correlation analysis between the frequency of PR-expressing epithelial cells and the vaginal epithelial thickness revealed a significant positive correlation in all COC users (*rho* = 0.48, *P* < 0.01).

ER-expressing cells were detected in the vaginal epithelium of all samples in the study. These cells were located in the basal cell layer of the epithelium, in which the majority of the cells were ER positive, and also in the parabasal and intermediate cell layers. Generally, the frequency of ER-expressing cells was clearly higher than PR expressing cells. In a previous human study on vaginal epithelium using MoAbs in immunohistochemistry and a semi-quantitative scoring system, PR showed a weaker staining intensity and a lower frequency than ER-expressing cells (Hodgins 1998), thus consistent with the results in the present study. ER-expressing cells in the lamina propria were detected in all four study groups but, in contrast to PR-*, ER-expressing cells in the stroma had a weaker intensity and were less abundant than the ER-expressing epithelial cells.

No significant difference was found between the follicular and luteal phase samples in the control group in regarding frequency of ER-expressing vaginal epithelial cells. This is consistent with previously published data on fertile women (Di Carlo 1985, Perez-Lopez 1993, Wiegerink 1980) although previous authors used techniques incorporating cytosol extracts, including stromal cells of vaginal tissue, thus precluding detailed information on distribution and quantification of ER expressing cells in the vaginal epithelium. In one study including eight ovulating women, the ER content in vaginal mucosa samples was increased in 3 and decreased in 5 subjects in the luteal compared with the follicular phase (Sjöberg 1989).

In DMPA users the ER expression was reduced significantly from 15.7% (13.6–17.0, median [IQR]) in the first sample, to 14.0 (10.2–15.9) in the second sample (*P* < 0.05), whereas in COC and LNG users there were no significant differences in ER expression between the first sample and the second sample.

No significant difference in ER expression between controls and COC users was seen, in agreement with the results from a recent study on *ERα* expression in vaginal epithelium of stress-incontinent fertile women (Fu 2003). Additionally there was no difference between controls and LNG users. Interestingly, ER expression was significantly higher in DMPA users than in LNG users, (*P* < 0.01). Although the percentage of ER-expressing cells was not significantly elevated in DMPA users compared with controls in the present study, the total amount of ER-
expressing cells in DMPA users was probably significantly higher since the epithelium per se exhibited hyperplasia and was significantly thicker in DMPA users than in controls (Paper I).

In the present study, the ER expression levels were obtained by utilizing an ERα specific MoAb in immunohistomorphometry. Previous studies suggest that the ER expression in vaginal epithelial cells consists predominantly of the ERα isoform while the ERβ expression is barely detectable in vaginal epithelium and very low compared with other hormone sensitive tissues (Fu 2003, Gebhart 2001, Pelletier 2000), thereby most probably allowing interpretations of the results in the present study.

The effects of endogenous progestins and exogenous gestagens on steroid receptor expression are apparently dependent on several factors including the biological difference between different organs with diverse embryological origin and epithelial morphology (i.e. squamous vs. glandular epithelium, and hyperplasia vs. atrophy). Previous studies have shown a failure to down-regulate ER after MPA treatment in hyperplastic compared with normal endometrium (Masuzawa 1994). The steroid receptor expression pattern may in addition be influenced by substances other than hormonal contraceptives (e.g. growth factors and cytokines) and by non-genomic regulation. Local regulation of ER and PR expression by MPA directly, or indirectly by the decreased E2 concentrations in serum in DMPA users is also possible. It is also notable that, LNG users did not show elevated ER expression in the first compared with the second sample, and ER expression in the first sample of LNG users was actually significantly lower than in DMPA users. In addition, the PR expression was significantly down-regulated in DMPA users but not in LNG users. The differences in steroid expression detected in LNG and DMPA users could reflect the structural differences between the compounds, e.g. steroid receptor affinity and other significant pharmacological and physiological distinctions. Compared with MPA given in hormone replacement regimes, DMPA is a high-dose gestagen with intra-muscular administration.

The effects on steroid receptor expression observed in other hormone-sensitive tissues (e.g. endometrium and breast) during different reproductive phases, steroid treatment, pathological conditions, as well as different methods in study design (i.e. in vivo / in vitro, cancer cell lines / biopsies, human / animal) could not clearly be interpreted to vaginal epithelial reactions in fertile women using DMPA as a contraceptive. This suggests that the regulation of the steroid receptors in the vaginal tissue is different from that in endometrial and perhaps also breast tissue. In the vaginal epithelium, the MPA concentrations arising from DMPA may have progesterone antagonistic actions due to functional inhibition of progesterone leading to up-regulation of ER (Christow 2002, Glasier 2002), which could induce vaginal proliferation and hyperplasia. The differences in steroid receptor expression patterns at different sites probably reflect the different functional roles, which may have important implications on human reproduction and immunity.
In previously published studies on PR and ER expression in the human vagina tissues were commonly obtained from patients as the opportunity arose, for example, during therapeutic surgical intervention to treat a disease, including heterogeneous groups of subjects, with little control over the anatomic origin of the tissue. The importance of defining the site of biopsy is clear, since the transition from vagina to vulva is marked by a decrease in PR and ER (Hodgins 1998). In the present study, inclusion criteria were strict, allowing only well-defined healthy subjects to participate and the biopsies were consistently taken from the lateral fornices. Thus comparisons could be performed between biopsies taken from subjects in all groups studied without bias resulting from different embryological origins at the sites of biopsy sampling.

In this study relations were found between PR and ER expression, epithelial thickness in vaginal mucosa and serum concentrations of steroid hormones. DMPA shows a pronounced effect on steroid receptor expression in the vaginal epithelium. The relation to the other types of contraceptives is more diffuse and no clear picture can be obtained. To explain the differences in the biological effects arising from different progestins/gestagens on human vaginal immunity, further studies on healthy subjects are needed.

**STEROID HORMONES (PAPER III)**

Serum concentrations of E<sub>2</sub> and progesterone were determined in both the follicular and the luteal phase of the menstrual cycle in 13 subjects in the control group and at both visits of all 15 subjects in the hormonal contraceptive user groups.

E<sub>2</sub> concentrations in serum showed no significant differences between the follicular and luteal phase samples in the eleven controls with verified ovulatory cycles (serum progesterone concentration ≥16 nmol/L in the luteal phase). For the proceeding analyses comparing steroid hormone levels between controls and hormonal contraceptive users, the results from all 13 subjects in the control group were included. No significant differences were seen in serum concentration of E<sub>2</sub> between the first samples and second samples within any of the hormonal contraceptive groups studied. In the DMPA user group E<sub>2</sub> concentration in serum was below the detection limit of the assay (72 pmol/L) in as many as 18 of the 30 samples taken from the 15 subjects, therefore the regular median concentration of E<sub>2</sub> in all DMPA users was set to <72 pmol/L in serum.

The median concentration of E<sub>2</sub> in serum had a wide range, from 72 (72–110) pmol/L in the DMPA user group samples to 336 (219–540; median [IQR]) in control samples. In the follicular phase/first sample E<sub>2</sub> concentration was significantly higher in controls (223 [187–514]) than in COC users (72 [72–193]; P < 0.001), LNG users (153 [124–267]; P < 0.001), and DMPA users (72 [72–80]; P < 0.001). Similarly, in the luteal phase/second sample the E<sub>2</sub> concentration was
also significantly higher in controls (379 [323–593]) than in COC (72 [72–126]; \( P < 0.001 \)), LNG (132 [72–350]; \( P < 0.006 \)), and DMPA users (72 [72–120]; \( P < 0.001 \)). Furthermore, in the first sample the \( E_2 \) concentration was significantly higher in LNG than in COC users (\( P < 0.003 \)) and DMPA users (\( P < 0.001 \)). The low \( E_2 \) concentration in DMPA users is consistent with previously published results (Bahamondes 2000, Clark 2001), and comparable to \( E_2 \) levels detected in postmenopausal women not under hormone replacement therapy (Rohr 1999), and significantly lower than in LNG users. Due to the detection limit (72 pmol/L) the absolute \( E_2 \) concentrations could not be obtained in the majority of the DMPA users, but there is a trend for the regular \( E_2 \) concentration in the first sample to be lower than in the second sample, which could explain the compensative up-regulation of vaginal ER expression seen in the first sample of DMPA users (Paper III, see above), resembling the ER levels detected in non-treated postmenopausal women (Perez-Lopez 1993). COC users also had very low concentrations of endogenous \( E_2 \) in serum, probably induced by the exogenous treatment with ethinyl-E2 included in COCs, which probably contributes to preventing the down-regulation of PR expression observed in DMPA users (Paper III, see above). The clinical relevance of low \( E_2 \) levels in long-term use of hormonal contraceptives without exogenous gestagens must be considered from several aspects. The role of \( E_2 \) in promoting immune protection against viral infection of the vagina has been suggested in animal models (Parr 1994, Smith 2000).

In ovulating controls the progesterone concentration in serum was significantly higher in the luteal phase than in the follicular phase. Following inclusion of three subjects without verified ovulatory cycles further statistical analysis nonetheless showed a significantly higher in the luteal (33.0 [17.0–40.5]) than in the follicular phase (2.7 [1.8–3.8]), as expected (\( P < 0.01 \)). There was no significant difference in progesterone concentration between the first and second samples in the COC or LNG user groups. In the DMPA user group there was a significantly higher progesterone concentration in the first sample (3.6 [2.6–4.7]) than in the second sample (2.6 [2.2–3.2]; \( P < 0.05 \)).

In the follicular phase/first samples there were no significant differences in progesterone concentrations between controls and hormonal contraceptive users, whereas in the luteal phase/second sample the progesterone concentration was significantly higher in controls than in COC (3.0 [2.3–3.5]), LNG (3.7 [2.7–4.7]) and DMPA users (2.6 [2.2–3.2]; \( P < 0.001 \)). This was expected since only one of the 6 subjects with regular menstrual cycles in the LNG user group had a progesterone level in serum indicating ovulation, whereas 7 subjects in the LNG user group had oligomenorrhoea and the remainder of the hormonal contraceptive users had amenorrhoea.

All DMPA users were amenorrhoic but each subject attended the clinic twice for biopsy sampling; the second visit took place on average 19 days (range 5–42 days) after the first visit. During the time interval between the two visits, the intra-muscular depot of MPA is expected to gradually decrease according to pharmaco
dynamic principles (Jain 2004). In the DMPA user group, the percentage of ER expressing epithelial cells in the first sample was elevated, complementary to the significant decrease in serum progesterone, and the proportion of ER expressing cells declined significantly during the time interval from the first to the second sample, in the direction of the median level of ER expression in the second sample of the other groups. The direct contribution from circulating MPA to the detected progesterone level in serum in DMPA users can be discussed. Given the fact that serum concentration of MPA in DMPA users generally reaches a plateau of approximately 2.5 nmol/L (Mishell 1996, Smit 2004), and that the cross-reaction of the progesterone assay with MPA was only 0.03%, the influence of MPA on the results obtained in the present study should be negligible.

Serum concentrations of E2 and progesterone showed a significantly positive correlation in all controls (\( \rho = 0.49, P < 0.01 \)), and significantly negative correlation in all COC users (\( \rho = -0.41, P < 0.05 \)). In the LNG and DMPA user groups no significant correlations between E2 and progesterone was found, probably displaying some of the heterogeneity of the groups caused by hormonal contraceptive use. Additionally, we found a significant positive correlation between E2 concentrations and epithelial thickness in the follicular phase samples in controls (\( \rho = 0.60, P < 0.05 \)), and in the first samples in the LNG (\( \rho = 0.59, P < 0.05 \)) and DMPA user groups (\( \rho = 0.63, P < 0.01 \)).

**INNATE IMMUNITY–EPITHELIAL DEFENSINS (PAPER IV)**

The vaginal epithelium of healthy fertile women was analysed for the presence of mRNA for 2 human \( \alpha \)-defensins (HD-5 and HD-6) and 4 human \( \beta \)-defensins (HBD-1 to 4). Levels of mRNA expression were determined in RNA extracted from freshly isolated vaginal epithelial cells using specific real-time qRT-PCR assays with RNA copy standards. Epithelial cells were isolated from biopsies obtained from the vaginal wall of 8 women, and purified by Percoll-gradient centrifugation. Four of the samples were additionally purified by depletion of leukocytes (CD45+ cells). To allow comparison between samples the mRNA level was normalized to the housekeeping gene 18S rRNA level determined in each sample.

All samples but one had detectable levels of at least one of the four \( \beta \)-defensins. HBD-1 mRNA was detected in two samples, HBD-2 mRNA in five samples, HBD-3 mRNA in six samples and HBD-4 mRNA in two samples. One sample expressed all four \( \beta \)-defensins and one sample expressed HBD-4 mRNA only. All samples expressing HBD-2 mRNA also expressed HBD-3 mRNA, and a significant correlation was found between the expression levels of HBD-2 and HBD-3 mRNAs (\( r_s = 0.9; P < 0.006 \)). No significant correlation was seen between the mRNA expression levels of the other \( \beta \)-defensins. There were interindividual differences in the \( \beta \)-defensin expression levels which may reflect variations in copy
numbers of the respective β-defensin gene since it was shown that individuals inherit different versions of chromosome 8 which contain varied numbers of repeated gene copies and that the genomic copy number was correlated to the level of mRNA expression (Hollox 2003). The cellular source of the defensin mRNAs is probably the epithelial cells, since obvious expression was also seen in cell preparations depleted of leukocytes. Cloning and sequencing of several HBD-3 and HBD-4 mRNA products verified the identification of the expected sequences and hence the correct mRNA species.

All samples showed a clear positive signal for HBD-3 and HBD-4 in the epithelial cells when using in situ hybridization on tissue sections from frozen vaginal mucosal biopsies utilising DIG-labelled antisense and sense probes for HBD-3 (n = 4) and HBD-4 (n = 3) mRNA, respectively. The strongest signal intensity was observed in parabasal and intermediate cell layers, while the majority of cells in the basal cell layer and some cells among the parabasal cells gave no or a very weak positive signal. As expected, the superficial third of the epithelium did not harbour any positive cells and contained mainly dead cells with pyknotic nuclei, typical of cells that are destined to be shed into the vaginal lumen (Forsberg 1995). The sense probes gave no signal.

Cells with fibroblast morphology and low staining intensity were scattered throughout the lamina propria. No variation between samples was detected microscopically in staining intensity and distribution of positive cells for the HBD-3 and HBD-4 mRNA positive cells, respectively, while the intensity of the HBD-3 signal was stronger than that of HBD-4.

This is to the best of the author’s knowledge the first demonstration of HBD-2, HBD-3 and HBD-4 mRNA in normal vaginal mucosa. Two previous studies have reported the presence of HBD-1 mRNA in vaginal mucosa (Bensch 1995, Valore 1998). HBD-1 was found to be constitutively expressed in other tissues (Fahlgren 2003, Fahlgren 2004, Lehrer 2004). HBD-1 was detected in 25% of the samples in the present study. However, the HBD-1 expression levels in nasolacrimal ducts are changed during inflammation (Paulsen 2001). Moreover, the HBD-1 gene sequence contains transcription factor regulatory elements for NF-IL-6 and IFN-γ, suggesting the possibility of gene regulation by external signals (Schroder 1999). However, other, yet unknown, inducers could also be operating in the vagina. In line with the present results, it was recently found that the concentration of HBD-1 in vaginal fluid was low (Valore 2002). There could also be differences in HBD-1 expression between epithelial cells at different vaginal sites. In the present study all biopsies were collected from the lateral fornices of the vaginal wall, while in previous studies unspecified tissue from vaginal repairs was used, often exhibiting histological changes indicative of inflammation, as exemplified in a recent study on surfactant protein A expression in the vagina (MacNeill 2004). Thus, it is possible that HBD-1 in vaginal tissue could be induced by inflammatory reactions caused
by the surgical procedure as well as by pathological conditions in vaginal repair tissue.

The detection of HBD-2 mRNA in human vaginal epithelial cells is compatible with the previous demonstration of HBD-2 in vaginal fluid of healthy women with regular menstrual cycles (Valore 2002). HBD-3 was the most frequently expressed defensin, detected in the majority of samples, and all but one of these samples also expressed HBD-2 mRNA. Interestingly, HBD-2 and HBD-3 have previously mainly been detected in pathological conditions, and were first described in psoriatic skin (Lehrer 2004). In normal intestinal epithelium no or low expression levels of HBD-2 and HBD-3 were detected, but levels were induced or upregulated in epithelial cells of inflamed colon (Fahlgren 2003, Fahlgren 2004), suggesting that the inflammatory state, as well as the tissue environment, seems to be important for the regulation of HBD-2 and HBD-3 expression in the gut. Studies on epithelial cells originating from skin, lung and intestine have shown that HBD-2 and HBD-3 are regulated differently in vitro. HBD-2 is induced by bacteria, IL-1β and TNF-α (Fahlgren 2003, Lehrer 2004), while IFN-γ seems to be the major stimulant for the expression of HBD-3 (Fahlgren 2004). HBD-2 could be induced by commensal Lactobacilli, but also by potentially pathogenic bacteria that are restrained by the defensin. Supported by the report that vaginal fluid is permissive for Lactobacillus crispatus and vaginals, but not for Escherichia coli or group B Streptococci (Valore 2002), the results in the present study suggest a distinctive regulation in the vagina: certain bacterial species in the commensal microflora of the vagina could mediate their beneficial effects indirectly by inducing defensin secretion and at the same time being resistant to its toxic actions.

The vaginal epithelium harbours a significant number of intraepithelial T lymphocytes (Paper I), capable of inducing HBD-3 production by IFN-γ secretion. Other, yet unknown, inducers could also be operating in the vagina. HBD-2 and HBD-3 activity against oral microorganisms is strain-selective, utilizing distinct pathways (Joly 2004), and their toxic actions against microbes seem to differ somewhat (Ganz 2003, Lehrer 2004), suggesting different reactivity patterns of HBD-2 and HBD-3. Thus, the combination of the two defensins may yield a broader spectrum of antimicrobial activity. Additionally, HBD-2 is salt-sensitive while HBD-3 is not, implying that both could be active in the vaginal fluid while HBD-3 could also be active within the mucosal tissue where there would be a physiological concentration of sodium chloride. Unfortunately, immunohistochemical analysis of the HBD-3 and 4 proteins in situ was precluded due to the absence of suitable commercially available specific antibodies (Fahlgren 2004).

mRNA for the two α-defensins HD-5 and HD-6 was not detected in any of the eight samples, which is consistent with the report that HD-5 was not detected in the vaginal fluid of healthy women (Valore 2002). Intestinal mucosal inflammation was shown to cause ectopic development of HD-5-producing cells (Cunliffe 2001, Fahlgren 2003). Whether inflammation can induce HD-5 production in vaginal
mucosa remains to be investigated, but a previous study has reported the presence of HD-5 in vaginal repair tissue (Quayle 1998).

Studies on defensin regulation by steroid hormones, i.e. regulation during the menstrual cycle, pregnancy, and menopause, may provide insight into their role in certain conditions with substantial impact on human health (e.g. STDs, preterm birth and chronic vulvo-vaginal candidiasis). The HBD-1 concentration in urine was found to be highest in pregnant women, intermediate in non-pregnant women and low in men (Valore 1998). A modulation of the cellular adaptive immunity parameters in the vaginal epithelium is seen in hormonal contraceptive users compared with healthy fertile controls (Papers I and II), and the influence on defensin expression is still unknown. None of the women in the present study used hormonal contraceptives including oral, subdermal or injectable administration forms. However five subjects used IUDs, whereof two used levonorgestrel-releasing IUD. In recent studies, expression of mRNA for antimicrobial components, including HBD-1 to 4, in the endometrium was found to vary in a cycle-dependent way (Fleming 2003, King 2003) and HBD-1 and HBD-2 were decreased at this site in users of COCs or levonorgestrel containing IUDs (Fleming 2003).

In conclusion, the presence of β-defensin mRNA-expressing cells in the human vaginal epithelium of healthy fertile women suggests that they contribute to the local immune defence within and at the surface of the epithelium.
Table III. Summary of Papers I–III

<table>
<thead>
<tr>
<th>Study group</th>
<th>Controls</th>
<th>COC</th>
<th>LNG</th>
<th>DMPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial thickness</td>
<td>↔</td>
<td>↑↑</td>
<td>↑</td>
<td>↑↑</td>
</tr>
<tr>
<td>CD45</td>
<td>↔</td>
<td>→</td>
<td>↑</td>
<td>↑↑</td>
</tr>
<tr>
<td>CD3</td>
<td>↔</td>
<td>→</td>
<td>↑</td>
<td>↑↑</td>
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<tr>
<td>CD4</td>
<td>↔</td>
<td>→</td>
<td>→</td>
<td>→</td>
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<tr>
<td>CD8</td>
<td>↔</td>
<td>→</td>
<td>→</td>
<td>↑</td>
</tr>
<tr>
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<td>↔</td>
<td>→</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>TcR-αβ</td>
<td>↔</td>
<td>→</td>
<td>↑</td>
<td>↑↑</td>
</tr>
<tr>
<td>TcR-γδ</td>
<td>↔</td>
<td>→</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>ER</td>
<td>↔</td>
<td>→</td>
<td>→</td>
<td>↑c</td>
</tr>
<tr>
<td>PR</td>
<td>↔</td>
<td>→</td>
<td>→</td>
<td>↓↓</td>
</tr>
<tr>
<td>S-E2</td>
<td>↔</td>
<td>↓↓</td>
<td>↓</td>
<td>↓↓</td>
</tr>
<tr>
<td>S-Progesterone</td>
<td>→↑↑b</td>
<td>→</td>
<td>→</td>
<td>↑↓d</td>
</tr>
</tbody>
</table>

* Combined oral contraceptive users (COC), levonorgestrel implant users (LNG), and depot-medroxyprogesterone acetate users (DMPA).

b significantly higher in the luteal than in the follicular phase in controls.

c significantly higher in the first than in the second sample in DMPA user and significantly higher compared with LNG users.

d significantly higher in the first than in the second sample in DMPA user

↔ No significant difference between the follicular and luteal phase samples in controls. Significantly higher (↑) and significantly lower (↓) compared with controls. No significant difference compared with controls (→)
Table IV. Summary of Paper IV

Frequencies of samples with defensin mRNAs expression as determined by real-time qRT-PCR and *in situ* hybridization on preparations from vaginal biopsies of healthy fertile women.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>β-defensin mRNA</th>
<th>α-defensin mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HBD-1</td>
<td>HBD-2</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>2/8</td>
<td>5/8</td>
</tr>
<tr>
<td><em>in situ</em> hybridization</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND = not determined
DISCUSSION FROM A CLINICAL PERSPECTIVE

This is the first cross-sectional study of human vaginal epithelial immune parameters conducted on coded vaginal mucosal biopsies and blood samples from a well-defined study population of healthy young women. The study population includes a control group and three groups of long-term users (1–5 years) of hormonal contraceptives (COC, LNG and DMPA). Vaginal epithelial immune parameters including epithelial thickness, total number of intraepithelial immune cells and all major immune cell types including T cell subtypes (CD4/CD8 and TcR-αβ/TcR-γδ utilization) and steroid receptor expression (ERα and PR) in vaginal epithelial cells, as well as serum concentrations of steroid hormones were analysed. Samples were collected on two occasions: in the follicular and luteal phase of the menstrual cycle in controls and at the same intervals in hormonal contraceptive users.

VAGINAL EPITHELIAL IMMUNE PARAMETERS IN CONTROLS

In controls, the average thickness of the stratified squamous epithelium (261 ± 16 µm) agrees well with earlier reported results (Bahamondes 2000, Mauck 1999, Witkin 1993). There was no statistically significant difference in epithelial thickness upon comparing the follicular and luteal phase samples. In a previous study the number of epithelial cell layers was found to decrease from 28 in the follicular to 26 in the luteal phase (Patton 2000), but it seems unlikely that this small difference could affect the ability of the epithelium to act as a mechanical barrier.

The vaginal intraepithelial immune cells (CD45+ cells; total leukocytes) were frequently located in the basal and parabasal cell layers, thus in the vicinity of the basal membrane and the underlying lamina propria. The majority, on average 75% of the intraepithelial immune cells consisted of T cells (CD3+), predominantly of T cytotoxic phenotype (CD8+ cells), followed by T helper cells (CD4+ cells). In addition, both TcR-αβ and TcR-γδ cells were detected and TcR-αβ+ cells dominated over TcR-γδ+ cells, giving an average ratio of 4:1. Thus, the majority of T lymphocytes are αβT cells although γδT cells also constitute a significant population. Approximately 10% of the T cells are CD4CD8 double negative, as suggested from the results in Paper I. Taken together, the results presented in Papers I and II indicate that the major T cell subset in the vaginal epithelium is CD8 αβT cells, and that the majority of γδT cells in controls are CD4CD8 negative.

Tissue macrophages (CD68+ cells) were also frequent, on average 15%. Unexpectedly, Langerhans cells (CD1a+ cells) were detected in only eight of the 15 women and constituted a minor population. Granulocytes (CD15+ cells) and NK cells (CD57+ cells) were detected at low density in occasional samples.
Intraepithelial B lymphocytes (CD19+, CD20+, or CD22+ cells) and monocytes (CD14+ cells) were not detected. This is the first demonstration of γδ T cells in human vaginal mucosa, while the presence of numerous CD8+ T cells in the vaginal epithelium has previously been observed in both humans (Johansson 1999, Patton 2000) and macaques (Miller 2003).

There were no significant differences between the follicular and luteal phase with regard to frequency of any of the intraepithelial immune cell types analysed. This is in agreement with previous studies in rhesus macaques (Ma 2001). Additionally, one previous study on humans (Patton 2000) reported consistency throughout the menstrual cycle with regard to the relative proportion of CD4+ to CD8+ cells, the presence of macrophages, and the absence of B cells, all in agreement with the results of the present study.

PR-expressing cells were distributed predominantly in the basal cell layer, and occasionally within more apical layers of the epithelium. Compared with PR-expressing cells, the frequency of ER-expressing cells was clearly higher; the majority of the cells in the basal layer were ER positive, and ER was also present in the parabasal and intermediate cell layers. This is consistent with previous results from a human study using semi-quantitative scoring of steroid receptor expression in vaginal epithelium (Hodgins 1998). The ER-expressing epithelial cells included the vast majority of the cells in the basal layer, most probably including some lymphocytes.

The epithelial cells seem to participate actively in the local immune defence of the vagina since mRNA for the antimicrobial β-defensins was detected in parabasal and intermediate cell layers. Notably, ER and PR expression showed an inverse relation to defensin expression pattern, possibly indicating that proliferating cells do not produce β-defensins. The mechanism for Ig secretion from the vaginal epithelium is not fully understood, but in the presence of defensins and T lymphocytes, both αβ and γδ T cells, within the vaginal epithelium the importance of innate and adaptive cell-mediated immune defence at this mucosal surface is apparent.

There was no significant difference between the follicular and luteal phase samples in the frequency of either PR- or ER-expressing vaginal epithelial cells. This is consistent with previously published studies on ER expression assayed in vaginal mucosal cytosol extracts from fertile women (Di Carlo 1985, Perez-Lopez 1993, Wiegerink 1980). Thus, the menstrual cycle changes in steroid receptor expression detected in the functionally different endometrium are not evident in the vagina. The importance of defining not only the tissue but also the site of biopsy sampling is clear, since the transition from vagina to vulva is marked by a decrease in PR and ER (Hodgins 1998). In the present study, biopsies were consistently taken from the lateral fornices, hence comparisons between the groups was performed without bias derived from different sites (e.g. embryological origins).
There was a wide range in E2 concentrations in serum in controls (336 [219–549]) and no significant difference was detected between the follicular (c.d. 8–13) and luteal phase (c.d. 20–25) samples in ovulating controls. Serum progesterone concentration in the luteal phase was estimated in 13 controls (33 [17.0–40.5] nmol/L) and the criterion for ovulation was serum progesterone concentrations ≥16 nmol/L in the luteal phase.

In conclusion, in controls the ovarian steroid hormone variations during a normal menstrual cycle did not affect the intraepithelial immune parameters studied. The importance of immune protection at this mucosal surface is apparent through the presence of T lymphocytes, both αβT cells and γδT cells within the vaginal epithelium, although the mechanisms governing this protection are not yet fully understood.

**EFFECT OF HORMONAL CONTRACEPTIVES ON VAGINAL EPITHELIAL IMMUNE PARAMETERS**

*Increased vaginal epithelial thickness in hormonal contraceptive users*

Among the three hormonal contraceptive user groups included in this study, DMPA users showed the most significant changes in the immune parameters studied. Compared with controls the vaginal epithelium was significantly thicker, and commonly associated with morphological changes, in all three groups of hormonal contraceptive users. This increase was most pronounced in COC and DMPA users. In a previous study no effect of long-term DMPA use on vaginal epithelial thickness was observed (Bahamondes 2000, Mauck 1999), probably due to methodological differences in tissue sampling and preparation. In any case, the use of exogenous gestagens is not associated with dramatic thinning of vaginal epithelium in humans, as was observed in progesterone-treated rhesus macaques and proposed as an important component in the increased susceptibility to vaginally inoculated SIV (Marx 1996, Hild-Petito 1998). However, increased epithelial thickness does not necessarily indicate an improved protective barrier since morphological changes seen in dermatoses (e.g. psoriasis vulgaris and eczema) are associated with increased susceptibility to secondary infections (Grossman 1989, Bikowski 1999). The proliferative effect of progesterone and gestagens is dependent on the tissue; progesterone induces differentiation in the endometrium while certain gestagens induce atrophy. In contrast, gestagens induce hyperplasia in the breast. Certain gestagens also seem to induce hyperplasia in the vagina, although the influence of E2 must also be considered. In COC and LNG users the proliferative effect on the vaginal epithelium of ethinyl-E2 and endogenous E2 in combination with gestagens may be associated with functional effects on the epithelium, e.g. epithelial integrity, in contrast to the effects in DMPA users. A common clinical sign in DMPA users and postmenopausal women not under hormonal replacement therapy is reduced rugae in the vaginal wall. In contrast to postmenopausal women, the macroscopic appearance may be caused by
morphological changes in DMPA users, with hyperplastic cells in the superficial epithelial layers facing the vaginal lumen, not atrophy.

**Increased frequency of intraepithelial immune cell types in gestagen-only users**
The number of intraepithelial leukocytes (CD45+) was significantly higher in LNG and DMPA users than in controls, which can be explained by the significantly higher frequency of T cells (CD3+) in gestagen-only users than in controls. Both αβ and γδT cell frequencies were significantly higher in gestagen-only users. Interestingly, no such changes were seen in the COC user group, suggesting an influence of progestin-only contraceptives on T-cell-mediated local immune defence of the vagina. CD8+ cells showed a statistically significant linear trend of increased frequency between controls and DMPA users.

CD8+ CTLs have been shown to be the major means by which the immune response eliminates virally infected cells and intracellular pathogens, but the role of CD8+ T cells in the vaginal epithelium was unknown until it was demonstrated that SIV-specific activity is present in the CD8+ T cell population in the vaginal epithelium of SIV-infected macaques (Lohman 1995). In line with this, human vaginal CD8+ cells have been shown to have cytotoxic capacity (White 1997a, White 1997b). Thus, most likely intraepithelial αβT cells are CTLs specific for virus-infected cells to a certain degree.

Recently, it was demonstrated that only a small proportion of vaginal intraepithelial cells in surgical specimens of normal vaginal tissue expressed TIA1, a marker of cytotoxic potential (Pudney 2005). This suggests that a proportion of CD8+ T cells in the vaginal epithelium instead represents a population of regulatory T cells such as have been described in the intestinal mucosa (Allez 2004). Interestingly, in the transformation zone (TZ) in non-inflammatory cervix samples TIA1-expressing cells were more abundant, indicating that the TZ may be a major inductive and effector site for cell-mediated immunity in the lower genital tract (Pudney 2005).

It is also possible that virus-specific CD8+ CTLs are homing to the vaginal epithelium as memory T cells, as part of the normal pattern of lymphocyte recirculation, unrelated to the presence of virus-infected cells (Kim 1999, Klonowski 2005). This could possibly reflect a history of subclinical viral infections. Human vaginal intraepithelial lymphocytes are predominantly CD8+ T cells (Paper I, Patton 2000) and it was very recently demonstrated that the majority of T lymphocytes in normal vaginal tissue were CD45R0+ (memory) phenotype (Pudney 2005). In any case, the presence of memory T cells indicates earlier immune response activated by virus penetration.

Interestingly, the CD4:CD8 ratio was significantly lower in DMPA than in LNG users, demonstrating selective expansion of diverse lymphocyte subsets in LNG and DMPA users. Notably, the absolute frequency of CD4+ cells was not significantly increased in LNG users. Other types of analysis are required to
determine whether these are helper cells for antibody production, cell-mediated immune responses, or even regulatory T cells with suppressive effects. Their frontline position makes them potential targets for vaginal transmission of HIV infection.

Results from animal studies have suggested Langerhans cells to be major targets for HIV infection (Hu 2000, Miller 1998, Turville 2001). The apparent species difference in frequency of CD1+ cells could have an effect on SIV/HIV infection mechanisms. In humans, the more frequent CD4+ lymphocytes would be expected to be more likely targets, although the expression of CCR5 (HIV co-receptor) was not determined in the present study.

It is likely that the γδT cells play a role in surveillance of the human vaginal epithelium. Mucosa Vδ1 γδT cells can be cytotoxic to stressed epithelial cells independent of Ag recognition (Allison 2001, Allison 2002, De Rosa 2001, Esin 1996, Groh 1998, Kabelitz 2005, Maeurer 1996, Sugita 2000, Wu 2002). However, they can also participate in anti-viral responses since it was recently shown that intraepithelial Vδ1+ cells in mice help protect against intravaginal infection with herpes simplex virus type 2 by promoting Th1 responses (Nishimura 2004). In periodontitis intraepithelial γδT cell frequency has been found to increase and start to express CD8 (Lundqvist 1993). In DMPA users, the frequencies of TcR-γδ+ cells as well as CD8+ cells were increased. Whether these changes are connected to stress or inflammation in the vaginal epithelium remains to be further investigated. Unlike αβT cells, it was recently shown that γδT cells can also act as potent APCs (Brandes 2005), allowing the possible induction of the adaptive immune reactions in the vaginal epithelium.

In conclusion, the demonstration of T lymphocytes in the vaginal epithelium of healthy women suggests that cellular immunity is important in the front-line defence of the lower genital tract. In this context, these new findings suggest the possibility of a different T cell response in controls, COC, LNG and DMPA users. Whether the changes observed in users of progestin-only contraceptives, especially in DMPA users, are associated with a change in the capacity of the immune system to offer protection against pathogen challenge must be further investigated.

**PR is down-regulated in DMPA users**

The PR expression was down-regulated and significantly lower in DMPA users than in controls, COC and LNG users. Low E2 levels could have indirect effects on PR expression levels. Additionally, DMPA might have its own down-regulating effect on PR expression in vaginal epithelial cells. This indicates a strong, perhaps stress-induced, hormonal effect on vaginal epithelium in DMPA users.

In DMPA users the ER expression in vaginal epithelial cells was higher in the first than in the second sample. This was not observed in LNG users, in fact, the ER expression was significantly lower in LNG users than in DMPA users. ER
expression was determined as a percentage of expressing cells. Hence the total amount of ER-expressing cells in the epithelium of DMPA users was probably significantly higher since the epithelium per se exhibited hyperplasia and was significantly thicker in DMPA users than in controls (Paper I). Hence, steroid receptor expression in DMPA users is remarkably different from that in LNG users.

**DMPA users are hypo-oestrogenic**

The E₂ concentration in serum was significantly lower in DMPA users than in controls and LNG users, and comparable to E₂ levels detected in postmenopausal women not undergoing hormone replacement therapy (Rohr 1999). In COC users the low endogenous E₂ production is replaced by ethinyl-E₂ in the COC pills. Low E₂ levels in DMPA users have been linked with a decrease in colonization of vaginal *Lactobacillus* (Miller 2000). Hydrogen-peroxidase *Lactobacillus* might directly kill free virus in the vagina during intercourse (Klebanoff 1991) and prevent bacterial vaginosis (Hawes 1996), which has been associated with an increased risk of HIV infection (Martin 1998, Taha 1998). It is obvious that the presence of E₂ or exogenous oestrogens increases the chances of the vagina to resist infection, as a result of an acidic environment and by promoting adaptive cellular immune responses. Thus, it is possible that DMPA increases the risk of HIV acquisition. Other local or systemic effects, direct or indirect, of the hypo-oestrogenic state or the high MPA concentration in long-term DMPA users cannot be excluded. Compared with DMPA users, the results, as well as the bleeding patterns, in the LNG user group are more heterogeneous.

**Future Studies**

The site of biopsy sampling used in our study is an area of the lower genital tract at elevated risk of exposure and invasion in the presence of infectious agents, e.g. STDs. The results suggest changes in the immune protection capacity at this site in gestagen-only users, especially in DMPA users. To define the effect of DMPA and LNG use on the vaginal immune system and to determine whether the use of hormonal contraceptives increases the risk of contracting STDs, additional in-depth studies should be conducted. It is important to stress that STD contraction is not caused by the hormonal contraceptive use per se, but rather depends on the sexual behaviour and exposure to pathogens, which may be facilitated by certain steroid hormones.

The description of these local immune components, the regulation of their presence under physiological hormonal variations during the menstrual cycle, and the influences of exogenous steroid hormones (e.g. hormonal contraceptives) are important topics for future functional studies on local vaginal immune responses aimed at:
• understanding the possible effect of hormonal contraceptives on innate immunity, e.g. defensin secretion,
• identifying risk factors for increased susceptibility to STDs,
• developing national and international guidelines for hormonal contraceptive prescription,
• the future development of effective contraceptives and
• the future development of effective treatment and vaccines against STDs.
CONCLUSIONS

The thickness of the human vaginal epithelium in healthy fertile women (controls) was on average 261 ± 16 µm, and there was no significant difference in thickness between the follicular and luteal phase of the menstrual cycle.

The epithelial thickness was significantly increased in hormonal contraceptive users compared with controls (333 ± 9 µm), especially in COC and DMPA users, and commonly exhibited hyperplasia.

Intraepithelial cells in the human vaginal epithelium of controls comprise, in order of abundance: T lymphocytes, whereof αβT cells were more frequent than γδT cells at a TcR-αβ:TcRγδ ratio of 4:1, and cytotoxic T cells (CD8⁺) were more frequent than T helper cells at a CD4:CD8 ratio of 0.7, followed by macrophages (CD68⁺) and Langerhans cells (CD1a⁺). There were no significant differences in the frequency of intraepithelial immune cells between the follicular and luteal phase of the menstrual cycle. In controls, B cells, NK cells, monocytes and macrophages were generally absent.

Increased frequencies of intraepithelial immune cells were detected in DMPA and LNG users compared with controls. This was explained by and increased frequency of T cells, both αβT cells and γδT cells, being most pronounced in DMPA users. This group showed a selective increase of the cytotoxic CD8⁺ T lymphocyte subset. There was no significant difference between controls and COC users.

In controls there were no significant differences between the follicular and luteal phase of the menstrual cycle concerning frequency of progesterone (PR) or oestrogen receptor (ER) expression.

In DMPA users the PR expression level was down-regulated to undetectable levels, and significantly lower than in controls and COC and LNG users.

In DMPA users the ER expression was significantly elevated in the first compared with the second sample, and significantly higher than in LNG users.

Important antimicrobial peptides, i.e. the β-defensins, are expressed by vaginal epithelial cells in fertile women, whereof mRNA from HBD-2 and HBD-3 was expressed most frequently. HBD-3 and 4 producing cells were
located in the parabasal and intermediate cell layers of the vaginal epithelium.

Thus, the epithelium has the potential to act as an effective, interior defence barrier locally in the vagina. The defence against potentially pathogenic agents positioned in the vaginal lumen normally involves cells of both the adaptive and innate immune system, as well as the epithelial cells, including the physical barrier and local production of innate constituents. Usage of gestagen-only contraceptives, especially DMPA, had an obvious effect on local cellular immune defence in increasing the frequency of intraepithelial T lymphocyte subsets within the epithelium, while usage of COCs did not.
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