

**Adenylate Cyclase in Normal  
and Leiomyomatous Uteri**

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**Penelope**

***Your persistence and love was the greatest encouragement.***

***The end of one task begins another.***

***My thanks and love.***

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## **Abstract**

Despite being one of the commonest pathologies of the uterus little is known regarding the aetiology of leiomyomata, however, recent evidence suggests an abnormality at the cellular level. This study was undertaken to examine the distribution of adenylate cyclase (AC) in myometrium and leiomyomata. Sections from normal and host myometria as well as from leiomyomata were stained with an antibody against AC V/VI using standard immunocytochemical methods. The percentage AC positivity in the tumours and in each region of the myometrium was calculated and statistically analysed. The tumours and the midmyometrium of normal myometria and host myometria had the highest levels of AC staining. Two of three characteristic cellular staining patterns showed significant differences between tissue types. The differences in staining may be related to the symptoms associated with the presence of leiomyomata and may also have an effect on their aetiology and/or the events occurring post-tumourigenesis.

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## INTRODUCTION

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Leiomyomata form one of the most common pathologies of the uterus. Despite their prevalence very little is known of their aetiology. Recent research has shown that the tumours arise in inherently abnormal myometrial tissue (Richards & Tiltman, 1996) and that they express several genes that are normally expressed in the gravid uterus (Andersen & Barbieri, 1995). Both of these findings suggest the occurrence of an abnormality at the cellular level. There are a vast number of intracellular events within any one cell. The initiation of many of these events is an extracellular signal impinging on the outer cell membrane. The attachment of an external stimulus to a cell surface receptor starts the second messenger cascade that forms the initial reaction of the cell to such events.

One of the most prominent of these systems is the cyclic adenosine 3' 5' monophosphate (cAMP) second messenger system. This second messenger system is initiated by the activation of a receptor by an extracellular signal. This in turn activates a member of the 'G' protein superfamily (Bourne *et al.*, 1991) which activates the enzyme adenylate cyclase (AC). Adenylate cyclase transforms its natural substrate adenosine triphosphate into cAMP. Both cAMP and the AC are membrane bound systems that control and are controlled by some of the factors that are thought to influence the aetiology of leiomyomata (Aronica *et al.*, 1994). Cyclic AMP itself has been observed to play a role in both morphogenesis and mitogenesis within the uterus (Seuwen & Pouyssegur, 1992), while AC is activated by hormones such as insulin like growth factor I which may have an influence on leiomyomata growth (Cho & Katzenellenbogen, 1993).

This study seeks to examine the distribution of the AC system in normal, non-neoplastic host myometrium and the tumour, using immunohistochemical techniques. To understand the role that AC and cAMP has in the normal myometrium and the possible role they play in tumourigenesis the following chapters outline: the anatomy

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of the normal myometrium, non-neoplastic myometrium and the leiomyomata that the latter contains and the underlying aetiology of leiomyomata, with reference to current evidence and proposed theories (Chapter 1); current knowledge regarding the AC system and cAMP (Chapter two); the microscopical localization of the various parts of this system (Chapter three) and the possible role the AC/cAMP system plays in tumourigenesis (Chapter four). Chapter five gives the justification, details of the tissue collection and methodology used for the experiments in this study. Chapter six details the results of the experimentation undertaken with the discussion and conclusions being given in chapter seven. The relevant references for each chapter appear at the end of the chapter.

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## Chapter One

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# **The Uterus and Its Leiomyomata**

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### *Morphology and Aetiology*

#### **Introduction**

Structure and function are related, one to the other, therefore prior to investigating cellular aspects of the normal, non-neoplastic host myometrium and leiomyomata that it harbours the structure of these tissues is described. Both, the macro and micro, anatomical appearance is well documented and the important features of the three tissues are briefly given below. As there are a number of proposals for the aetiology of these tumours, the current evidence and aetiological theories regarding the leiomyomata are discussed below with reference to the literature.

### **1. Morphology of the Normal Uterus**

#### **1.1 Macro Anatomy**

The uterus is a pear shaped muscular organ lying in the pelvis, anterior to the rectum and posterior to the urinary bladder with a cavity which conforms to its outer shape. It is divided into two distinct anatomical portions; the upper expanded two thirds forms the body or corpus of the uterus, whilst the lower third forms a small cylindrical structure, the cervix (Figure 1.1-1.2).

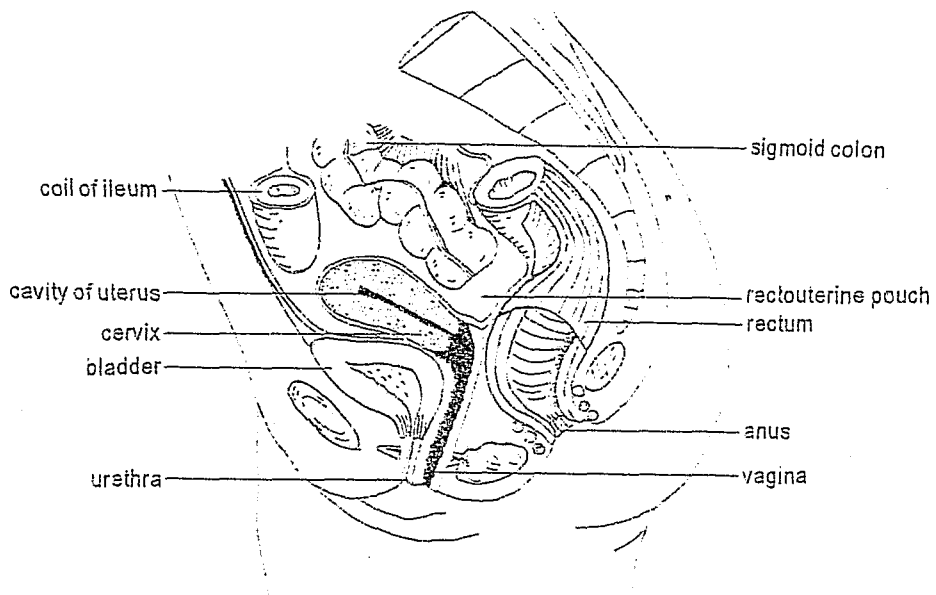


Figure 1.1: Schematic diagram of a sagittal section of the pelvis to show the anatomical position of the uterus and some of the pelvic content relationships.

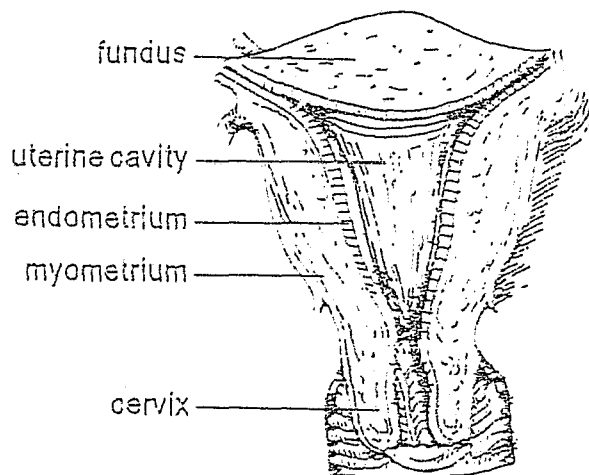


Figure 1.2: Schematic diagram of the uterus showing the main uterine subdivisions.

### **1.1.1 The Uterine Wall**

The uterine wall can be divided into three layers. An external serosa or perimetrium, which consists of peritoneum supported by a thin layer of connective tissue. The bulk of the wall, the myometrium, consists of bundles of smooth muscle cells with a loose connective tissue matrix. The inner layer or endometrium is the mucosal layer lining the uterine cavity which undergoes cyclical changes under the influence of the reproductive steroid hormones. In this study, the tissue of interest is the myometrium and this is described in more detail below.

The myometrium forms the bulk of the uterine wall. The loose connective tissue matrix contains a number of blood and lymph vessels, as well as nerve fibres. In humans the muscle can be divided into three layers of variable distinction:

- 1) An inner or subendometrial layer in which the muscle bundles are arranged longitudinally and circularly in relation to the deep pits of the endometrial glands. The portion immediately below the endometrium is sometimes referred to as 'the junctional zone' (Scoutt *et al.*, 1991).
- 2) The midmyometrial layer, making up the bulk of the myometrium, where the muscle bundles have a random orientation.
- 3) The outer or subserosal layer where the muscle fibres are predominately arranged longitudinally.

## **1.2 Micro Anatomy of the Myometrium**

### **1.2.1 Light Microscopy**

The muscle of the myometrium consists of typical blunt ended spindle shaped smooth muscle cells with central fusiform nuclei (Figure 1.3). The size of the smooth muscle cells is dependent on the physiological state, ranging from 20  $\mu$ m in length in the non-

gravid state to several hundred microns in the gravid uterus (Schoenberg, 1977). The subserosal layer contains elastic fibres that extend into the midmyometrial layer but do not extend as far as the subendometrial layer. The percentage muscle content is known to vary with each region of the uterus, such that the highest percentage is found in the fundal region of the uterus (28%). The muscle content steadily reduces in the lower segment (15%) but remains high in comparison to the cervix which has a muscle content of less than 7.5% the remainder being made up of elastic and connective tissue (Schwalm & Dubrausky, 1966). These structural differences reflect the functional differences apparent between these three areas during parturition (Llewellyn-Jones, 1982a).

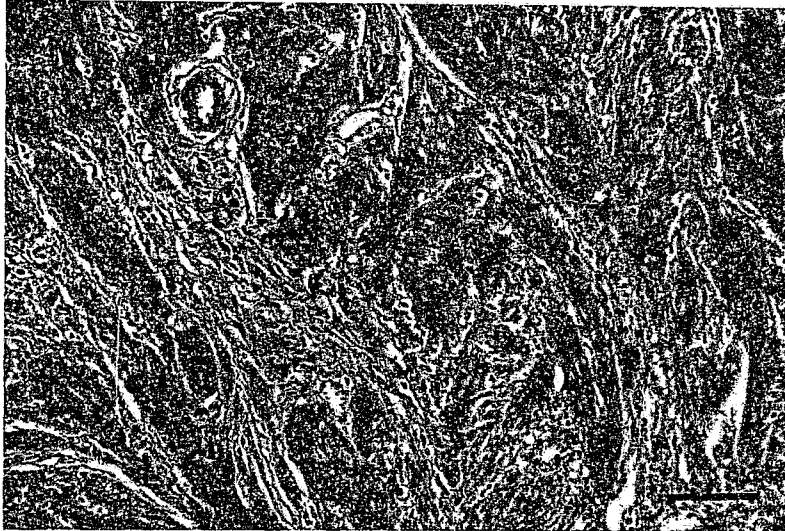


Figure 1.3: Light micrograph of an haematoxylin and eosin stained section of the myometrium. Scale bar = 50  $\mu$ m.

### **1.2.2 Electron Microscopy**

The myocytes forming the myometrium have a similar structure to other smooth muscle cells (Figure 1.4). Three distinct types of filament have been reported:

- 1) Thin filaments (6-8 nm in diameter) which are helical strands of predominantly  $\beta$  actin, with tropomyosin decorating the filaments.

2) Thick filaments (12-18 nm in diameter), composed of aggregates of myosin molecules.

3) Intermediate filaments (10 nm diameter), the majority of which are desmin and vimentin.

The bulk of the filaments seen within the cell are thin filaments which are believed to run obliquely across the cell (Bagby *et al*, 1971). Dense bodies, 1  $\mu\text{m}$  in length and between 0.25 - 0.5  $\mu\text{m}$  in width, are randomly arranged across the filaments. These dense bodies and sarcoplasmic dense bands are analogous to the z-lines of skeletal muscle. The actin filaments have been shown to be inserted into the dense bodies and bands, in a similar way to which they insert into the z-disks of skeletal muscle. Actin filaments interdigitate and crossbridge with the myosin filaments in these dense bodies/bands in what are termed 'mini-sarcomeres' (Jiang & Stephens, 1994). The ratio of actin:myosin is 15:1 in smooth muscle and as such the myosin is rarely seen with the electron microscope.

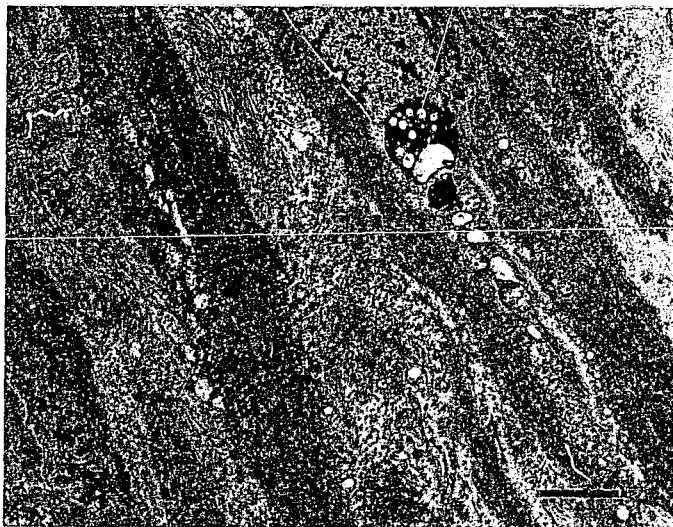


Figure 1.4: Electron micrograph of myometrial muscle cells. Scale bar = 5  $\mu\text{m}$

A cytoskeletal network is thought to be formed by the intermediate filaments. The filaments help to distribute tension throughout the cell, which assists in the maintenance of cell shape (Jiang & Stephens, 1994). The intermediate fibres have also been shown to be involved with the dense bodies, that lie across the thin filaments and as such they assist in retaining the structural integrity of the 'mini sarcomeres' (Jiang & Stephens, 1994).

The sarcolemma is a trilaminar structure approximately fifteen nanometres thick with dense bands located randomly along the length of the membrane. These dense bands are similar to the dense bodies found lying across the filaments and are believed to be the termination point of the actin filaments. A disrupted membrane or one in which breaks occur is thought to be artifactual or represent a possible syncytial nature for the myocytes (Mark, 1956). Myocytes often exhibit gap junctions which are composed of trans-membrane proteins termed connexins. These proteins are thought to create pores in the membrane allowing cell to cell communication by the passage of ions, such as calcium. Connexins are known to be increased during term and pre-term labour (Andersen *et al*, 1993) probably a result of their role in ensuring synchronous contraction of the muscular uterine wall (Carsten & Miller, 1987).

The nucleus is centrally located in the cell and has an irregular outline. Dense heterochromatin is usually peripherally located and there are one or two nucleoli situated in the mid third of the nucleus. Mitochondria, Golgi apparatus, endoplasmic reticulum and ribosomes make up the normal complement of cell organelles that are located at the nuclear poles. Rough endoplasmic reticulum has been shown to be involved in the renewal of contractile proteins, as well as in the synthesis of the collagen matrix and elastic fibres (Ross, 1971). The cytoplasmic vesicles, commonly seen in smooth muscle, have been shown to play a role in the export of extracellular matrix proteins (Ross & Klebanoff, 1971).

The smooth endoplasmic reticulum is not well developed and the quantity present in the cell will depend on the state of uterine gravidity (Mark, 1956). It has been suggested that the smooth sarcoplasmic reticulum is an intracellular source of calcium ions which are released during the contractile process (Jiang & Stephens, 1994).

Tubular structures are seen near the cell surface which engulf flask shaped caveolae of the plasmalemma. These invaginations are approximately sixty nanometres in diameter and alternate along the membrane with the dense bands previously mentioned. These caveolae are believed to be sites of calcium ion pumps (Fujimoto, 1993) which remove calcium ions from the cell during the contraction/relaxation process, using calcium-adenosine triphosphatase as the energy source.

## **2. Morphology of Leiomyomata**

There are two distinct entities that have to be considered in this section. First the host myometrium in which the tumour is found and secondly the tumour itself. Both of these will be described below with detail being given only where the structure differs from that of the normal.

### **2.1 Macro Anatomy**

The presence of fibroids increase the size and weight of the uterus, which may also be deformed if the tumour is large or subserously located. Leiomyomata are found generally in three locations within the uterus, submucosal, intramural and subserous (Figure 1.5). The submucous tumour is found in approximately 5% of the cases seen (Novak & Woodruff, 1974) and are usually solitary, however, in an earlier report, an incidence of 24% was recorded as being common for these tumours (Mahfouz & Magdi, 1941) The majority of tumours (70%) are found in the intramural position, mainly in the posterior wall of the uterus (Llewellyn-Jones, 1982b). They can be either solitary or as numerous small entities. The subserous tumours are found in the remaining 25% of cases and maybe pedunculated; adhering to intra abdominal organs, from which they may derive a second blood supply (Novak & Woodruff, 1974).

Solitary tumours are uncommon and as many as 225 have been found in one uterus (Lapan & Soloman, 1979). They can range in size from a diameter of less than one millimeter to observed diameters of over twenty centimeters, with an individual weight that can become as great as 10 Kg. The tumours are typically spherical but can attain any shape where they protrude from the wall of the uterus. They are well

demarcated by a line of cleavage, which is formed by a pseudocapsule of compressed muscle and areolar tissue. Usually they are easily distinguished from the surrounding tissue by their pearly white to tan colouration and whorled appearance (Llewellyn-Jones, 1982b) (Figure 1.6) but this may be altered by the processes calcification and necrosis.

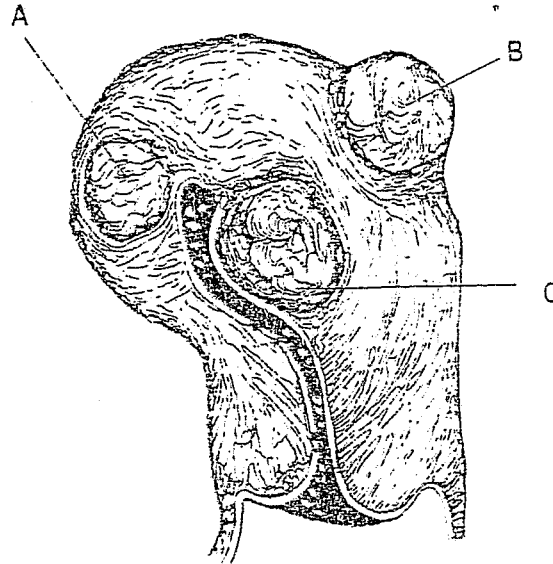


Figure 1.5: Schematic diagram of a uterus showing the three common positions for leiomyomata. a) Intramural, b) subserosal and c) subendometrial.



Figure 1.6: Macrograph of a host uterus with leiomyomata (arrows).

## **2.2 Micro Anatomy**

### **2.2.1 Light Microscopy**

No histological abnormalities can be observed in the host myometrium, using the light microscope. Tumours show a nodular circumscription with characteristic anastomosing whorled fascicles of fusiform cells. The cells are of uniform size and are not aligned with any of the muscle fascicles in the neighboring myometrium. These tumorous areas appear more cellular but rarely demonstrate nuclear atypica. The cells have a fibrillar, eosinophilic cytoplasm; the nuclei are usually small and elongated with finely dispersed chromatin. The amount of intercellular connective tissue in each tumorous region is variable and has been shown to contain a predominance of type I and type III collagen, with focal areas of fibronectin around individual smooth muscle cells (Stewart *et al*, 1994).

### **2.2.2 Electron Microscopy**

At the ultrastructural level a difference between the host myometrium and normal has recently been noted. Richards (1995) observed that the length of the plasmalemmal dense bands found in the cells of the host myometria and the tumours were longer than those seen in normal myometria, though no quantification of this difference was undertaken. There appeared to be a consequential decrease in the number of plasmalemmal caveolae associated with these myocytes. As these caveolae are the location of the active calcium extrusion pumps this may effect the contractile processes of these areas (Fujimoto, 1993).

The tumours have also been observed to contain structurally abnormal mitochondria and sarcoplasmic reticulum (Richards, 1995), which may relate to further abnormalities in the calcium uptake and release system in these areas. There is an apparent increase in the numbers of mitochondria, though this is variable inter- and intra-tumour. Signs of cellular degeneration have been noted in some tumours, this is often seen as myelin figures and swelling of the sarcoplasmic reticulum (Richards, 1995). The arrangement of the myofilaments is similar to that of normal myometrial myocytes but there is an apparent increase in the amount intermediate filaments observed. These filaments have been shown to occasionally fill substantial portions of the cytoplasm (Eyden *et al*, 1992; Richards, 1995) and appears to be due to an increase in the vimentin component (Eyden *et al*, 1992) or a general decrease in the amount of actin filaments in the cell. Nuclear atypia, as seen at the ultrastructural level, often takes the form of membrane infoldings (Richards, 1995). Few mitotic figures are seen, though Tiltman (1985) and Kawaguchi *et al* (1989) do show an increase in their numbers when compared to the surrounding tissue.

## **3. Aetiology of Leiomyomata**

Despite the commonality of these tumours in humans and their occurrence in other species such as the Asian rhinoceros (*Dicerorhinus sumatrensis*) (Schaffer *et al*, 1994) and the woodchuck (*Marmota monax*) (Foley *et al*, 1993) little is established of the aetiology of leiomyomata. Research, using the X-chromosome-linked marker enzyme

glucose-6-phosphate dehydrogenase, has established that the tumours arise from a unicellular progenitor (Townsend *et al.*, 1970). This marker is found only in a small minority of women who have the heterozygous gene, which is found most often in black populations. Recent genetic studies, using the X-chromosome-linked phosphoglycerokinase gene, have confirmed the monoclonality of these tumours in a wider population (Hashimoto *et al.*, 1995). What the progenitor cell is or how it becomes activated to form the tumours is unknown. A survey of the literature reveals a large number of proposed aetiological mechanisms, which can be embraced by two major schools of thought. The first, is a belief that the tumours have a hormonal origin and the second school hold that there is an underlying genetic or cytogenetic abnormality. These two major aetiological mechanisms are explored in greater detail below.

### **3.1 Hormonal Influences**

The most prevalent theory regarding the aetiology of leiomyomata is that of a hormonal origin. Investigations looking for a hormonal cause of these tumours have shown that the reproductive steroid hormones, oestrogen and progesterone, have the greatest influence, with possible secondary influences from growth factors, such as epidermal growth factor and insulin-like growth factors. The origins of these hormonal theories are twofold and the evidence to support this view point arises as a result of these origins.

1) The majority of tumours arise during the reproductive years. It has been shown that at 20 years of age 17.5% of autopsy specimens show evidence of tumours (Haines & Taylor, 1975), rising to an incidence of 40% by 45 years of age (Gompel & Silverberg, 1994). Following menopause, there is evidence to suggest that there is regression or at least cessation of growth of leiomyomata (Norris & Zaloudek, 1982).

2) The formation of tumours has been associated with continuous unchallenged oestrogen (Lipschütz, 1942; Ross *et al.*, 1986) and the positive

mitogenic effects of oestrogen have been established in breast tumour growth (Sheffield & Welsch, 1985).

Vollenhoven *et al* (1990) has shown that nulliparous women have a greater likelihood of developing leiomyomata than those of high parity. A possible mechanism put forward for this, is that pregnancy disrupts a situation where there is unchallenged circulating oestrogen. It has also been reported that the use of drugs with oestrogenic potential, such as clomiphene, induce massive growth surges in existing tumours (Prakash & Scully, 1964; Frankel & Benjamin, 1973; Felmingham & Corcoran, 1975). Animal models have been used to try and elicit tumour growth using unchallenged oestrogen but results from these experiments, although showing the tumorigenic potential of unchallenged oestrogen, have not definitively shown the induction of uterine leiomyomata (Nelson, 1937; 1939; Lipschütz, 1942).

Data on the numbers of oestradiol receptors present in myometrial and tumorous tissue obtained using radioimmunoassay techniques have been equivocal. Most of the studies undertaken have shown little or only slight increases in the quantity of available receptors (Farber *et al*, 1972; Pollow *et al*, 1978; Tamaya *et al*, 1979). Only Wilson *et al* (1980) have shown that there is a significant increase in the available oestradiol receptors present in leiomyomatous tissue.

Oscillations in the available receptor content of leiomyomatous tissue during the endometrial cycle, with receptor numbers being higher in the tumour than the surrounding non-neoplastic host tissue, have been demonstrated using both radioimmunoassay (Soules & McCarty, 1982) and immunostaining (Lessey *et al*, 1988) using a contrast dependent light microscopy technique. Using a contrast independent microscopical method (Richards *et al*, 1994), quantitative immunocytochemistry of the total receptor content has shown that these levels do not vary during the endometrial cycle in normal myometrial tissue (Richards & Tiltman, 1995). Investigations of the non-neoplastic host myometrium of leiomyomatous uteri have demonstrated a higher total oestrogen receptor content to that of normal (Richards & Tiltman, 1996), thus inferring an abnormality of the tissue in which these tumours arise. The tissue from leiomyomata has been observed to have a similar

number of receptors to that found in the subendometrial region of the non-neoplastic host myometria (Richards, 1995).

Current therapy, other than surgical intervention, revolves around the creation of a hypoestrogenic state in the patient, in an attempt to ameliorate the symptoms associated with the presence of these tumours. The use of gonadotropin hormone-releasing hormone (GnRH) agonists, although initially stimulating the release of luteinising hormone and follicular stimulating hormone, desensitises the gonadotrope receptors and reduces gonadotropin release (Schriock, 1989). The artificial menopause produced has been shown to reduce the size of the tumours by 25-80%, mostly in the first month (Crow *et al.*, 1995). However, if therapy is withdrawn the tumours regrow rapidly in a similar manner to that seen with progestin (Mixson & Hammond, 1961).

These studies relating to oestrogen and the myometrium have suggested that the tumours have an oestrogen dependency but have not proved conclusively that they arise as a direct result of excessive oestrogen. Other studies on progesterone and the growth factors tend to support this hypothesis rather than suggesting alternative hormonal aetiologies (Anderson & Barbieri, 1995).

Progesterone has been shown to have a mitogenic effect on the tumours especially where progestins are being taken as oral contraceptives (Fechner, 1968; Tiltman, 1985). Mixson and Hammond (1961) demonstrated an increase in tumour size following progestin treatment, though this effect was a temporary one, as once the progestin treatment was withdrawn the tumour returned to its original size. As progesterone is usually thought to mitigate the effects of unchallenged oestrogen (Lipschütz, 1942; Buttram & Reiter, 1981), Mixson and Hammond's (1961) finding may reflect a 'pseudo-pregnancy' response by the leiomyomata. Harrison-Woolrych and Robinson (1995) contend that high-dose progesterone may play an important role in the growth of the tumours and that GnRH agonist therapy may be successful as a result of lowering of progesterone levels rather than the creation of a hypoestrogenic state. Available progesterone receptors are thought to be over expressed by the tumour, when compared to their 'normal' host tissue (Brandon *et al.*, 1993) and respond in a cyclic manner during the menstrual cycle (Andersen & Barbieri, 1995).

However, there have been no studies regarding the total progesterone receptor content of either normal myometrial tissue, non-neoplastic host myometrial tissue and the tumour in a similar manner to that done for oestrogen (Richards, 1995; Richards & Tiltman, 1995; 1996).

Polypeptides, such as epidermal growth factor, insulin-like growth factor and parathyroid related peptide, have been associated with neoplastic growth and mitogenicity (Andersen & Barbieri, 1995). Research has shown that epidermal growth factor receptors are expressed to the same extent in both, non-neoplastic host myometrium and leiomyomata, however no work has been done on normal myometrium (Hoffman *et al*, 1984). Differences in the levels of insulin-like growth factor in the tumours in comparison to the non-neoplastic host myometrium have been shown (Vollenhoven *et al*, 1993). In agreement with others, Vollenhoven and colleagues showed an increase in insulin-like growth factor II in the tumour while insulin-like growth factor I was reported to be present in similar quantities. The binding proteins for insulin-like growth factor were found to be present while binding protein III was decreased in the tumour. They thought that this depression in the binding protein allowed the increased expression of insulin-like growth factor II to be bioactive and promote tumour growth (Vollenhoven *et al*, 1993). Parathyroid hormone related peptide has been shown to be present in tumours associated with humoral hypercalcemia of malignancy (Stewart & Broadus, 1990). Though normally expressed in the myometrium, this factor has been described as being elevated in neoplastic tissue in comparison with the host tissue (Weir *et al*, 1994). Parathyroid hormone related peptide has been shown to play a role in the modulation of mitogenesis and has been shown to stimulate cAMP (Weir *et al*, 1994).

### 3.2 Cytogenetic and Genetic Influences

Cytogenetic analyses of leiomyomata have shown that at least 30% of tumours demonstrate a clonal chromosomal abnormality or abnormalities (Hu & Surti, 1991). There have been a number of attempts to classify the various abnormalities and thus subdivide the tumours into specific cytogenetic sub-types (Hu & Surti, 1991; Meloni *et al*, 1992), however there is little consensus between the groups, as to the number of

insertions, deletions and translocations found in the tumours. The number of abnormalities varies both *intra* and *inter* patient sample (Rein *et al*, 1991) and the sample size in each of the studies undertaken has been small in comparison to immunological studies.

The main cytogenetic theories arising from these studies are based on the more common abnormalities observed. Three of those observed involve chromosome bands 7q31-32 (Fan *et al*, 1990), 12q13-15 (Sait *et al*, 1989) and 14q23-24 (Rein *et al*, 1991). All three of these positions are associated with oncogenes and sites known to be involved with other benign tumours (Bullerdiek *et al*, 1987; Rein *et al*, 1991). The chromosome rearrangement at 7q31-32 may involve the oncogene *met*, which is at this location, but no tumourigenic potential has been assigned to this oncogene. The 12q13-15 abnormality has been associated with other benign neoplasms, such as pleomorphic tumours of the salivary gland (Bullerdiek *et al*, 1987) and it has been suggested that the oncogenes, *int1* and *gli* may be involved in the pathogenesis of leiomyomata (Sait *et al*, 1989). The chromosome band 14q23-24 is associated with the oncogene *fos* but again this oncogene has not been reported in other neoplasms (Rein *et al*, 1991).

There has been no one specific cytogenetic abnormality found within the leiomyomata samples investigated and as has already been stated there is *inter* and *intra* sample variation. As leiomyomata are benign tumours, the cytogenetic abnormalities observed in the samples may be a result of the tumours abnormal growth rather than the initiating factor for the transformation of 'normal' tissue.

Leiomyomata have been shown to be a significant cause of morbidity in women (Vollenhoven *et al*, 1990); however, its commonality across population groups appears to preclude heredity within the population as being a significant aetiological event. However, other genetic influences have been shown to be of importance in leiomyomatous tissue. The genes that are expressed as the polypeptide factors mentioned above, are regulated by oestrogen (Andersen & Barbieri, 1995). The increased expression of genes for these and other polypeptides in leiomyomata, have led to the conclusion that these tumours represent dysregulated differentiation, as

opposed to the dysregulated cell cycle progression, found in other neoplastic tissues (Andersen & Barbieri, 1995). Further, as most of these receptors have been shown to respond to the endometrial cycle, disturbances in the regulation of gene expression during the hormonal quiescent phases of the cycle, maybe of aetiological significance.

### **3.3 Aetiological Conclusions**

Though both theories as presented above seem to be disparate at first glance, the more recent work in both fields, that of Richards and Tiltman (1995; 1996), Richards (1995) and Andersen and Barbieri (1995), suggest that an underlying abnormality may be found within the cells involved in the formation of leiomyomata. Richards and Tiltman (1996) demonstrate that the non-neoplastic tissue of the host myometrium is essentially abnormal with regards its total receptor content, when compared to normal myometrium. As the receptor levels are higher, the tissue is probably more susceptible to oestrogen which would then lead to the abnormal expression of the growth factors and other compounds, such as cyclic adenosine monophosphate, which may have a tumorigenic effect on more susceptible cells within the myometrial population.

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## Chapter Two

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# **The Second Messenger System**

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### *Adenylate Cyclase and Cyclic AMP*

#### **Introduction**

The internalisation of critical systems by early eukaryotes, such as gene transcription and energy transduction, required the development of a multi-purpose signaling system on the external cell membranes (Whitfield *et al*, 1987). This signaling system has several recognisable components, collectively known as the second messengers. Researchers are now beginning to realize that the various second messenger systems, which include cyclic 3' 5' adenosine monophosphate (cAMP), calcium ions and inositol triphosphate (Alberts *et al*, 1989), interact with each other, to control internal cell systems. A second messenger, can be defined as a molecule or ion whose cellular concentration is modified by external hormonal or electrical signals effecting the cell surface. The modification of the concentration of the second messenger results in changes in internal cellular mechanisms otherwise unreachable by the extracellular hormone/signal.

Cyclic 3' 5' adenosine monophosphate has been recognised as a second messenger since its discovery in 1958 (Sutherland & Rall, 1958) and, as such, has been extensively researched (Steer, 1976). Recent studies have indicated that cAMP has a synergistic effect on the transcription of estrogen regulated genes, such as insulin-like growth factor I (Cho & Katzenellenbogen, 1993) and some of these are abnormally expressed in leiomyomata (Chapter One). Cyclic 3' 5' adenosine monophosphate and its influence within the cell is controlled by hormonal switching, either stimulatory or inhibitory, of the enzyme adenylate cyclase. Adenylate cyclase (AC) is a membrane bound lyase, that synthesizes cAMP from adenosine triphosphate (ATP) (Sutherland *et al*, 1962). Adenylate cyclase can be considered to be part of a tripartite system, each sub-component of which interacts to attain the synthesis of cAMP. The components of this system include the receptor on the external surface of the membrane, a guanosine

triphosphatase (GTPase) and the enzyme, AC. Current knowledge of this tripartite system and the mechanisms of cAMP action are detailed below.

## 1. Adenylate Cyclase, the system

As briefly outlined above, the AC system, as opposed to the enzyme, can be thought of as being a tripartite system, the three components of which combine to control the production of the second messenger, cAMP. This interface between the extracellular milieu and the modification of intracellular processes begins with the activation of a cell surface receptor.

### 1.1 The Receptor

Structurally the receptors are a very similar group of trans-membrane glycoproteins. They are all thought to have seven hydrophilic membrane spanning domains (Levitzki & Bar-Sinai, 1991) and may be activated by several circulating compounds (Table 2.1).

**Table 2.1:** Some of the stimulatory and inhibitory receptors effecting the adenylate cyclase system.

| Stimulatory         | Inhibitory           |
|---------------------|----------------------|
| $\beta$ -adrenergic | $\alpha$ -adrenergic |
| Histamine           | Prostaglandins       |
| Glucagon            | Opiates              |

Experiments with chimeric receptors have demonstrated that the receptors have two structurally separated binding domains, the ligand (hormone) binding domain and the GTPase binding domain (Wong *et al*, 1990). Thus, the ligand binding domain of an inhibitory receptor was separated from its natural GTPase binding domain and rejoined to the GTPase binding domain of a stimulatory receptor. This inhibitory/stimulatory chimeric receptor will give a stimulatory response to an inhibitory hormone (Wong *et al*, 1990). It is therefore the combination of the two domains that is responsible for the stimulatory or inhibitory response.

## 1.2 'G' Proteins

The GTPases of the AC system are known as guanosine binding proteins or 'G' proteins. They belong to a super family of GTPases (Bourne *et al*, 1991), members of which include the transducins from the retina, p21<sup>ras</sup>, G<sub>s</sub> and several others. The structure of these proteins has been shown to be conserved within the family, with the majority of them being of the single protein type (Bourne *et al*, 1991). Those that interact with AC are unusual, in that they tend to have a heterotrimeric structure (Bourne *et al*, 1990). The two main 'G' proteins that are of specific interest to AC systems are the G<sub>s</sub> (stimulatory 'G' protein) and G<sub>i</sub> (inhibitory 'G' protein).

The heterotrimeric structure consists of an  $\alpha$ ,  $\beta$  and  $\gamma$  subunit (Gilman, 1987); the relative molecular weights of these subunits vary with type and function. The  $\alpha$  units are between 41 and 45 kd ( $\alpha_i$  and  $\alpha_s$  respectively) and the  $\beta$  and  $\gamma$  subunits are much smaller at approximately 35 and 10 kd respectively (Gilman, 1987). 'G' proteins are thought to be situated on the inner leaflet of the plasma membrane. The  $\alpha$  subunit is hydrophilic and the  $\beta\gamma$  subunits are hydrophobic, as a result of this arrangement it is possible that the  $\beta\gamma$  subunits anchor the  $\alpha$  subunit to the membrane (Gilman, 1987; Watson & Arkinstall, 1994).

Alpha subunits are structurally similar to the single structural unit GTPases (e.g. Ras H, p21<sup>ras</sup>) and are thought to contain the receptor and AC binding domains. Both of these domains appear to reside in the C terminal end of the subunit (Masters *et al*, 1988). There is a large amount of homology between the  $\alpha$  subunits of the heterotrimeric 'G' proteins, even though they differ in their responses to certain agents (Watson & Arkinstall, 1994).

A large number of genes code for the  $\beta\gamma$  subunits (Gilman, 1987; Gautam *et al*, 1990). Through this genetic diversity the  $\beta\gamma$  units may determine the specificity of the 'G' proteins (Levitzki & Bar-Sinai, 1991). Despite this diversity, the  $\beta\gamma$  subunits from G<sub>s</sub> have been shown to be interchangeable with those from G<sub>i</sub> (Cassey & Gilman, 1988). The  $\beta\gamma$  subunits may also facilitate the activation of 'G' proteins by hormone-receptor

complexes and play a role in the regulation of potassium channel regulation (Watson & Arkininstall, 1994).

### 1.2.1 Activation and Inhibition of AC

Figure 2.1 shows the basic stimulatory cycle of the 'G' proteins. The 'G' protein is non-functional when it binds guanosine diphosphate (GDP). Following stimulation, by hormone-receptor complexes or other stimulants, the bound GDP is released and replaced by guanosine triphosphate (GTP) at the binding domain. This activates the 'G' protein into a form that will interact with AC. 'G' proteins are members of a GTPase super family, therefore once activated, hydrolysis of the GTP to GDP starts. This returns the 'G' protein to the inactive form, thus completing a self regulatory cycle (Figure 2.1) (Bourne *et al*, 1990).

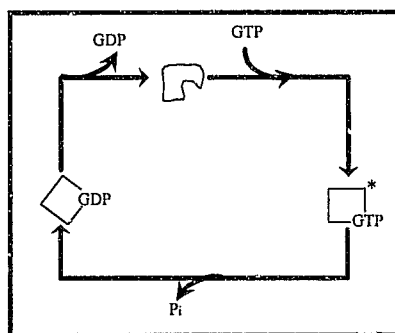
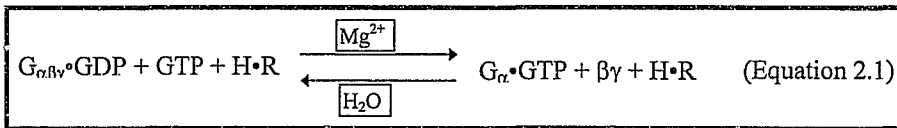


Figure 2.1: A generalized 'G' protein stimulatory cycle. The binding of GTP activates the G protein which hydrolyses the GTP to GDP returning the 'G' protein to its inactive form. P\* - active form, P<sub>i</sub> - phosphate ions.

There are two theories regarding the activation/inhibition of AC. The first or 'shuttle' theory (Citri & Schramm, 1980; De Lean *et al*, 1980), proposes that the 'G' protein shuttles between the hormone-receptor (H•R) complex and the enzyme. However, experiments have shown that the activation of AC is dependent on the receptor concentration (Tolkovsky & Levitzki, 1978), whereas the 'shuttle' mechanism predicts a dependence on the concentrations of G<sub>s</sub> and AC for the activation (De Lean *et al*, 1980). The alternative to the 'shuttle' theory is the 'collision coupling' theory

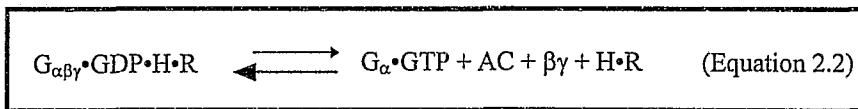
proposed by Levitzki (1982; 1987) who suggested that the hormone-receptor unit acts in a catalytic fashion (Tolkovsky & Levitzki, 1978). It has also been proposed that the 'G' protein is bound to the enzyme (Levitzki & Bar-Sinai, 1991) rather than moving backwards and forwards as in the 'shuttle' theory. The 'collision coupling' theory predicts that the activation of AC will be dependent on receptor concentration and such predictions using the theory have been verified by experimental results (Levitzki & Bar-Sinai, 1991)

Gilman (1987) showed that the  $\beta\gamma$  subunits dissociated from the  $\alpha$  subunit,  $G_i$  or  $G_s$ , in the presence of magnesium ions. The three subunits reassociate when the GTP hydrolysed (Equation 2.1). The  $\beta$  and  $\gamma$  units being interchangeable reassociate with either  $\alpha$  subunit (Cassey & Gilman, 1988).



H·R - Hormone:Receptor Combination

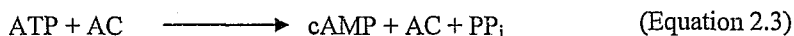
Thus the activation of a  $G_i$  unit induces the dissociation of the  $\alpha_i$  subunit from the  $\beta\gamma$  subunits (Equation 2.2). This increases the local pool of  $\beta\gamma$  subunits, which then compete with AC for the available  $\alpha$  subunits. An overabundance of  $\beta\gamma$  subunits will drive equation 2.2 to the left, thus inhibiting the stimulation of AC, as the  $G_{\alpha}$  subunit is no longer able to accept a GTP molecule.



H·R - Hormone:Receptor Combination

### 1.3 Adenylate cyclase the enzyme

Adenylate cyclase is the workhorse of the signal transduction process. It transforms ATP into cAMP (Equation 2.3), thus increasing the levels of the second messenger in the cell. Biochemical investigations of the actions of AC have been extensive since its first description (Sutherland *et al*, 1962). In 1989 the structure of AC was elucidated using cDNA cloning techniques (Krupinski *et al*, 1989). Since the cloning of the first AC isoform, eight other isoforms have been described (Tang & Gilman, 1992). The following section details what is known regarding the structure, stimulation and inhibition of these isoforms.



#### 1.3.1 Structure

Initial evidence suggested that there was a range of AC forms and hydrodynamic studies in the late seventies hypothesized that the molecular weight of AC ranged between 160 and 230 kd (Ross & Gilman, 1980). Genetic (Livingstone *et al*, 1984) and biochemical experiments (Mollner & Pfeuffer, 1988) increased the body of evidence suggesting the existence of multiple forms of AC. Determination of the molecular weight of the protein, following improved purification techniques, indicated a weight of between 120 and 150 kDa, the earlier higher figures may have been the result of co-purification of the enzyme with the associated 'G' protein (Smigel, 1986). The sequencing of bovine brain AC, designated type I, by Krupinski *et al* (1989) and other isoforms has established a molecular weight of between 110 and 200 kDa depending on the isoform (Watson & Arkinstall, 1994).

The structure of the AC type I isoform is highly unusual (Figure 2.2) with its two trans membrane and corresponding cytoplasmic domains. Krupinski and colleagues (1989) suggested that AC acted as a transporter molecule or channel because of its similarity to the transporter molecule P-glycoprotein. However, there are no sequence

homologies between AC and other channel forming molecules, therefore this function is an unlikely one for AC (Tang & Gilman, 1992).

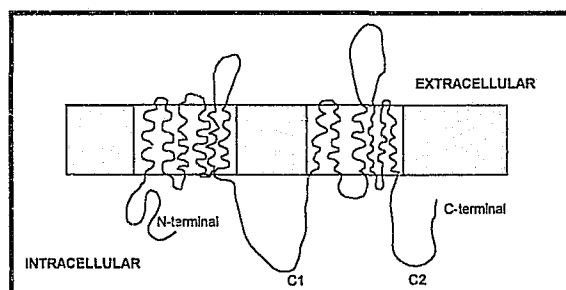


Figure 2.2: Structure of the AC type I isoform after Krupinski *et al* (1989). C1 and C2 are two cytoplasmic domains.

Other isoforms of AC have been subsequently sequenced, the majority from neural tissues (Tang & Gilman, 1992) (table 2.2). All of those that interact with the trimeric 'G' proteins, types I - VI, have a structure similar to the type I form (Watson & Arkinstall, 1994). There is a large amount of homology between all the isoforms and the two cytoplasmic domains (C1 and C2 in figure 2.5) are well conserved with a 50-92% sequence similarity in types I - VI (Watson & Arkinstall, 1994).

**Table 2.2:** Adenylate cyclase isoforms and the tissues in which they are predominately expressed.

| Type | Site of Expression   |
|------|----------------------|
| I    | Brain                |
| II   | Brain, Lung          |
| III  | Olfactory            |
| IV   | Brain, others        |
| V    | Heart, Brain, others |
| VI   | Heart, Brain, others |
| VII  | Drosophila           |
| VIII | Dictostyleum         |

The two cytoplasmic domains are thought to be responsible for the catalytic response of the enzyme. Both the domains are required for the binding of the substrate and may also be the location of P-site inhibition (Londos & Wolff, 1977; Tang & Gilman, 1992). There is little homology between the isoforms in the intermembranous portion of the molecule. The stimulation of the enzyme with forskolin may occur through this hydrophilic region (Krupinski *et al*, 1989).

### **1.3.2 Stimulation and Inhibition**

The stimulation and inhibition of all the AC isoforms is a result of the 'G' protein cycle, discussed briefly above. However the isoforms of AC can be divided into sub-groupings as a result of their interaction with calcium and the calcium binding protein calmodulin (Watson & Arkininstall, 1994). Table 2.3 shows the groupings of the AC isoforms according to this classification and their sensitivity to  $\text{Ca}^{2+}$  and calmodulin.

**Table 2.3:** Classification of AC isoforms according to their reactivity to  $\text{Ca}^{2+}$  and calmodulin.

| $\text{Ca}^{2+}$ /Calmodulin sensitivity                    | AC Isoform                  |
|-------------------------------------------------------------|-----------------------------|
| Stimulatory also by $\text{G}_s$ , $\text{G}_{olf}$         | I, III                      |
|                                                             |                             |
| No response                                                 | II (but PKC* activated), IV |
|                                                             |                             |
| Insensitive but inhibited by $\mu\text{M}$ $\text{Ca}^{2+}$ | V, VI                       |

\*PKC - Protein kinase C

The varying sensitivity of the AC isoforms may reflect the enzymes role in the cells from which these isoforms are found. For example, type I has been associated with memory and learning processes while type II is thought to play an integratory role in signals from a number of receptors subtypes (Watson & Arkininstall, 1994)

## 2. Cyclic Adenosine Monophosphate

Cyclic adenosine monophosphate (Figure 2.3) was first described by Sutherland and Rall (1958) who purified the compound from dog liver and described its breakdown by phosphodiesterase (PDE). Since then there have been many studies on the actions of cAMP within the cell (Friedman, 1976; Steer, 1976). Cyclic adenosine monophosphate is formed from the reaction of AC with ATP, as shown in equation 2.3. Once formed the cAMP is either degraded by PDE to adenosine monophosphate (equation 2.4) or activates cAMP-dependent protein kinase (protein kinase A (PKA)) (Stryer, 1981).

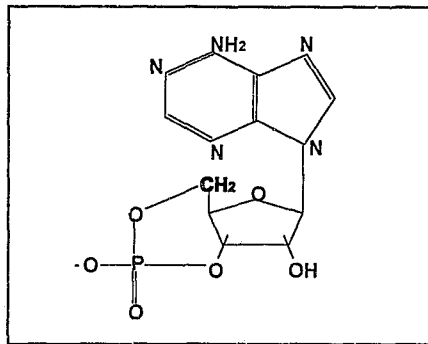
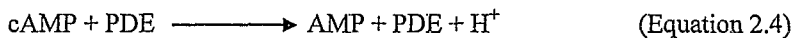


Figure 2.3: Structural formula of cyclic adenosine 3' 5'-monophosphate.



Protein kinase A is composed of two dimer subunits, the regulatory (R) subunit and the catalytic (C) subunit (Cho-Chung *et al*, 1989). The action of cAMP in activating this kinase is shown in equation 2.5. The free C subunits interact further within the cell modifying other reactions, this is the cAMP cascade, the interactions of the second messenger system (Cho-Chung *et al*, 1989).



## 2.1 cAMP the Second Messenger

Cyclic adenosine monophosphate affects many cell reactions through its actions on PKA, including the influence of growth and differentiation (Friedman, 1976), smooth muscle relaxation (Lincoln & Cornwell, 1991) and genetic transcription (Borrelli *et al*, 1992). Cyclic adenosine monophosphate interacts with PKA and frees the C subunit (Equation 2.5), which either phosphorylates proteins within the cytoplasm, thus activating them or else it migrates to the nucleus, where it activates transcription by binding to cAMP-responsive element binding protein (CREB) (Walton & Rehfuss, 1992) (Figure. 2.4). These responses are inactivated by the lowering of cAMP levels and the degradation of the C subunits.

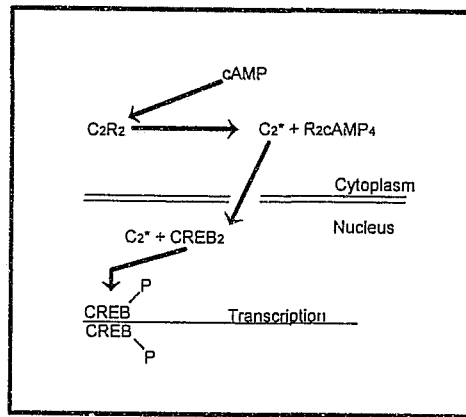


Figure 2.4: Cyclic adenosine monophosphate stimulated PKA action in gene transcription.

Cyclic adenosine monophosphate has both an inhibitory and stimulatory affect on cell growth (Ralph, 1983; Silberstein *et al*, 1984). The inhibitory affects are thought to relate to cAMP's control of protein kinases and the subsequent phosphorylation of proteins, as alterations in this path have inhibited growth (Ralph, 1983; Cho-Chung *et al*, 1989). Cyclic adenosine monophosphate has a mitogenic affect on certain tissues; in particular mammary duct size and uterine wet weight will increase following the elevation of cAMP (Silberstein *et al*, 1984; Stewart & Webster, 1983).

In smooth muscle, cAMP promotes relaxation through the action of hormones and drugs targeting  $\beta$ -adrenergic receptors and other activators of AC (Krall & Korenman, 1979). Calcium ions ( $\text{Ca}^{2+}$ ) directly influence muscle contraction and relaxation but also interact with cAMP to achieve the relaxed state (Ishine *et al.*, 1993).

CREB regulation of gene transcription affects the gene's coding for somatostatin, parathyroid hormone and  $\beta_2$ -adrenergic receptor (Borrelli *et al.*, 1992). Cyclic adenosine monophosphate has an affect on oestrogen receptors and oestrogen receptor mediated transcription (Aronica & Katzenellenbogen, 1993; Cho & Katzenellenbogen, 1993)

### **3. Significance**

It is clear, from the foregoing discussion, that cAMP and its regulation by the AC system is of importance to diverse operations of the cell and in smooth muscle cAMP plays a part in the regulation of contraction and  $\text{Ca}^{2+}$  influx. The recent work on CREB transcription and the genetic expression of other compounds implies that any disruption to this cycle of events may increase the expression of genetic material which would otherwise not be observed. A number of unusual genetic messages are expressed in tissue from leiomyomata (see chapter one) and these may be influenced by the cAMP cascade.

Evidence of an abnormality in the workings of the system, that has been described above, may have a direct bearing on the growth and biochemistry of these tumours. The cellular localization of the various components of the tripartite AC system in normal myometrium, the non-neoplastic host myometrium and the tumours themselves, will give an indication of any abnormalities in the control sites for cAMP production within these tissues.

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## Chapter Three

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# **Microscopical Localization of the Adenylate Cyclase System**

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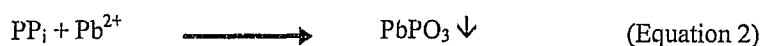
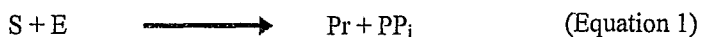
### *Microscopical Localization*

#### **Introduction**

The microscopical localization of any enzyme has been traditionally the province of the histochemist but with the advent of immunocytochemistry and *in situ* hybridization a greater specificity appears to be attained. These techniques are briefly outlined below along with their advantages and disadvantages.

#### **1. Histochemistry**

Initial localization of enzymes, particularly those that had phosphorylated substrates, was carried out using a heavy metal precipitation technique (Gomori, 1939). This technique utilizes the fact that phosphate salts of lead are insoluble and precipitate out of solution. Thus, the enzyme (E) and its phosphorylated substrate (S) react to form the dephosphorylated/cyclicized product (Pr), releasing phosphate ions (P<sub>p</sub>i) into solution (Equation 1). The phosphate ions react with lead, the capture agent, to form lead phosphate, which precipitates out of solution (Equation 2).



As with any technique there are several areas that have to be carefully assessed before accurate localization can be determined. For example, the enzyme under investigation must be in a functional state during the various stages of the experiment otherwise localization will not take place. This being the case fixation of the tissue must be carefully considered and in localizing AC in some tissues it is better to fix after localization rather than before (Richards, 1994). The enzyme may also need its activity enhanced and therefore the incubation medium must be carefully formulated so that the various components have an overall stimulatory effect on the enzyme. The incubation solutions that are used can be extremely complex and the methods that have been employed by AC histochemists to overcome these difficulties since the enzymes initial localization (Howell & Whitfield, 1972) have been reviewed by Richards and Richards (1998). This technique will only localizes the active enzyme, therefore no information is garnered regarding the inactive enzyme or the protein's production sites within the cell, i.e. mRNA or latent pools of the enzyme.

## **2. Immunocytochemistry**

Since the 1960's developments in immunology have allowed the cell biologist to localize specific protein moieties using the antibody - antigen reaction (Polack & van Noorden, 1987). The immunocytochemical technique utilise an organism's natural defence system to produce antibodies against the 'invading' protein or amino acid sequences. Injection of a host animal with the chemical of interest, the antigen, will induce the production of antibodies. The antibody, specific for the antigen, can then be harvested from the animal's serum and purified, polyclonal, or a culture of lymphocytes, from the host (mouse) animal's spleen, fused with myeloma cells is used to obtain monoclonal antibodies (Polack. & van Noorden, 1987). Once purified the manufactured antibody will attach to the initiating antigen if it is present in the tissue of interest (Fig 3.1). By tagging the antibody with a visual marker, such as the enzyme peroxidase which reacts with diaminobenzidine (DAB) to form a brownish deposit, the site of reaction can be detected (Burns, 1989).

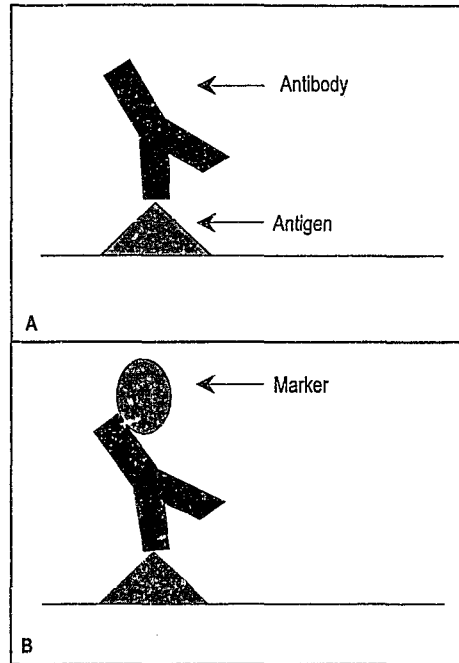


Figure 3.1: Schematic representation of a) antibody : antigen reaction on tissue section and b) same reaction with the antibody tagged with a visible marker.

The method as described above may not detect the target antigen in the tissue for two possible reasons. Firstly, if the antigen is in too small a quantity too little DAB reaction product will form. In this case the deposit will not be observed using the light microscope and result in a false negative. To overcome this it is possible to make the technique more sensitive by using the high affinity between avidin and biotin. If a second biotinylated antibody, raised against the animal in which the first antibody was raised, is attached to the first antibody then avidin with a larger number of peroxidase molecules can react at the site thereby increasing the amount of DAB reaction product at the site (Fig. 3.2).

In the second case the antigen may be 'masked' as a result of the tertiary structure being rearranged during fixation or processing. If the antigen is masked, for one reason or another, it can be retrieved by using protease digestion (Finley & Petrusz, 1989), microwave techniques (Shi *et al.*, 1991) or ultrasound (Podkletnova & Alho, 1993).

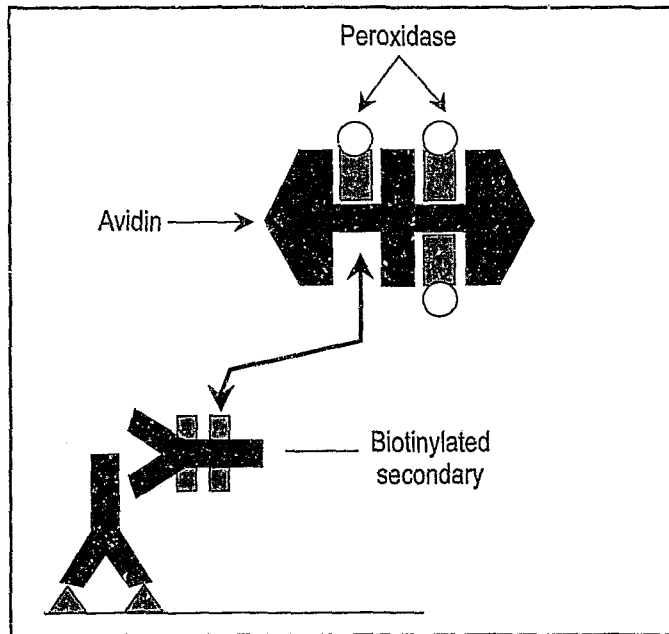


Figure 3.2: Schematic representation of a primary antibody tagged with biotin which will react with a molecule of avidin. The avidin carries more peroxidase reactive sites and thus enhances the amount of end product visible at the reaction site.

This method of detecting target proteins is dependent on the protein or amino acid sequence being present in the tissue and available for the reaction in figure 3.1 to take place. Such a technique will provide information with regards the protein's presence or absence within the tissue but will not give information on its activity or any part of the production sites for the enzyme.

### **3. *In Situ* Hybridization**

In recent years the immunocytochemical technique has been taken one step further. If the genetic sequence for the protein rather than the protein itself is used as the antigen then the corresponding transcription sequence, labeled with a visual marker, will attach and hence localize the protein's genetic sequence. The main drawback to this technique is that the amount of genetic code available for detection is very limited and

can easily be destroyed during processing. In either case steps must be taken to enhance the visualisation process and prevent the removal or destruction of the piece of DNA or RNA being sought (Leitch *et al.*, 1994)

*In situ* hybridization techniques will only detect the presence of the message and not the protein. This means that no information regarding the protein's presence / absence or activity status in the tissue can be determined.

#### **4. Controls**

All the foregoing techniques require the presence of rigorous controls to ensure that the reaction being observed is the specific reaction being investigated. Both negative and positive controls need to be undertaken in order to be certain of the result.

Negative controls include the use of enzyme inhibitors, for AC the chemical alloxan (Cohen & Bitensky, 1969); normal serum in place of the primary antibody (van Leeuwen, 1989); the use of a DNA/RNA sequence that is known not to be present in place of the correct transcription sequence (Leitch *et al.*, 1994). These are specific controls for each of the techniques mentioned above but for all of these techniques a positive control, consisting of known positive tissue, will allow the investigator to draw conclusions regarding the experiment. It is useful if the positive tissue is a separate cell type to that under investigation but occurring in the same section, for example muscle and nerve tissue, as this negates the need for a separate section of tissue that has to be processed at the same time as the other sections (Polack & van Noorden, 1987).

#### **5. Tissue Localization**

Adenylate cyclase has been localized within a large variety of tissues (table 3.5). The table shows a representative number of tissues from several species. No one technique of those mentioned above can be regarded as being better or worse than any other.

Each provide information regarding the location of the AC enzyme system. Histochemistry is a valuable method for the cell biologist, as it is with this technique

that the active enzyme is localized. This may or may not relate to the localization obtained by immunocytochemical techniques, which will localize specific amino acid residues irrespective of the enzyme's state of activity. The newest technique available to the cell biologist, *in situ* localization, provides information regarding the genetic precursors to AC but does not provide information regarding the tissues capability of expressing the message.

## **5.1 Histochemical Localization**

The vast majority of histochemical localizations of AC have located the working enzyme to the plasma membrane. However, AC has been localized to internal membranous structures in a variety of intact tissue samples and fractionated samples (Cheng & Farquhar, 1976; Fine *et al*, 1982; Poeggel *et al*, 1982), although the significance of these findings is debatable. It is believed that the localizations to the golgi and endoplasmic reticulum may be pre-cursor sites of the working enzyme though no immunolocalization or *in situ* method to date has shown this to be the case (Cheng & Farquhar, 1976). There does not appear to be any fluctuation in AC activity with age, as the earliest it has been demonstrated is in the membranes of neuroectoderm cells in *Bufo bufo* (Farnesi *et al*, 1993) through to adult frogs (Richards, 1994).

## **5.2 Immuno- and *In Situ* Localization**

The localization of the AC system using immunocytochemical and *in situ* methods were initially undertaken in neural tissue. As most of the AC isoforms thus far cloned are of neural origin this investigative approach is not surprising. The initial G protein localizations were carried out on olfactory tissue (Jones, 1990) and was taken to the ultrastructural level by Menco and colleagues (1994). Others have investigated the numerous aspects of the G proteins using immunocytochemistry in a variety of tissue including liver (Cadrin *et al*, 1996), cochlear (Mizuta *et al*, 1996) and myometrium during pregnancy (Europe-Finner, *et al*, 1994). Workers localizing AC itself have only recently started to use the immunocytochemical approach. Most of the AC localizations to date have involved AC and its interaction with calcium ions

Table 3.1: Some of the tissues in which AC has been localized.

| Organism                 | Tissue          | Author                     | Year |
|--------------------------|-----------------|----------------------------|------|
| Human                    | Platelets       | Gonzalez-Utor <i>et al</i> | 1992 |
|                          | Marrow          | Krzysztofowicz & Dabrowski | 1984 |
|                          | Placenta        | Matsubara <i>et al</i>     | 1987 |
|                          | Heart           | Yamamoto <i>et al</i>      | 1991 |
|                          | Sweat glands    | Tainio                     | 1987 |
|                          | Brain           | Stengel <i>et al</i>       | 1992 |
| Rat                      | Kidney          | Araki & Saito              | 1979 |
|                          | Olfactory       | Bakalyar & Reed            | 1990 |
|                          | Brain           | Cali <i>et al</i>          | 1994 |
|                          | Pancreas        | Howell & Whitfield         | 1972 |
|                          | Fat cells       | Rechardt & Hervonen        | 1985 |
|                          | Oral mucosa     | Fine <i>et al</i>          | 1982 |
|                          | Liver           | Mayer <i>et al</i>         | 1985 |
| Mouse                    | Teeth           | Osman <i>et al</i>         | 1981 |
|                          | Vagina          | Kvinnslund                 | 1979 |
|                          | Ovary           | Hiura & Fujita             | 1977 |
| Hamster                  | Adrenal         | Carmichael                 | 1984 |
| Rabbit                   | Taste bud       | Asanuma                    | 1990 |
|                          | Eye             | Palkama <i>et al</i>       | 1986 |
|                          | Platelets       | Spreca <i>et al</i>        | 1991 |
| Guinea Pig               | Macrophage      | Dini & Del-Rosso           | 1983 |
|                          | Testis          | Pascolini <i>et al</i>     | 1983 |
| Cow                      | Oocyte          | Kuyt <i>et al</i>          | 1988 |
| Chicken                  | Bone            | Fukushima <i>et al</i>     | 1991 |
|                          | Embryo          | Sanders                    | 1987 |
| Trout                    | Eye             | Athanassious <i>et al</i>  | 1984 |
| <i>Torpedo marmorata</i> | Electric organ  | Muller <i>et al</i>        | 1985 |
| Toad                     | Urinary bladder | Davis <i>et al</i>         | 1987 |
|                          | Embryo          | Farnesi <i>et al</i>       | 1993 |
| <i>Rana fuscigula</i>    | Epithelium      | Richards                   | 1994 |
| Locust                   | Flight muscle   | Swales & Evans             | 1988 |
| Tetrahymena              |                 | Csaba & Sudar              | 1985 |
| Brassica                 | Stigma          | Gaude & Dumas              | 1986 |

particularly in cardiac tissue (Schulze *et al.*, 1996; Gao *et al.*, 1997) and myometrium (Richards *et al.* 1998). Recent *in situ* experiments have also focused on AC's interaction with calcium ions (Charbardes *et al.*, 1996) in tissues other than those of neural origin.

## 6. Significance

As can be seen from the preceding discussion, much effort has gone into the localization of AC in a large number of tissues. That many of these reports have shown the localization, using histochemical techniques, to be on the plasma membrane is to be expected with regards to its known function (Chapter 2). The advent of *in situ* and immunocytochemical techniques have opened up the possibility of investigations into the localization of the pre-cursor amino acids, the sites of synthesis and the routes of insertion into the plasma membrane. At present, most of the more recent investigations have been aimed at the location of neural AC isoforms and it is surprising, considering the large variety of non-neural tissue to which AC has been localized (table 3.1), that few of the cloned isoforms have been detected in non-neural tissues. The importance of AC in cellular interactions makes it an important enzyme to localise in any tissue using as accurate a methodology as possible. Initial localization studies in the myometrium suggest a change across the wall (Richards *et al.*, 1998) which may play a role in tumourigenesis or the promotion of tumour growth and thus a more detailed study of normal, host and tumour tissue is indicated.

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## Chapter Four

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# **Aspects of Tumours, Adenylate Cyclase, cAMP and the Uterus**

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### *Tumours, AC, cAMP and the Uterus*

## **Introduction**

The AC system and cAMP play a major role in the control of cellular mechanisms in normal cells. Any perturbations in the control system are likely to play a significant role in abnormal biological phenomena. Initiation of neoplastic growth, whether benign or malignant, can be considered to be the breakdown in control of the cells growth patterns and as such may influence or be influenced by these interlinked systems. The AC system has been implicated in tumourigenesis as a result of alterations to the genetic material coding for parts of the system (Lyons *et al*, 1990). Stimulation of the enzyme AC and the consequential rise in cAMP induced by hormones and other pathways has a stimulatory effect on tumour growth and the metastatic potential of neoplastic cells (Shah *et al*, 1994; Guidotti *et al*, 1972). These and the effects of increases in cAMP on uterine tissue are discussed below.

### **1. 'G' proteins**

There is a growing body of evidence for the involvement of the GTPase superfamily pathways in cell proliferation (Seuwen and Pouysségur, 1994). The involvement of the GTPases is often the result of point mutations in the genes coding for these proteins (Lyons *et al*, 1990). The effects of these mutations on the 'G' proteins of the AC system are discussed below.

## 1.1 The Oncogenic Mutations

The mutations involved occur on genes coding for amino acids that belong to a part of a highly conserved region of the 'G' protein (Lyons *et al*, 1990) which plays a role in the GTPase-catalysing function of the 'G' protein (Lyons *et al*, 1990). The mutations of the  $\alpha_s$  subunit, called *gsp*, substitutes arginine for glutamine (Gln<sup>227</sup>) (Fig. 4.1a) or else there is a substitution of cysteine/histidine for arginine (Arg<sup>201</sup>) (Fig 4.1b). The substitution of Gln<sup>227</sup> is the equivalent of the Gln<sup>61</sup> oncogene mutation in p21<sup>ras</sup> which inhibits its ability to hydrolyse GTP (Landis *et al*, 1989). The arginine that is substituted at Arg<sup>201</sup> is the site of cholera toxin catalysed adenosine diphosphate ribosylation (Levitzki & Bar-Sinai, 1991) which inhibits GTPase activity (Lyons *et al*, 1990). Thus both point mutations affect the GTPase activity of the G<sub>sα</sub> subunit in such a way that it permanently turns on the enzyme, thus overproducing cAMP. The mutation on the G<sub>iα</sub> subunit, called *gip2*, is similar to the *gsp* mutation at Arg<sup>201</sup> in that an arginine (Arg<sup>179</sup>) is replaced by cysteine or histidine (Lyons *et al*, 1990) (Fig 4.1c). This also turns on the subunit, which although inhibiting AC, stimulates other signaling mechanisms, such as potassium channels and phospholipase D (Selzer *et al*, 1993).

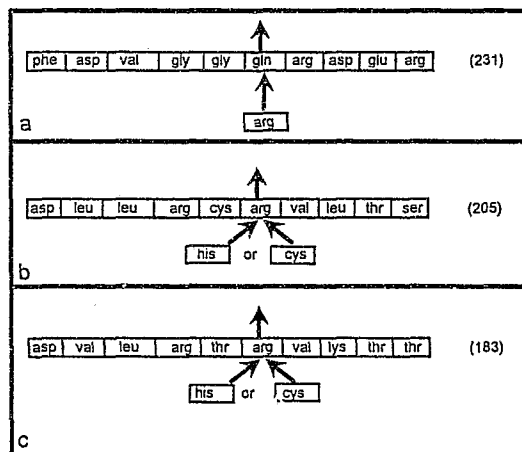


Figure 4.1: Partial amino acid sequence of the 'G' proteins showing the position of the point mutations a - b) *gsp* and c) *gip2*.

## 1.2 Effects of G<sub>s</sub> Mutations

The *gsp* mutations were detected in a sub-population of growth hormone producing pituitary tumours (Vallar *et al*, 1987). The mutation increases the secretion of growth hormone from these tumours by over 1000% (Landis *et al*, 1989) through increases in the amount of intracellular cAMP (Seuwen & Pouysségur, 1994). This mutation is classed as oncogenic, as it stimulates a mitogenic response in cell lines other than the pituitary cells in which it was first found (Seuwen & Pouysségur, 1994). The *gsp* mutation has been identified with other disease conditions and tumours, most notably in McCune-Albright syndrome, a disease of unknown cause with multiple endocrinopathies, including pituitary adenomas and hyperthyroidism (Whitfield *et al*, 1987) and tumours of the thyroid (Lyons *et al*, 1990). Lyons and colleagues (1990) hypothesized that the *gsp* may also be found in other cell types which have a mitogenic response to cAMP.

## 1.3 Effects of the G<sub>i</sub> Mutation

The G<sub>i</sub> mutations were first described by Lyons and colleagues (1990) and have since been shown to have neoplastic growth effects in fibroblast cell cultures and to promote tumour growth *in vivo* (Pace *et al*, 1991). The mutation is a similar one to the G<sub>s</sub> mutation, in that it is located on the  $\alpha$  subunit of the G<sub>12</sub> protein, hence its name *gip2*. Another mutation of the G<sub>i</sub> proteins is found in benign autonomous adenoma (independent of pituitary control) of the thyroid (Selzer *et al*, 1993). These tumours secrete thyroid hormone which is under the control of G proteins as is cellular proliferation in the thyroid (Dumont *et al*, 1989). The exact method by which G<sub>i $\alpha$</sub>  exerts its mitogenic effect is not known. It is thought that the G<sub>i</sub> proteins not only exert an inhibitory effect on AC but also through alternative G<sub>i</sub> effector pathways which may cooperatively stimulate the tyrosine phosphorylation of mitogen activated protein kinases (Selzer *et al*, 1993; Seuwen & Pouysségur, 1994).

## 2. Adenylate Cyclase and cAMP

The effects of the G proteins on the AC-cAMP system noted above have been found to be highly tissue specific, mainly occurring in endocrine and hormone dependent tissues (Seuwen & Pouysségur, 1994). The stimulation of AC by hormones such as insulin-like growth factor II and oestrogen, rather than as a result of G protein mutation, with the concomitant rise in cAMP as a promoter of growth, has been well established (Seuwen & Pouysségur, 1994).

Both morphogenic and mitogenic effects of hormone induced rises of cAMP have been reported in several tissues (Table 4.1). Increases in cAMP levels have also been implicated in the activation of the mitogen-activated protein kinase cascade, via protein kinase A (Frödin *et al*, 1994).

**Table 4.1:** The effect of hormone induced rises in cAMP content of various tissues

| Tissue   | Effect            | Author                          |
|----------|-------------------|---------------------------------|
| Parotid  | ↑DNA, ↑Wet weight | Guidotti <i>et al</i> , 1972    |
| Prostate | ↑Growth           | Shah <i>et al</i> , 1994        |
| Uterus   | ↑DNA, ↑Protein    | Stewart & Webster, 1983         |
| Mammary  | morphogenesis     | Silberstein <i>et al</i> , 1984 |

Stimulation of the AC-cAMP system by cholera toxin, in a similar manner to that achieved by the G<sub>s</sub> mutation, the proliferation of neoplastic mammary tissue *in vivo* has been observed (Sheffield & Welsch, 1985). The proliferative effect was enhanced when oestrogen was administered in conjunction with the cholera toxin and was found to be transferable to the *in vivo* situation (Sheffield & Welsch, 1985). The increase in uterine wet weight observed by Stewart and Webster (1983), with cholera toxin administration, was similar to and greater than that seen when oestrogen alone was administered. These increases in protein synthesis which result from increases in cAMP levels may be the result of the activation of dephosphorylating activation of synthetases (Berg, 1991).

### **3. Significance: A Role for the AC system and cAMP in Uterine Tumourogenesis?**

It is clear from the foregoing discussion that the AC system and cAMP have growth inducing effects on various tissues and neoplastic cells. Genetic mutations have been found that effect the production of AC and cAMP, as well as the stimulation of other second messenger pathways. The response of cAMP to hormonal stimulation of AC is mitogenic in some tissues but has on occasion been shown to have inhibitory effects in metastasizing cells (Suh *et al*, 1992), possibly as a result of its ability to elevate tissue inhibitors of metalloproteinases (Tanaka *et al*, 1995).

In the uterus, increases in cAMP levels appear to stimulate growth and this effect is increased in the presence of oestrogen (Stewart & Webster, 1983). Transcription of genes, controlled by CREB and the oestrogen receptor, in breast cancer cells (Cho & Katzenellenbogen, 1993; Cho *et al*, 1994) and in myometrial cells (Aronica & Katzenellenbogen, 1993; Aronica *et al*, 1994) are synergistically effected by oestrogen and cAMP. Uterine cAMP may itself be partially controlled by oestrogen (Bekairi *et al*, 1984), possibly through the induction of histamine formation (Salganik *et al*, 1980). Results from recent work on oestrogen receptors show that the outer portion of the myometrium from uteri harbouring leiomyomata have increased levels of oestrogen receptors (Richards & Tiltman, 1996). Though AC has not been localized in the uterus using histochemical methods (Chapter 3), biochemical localization suggests that there are greater quantities present in the outer portion of the uterus (Fortier & Krall, 1983). The factors of tumourigenesis mentioned above suggest a line of investigation for the possible underlying aetiology of myometrial tumours.

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## Chapter Five

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# **Study Justification and Tissue Collection**

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### *Justification, Outline and Collection*

#### **Study Aim**

To determine the localization of adenylate cyclase (AC) in the myometrium of the lower segment obtained from normal, host and leiomyomatous tissue.

#### **1. Introduction**

Despite the commonality of the uterine leiomyomas, very little is known regarding the cellular mechanisms taking place. The studies that have been carried out at the cellular level have been concerned with the presence or absence of various hormone receptors which may or may not have a role to play in the tumourigenesis of leiomyomata. As the AC/cAMP second messenger system has been shown to play a role in tumourigenesis in other tissue and affects myometrial tissue, this system's localization and functional characteristics need to be established. Biochemical studies have provided evidence to suggest that AC is differentially distributed through the wall of the myometrium (Fortier & Krall, 1983) but this type of study does not show specific area and cell distribution. Studies of the fundal region of the myometrium have demonstrated the distribution of AC in normal human myometrium (Richards *et al.*, 1998). The study by Richards *et al.* (1998) demonstrates a higher AC activity in the midmyometrial sector of this tissue, when compared to the other sectors (subendometrial and subserosal). The authors further stated that in the sample examined there was no variation with the stage of the endometrial cycle but age decreased the amount of AC present in the tissue (Richards *et al.*, 1998). Neither in this latter study nor in any other has the distribution of AC type V/VI been examined in the lower segment region of the myometrium from host uteri. Richards and Tiltman

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(1996) have shown that the localization of oestrogen receptors are changed in the host myometrium, with an increase in oestrogen receptors across the muscle wall. It has been suggested that this increase makes the muscle bulk in these myometria more susceptible to normal circulating levels of oestrogen and therefore providing a nidus for the occurrence of leiomyomata. Adenylate cyclase and oestrogen have been shown to have a synergistic relationship (Aronica *et al.*, 1994). It is therefore possible that the localization of AC may be affected by an increase in oestrogen susceptibility of the myometrium. Similarly Richards (1995) has demonstrated that leiomyomata have an increase in oestrogen receptors. Increases in other hormones and chemicals that effect the AC system have also been demonstrated in leiomyomata (see chapter 2). However, the localization of AC has not been determined in leiomyomata. AC is characterized as a membrane bound enzyme but it has also been localized to other cell organelles (Chapter 3). Therefore localization using immunocytochemical techniques, which do not normally give information regarding activity status, may suggest activity status if the localization is membrane specific.

## **2. Study Justification**

Adenylate cyclase and the cAMP system undoubtedly play an important role within the cell. In the myometrium this role includes a control of the contraction:relaxation cycle and morphogenesis. Abnormalities in both these activities are found when leiomyomata are present in the myometrium (dysmenorrhea and smooth muscle growth). Answers to several basic questions regarding the distribution and operation of this system in normal and host myometrium and the tumours themselves need to be obtained.

- Where is AC localized in the normal lower segment myometrium and
- Is there a difference in localization between normal, host myometrium and leiomyomata?

- If the distribution is different between these tissues, does AC have a role in the aetiology of the tumour or the symptoms associated with their presence?

In order answer to some of these questions the following study was undertaken

### **3. Study Outline**

Sections of tissue of the lower segment myometrium from surgically removed uteri were processed using immunological techniques to obtain cellular localization of the adenylate isoform (Type V / VI). The amount of isoform present in the tissue is assessed by counting the number of positive cells vs. the number of negative cells in randomly selected areas of the sections. The amount of positivity will be related to the phase of the menstrual cycle, age of the patient, the status of the myometrium (normal or host [harboring leiomyomata]) and within the tumour itself.

### **4. Tissue Collection**

This study used the tissue obtained from a collection of 191 surgically resected uteri that had been collected by Prof. P. A. Richards at Groote Schuur Hospital, Cape Town, Republic of South Africa, which is presently housed at the Department of Anatomical Pathology, SAIMR, Johannesburg, Republic of South Africa (Richards, 1995). The collection represents women from all races with an age range of 13 years to 76 years of age.

The uterus once surgically removed was immediately sent to the pathology laboratory for expeditious tissue sampling. All the uteri sampled arrived in the laboratory within ten minutes of removal. In the laboratory the uteri were sectioned in the sagittal plane. Parallel transmural blocks of tissue were dissected from the fundus and lower segment from one half of each uterus (Fig. 5.1). The blocks were fixed in 10% buffered formalin overnight and routinely processed through alcohols and xylol to wax. When leiomyoma were present the 'normal' tissue was sampled and processed in the same

manner. The tumours were sampled and designated as being large ( $>3$  cm) or small ( $<3$  cm but  $> 3$  mm).

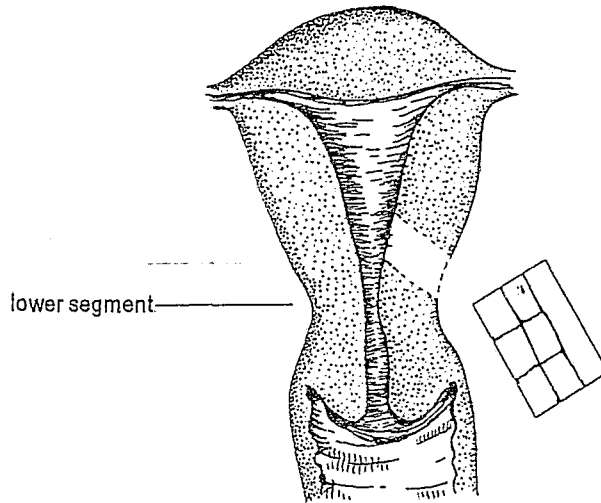


Figure 5.1: The coronal section of a uterus, indicating the position of the lower segment sample taken during the collection by Richards (1995).

The majority of hysterectomies were performed for menorrhagia, often in the absence of clinical anaemia with a small proportion being removed for other pathologies such as pelvic inflammatory disease. Over three quarters of the sample had leiomyomata present.

The tissue collection used represents a valuable resource for research into leiomyomata. Much of the previous work on this tissue has been undertaken using the tissue samples obtained from the fundal region. In order to conserve this material and to examine a region of the uterus, which is not as well researched, blocks of the lower uterine segment from 44 patients were sectioned for this study. The number of samples and the stage of the menstrual cycle are shown in table 5.1. Of these 45% had leiomyomata and either a small or large tumour was sectioned from these specimens. The age range for the patients in the sample population was from 13 years to 71 years

of age with a median of 43 years. Gravidity and parity for these patients ranged from 0 to 8 (median 2).

**Table 5.1:** Numbers of blocks sectioned from each stage of the menstrual cycle for this study.

|        | Menstrual | Proliferative | Secretory | Total |
|--------|-----------|---------------|-----------|-------|
| Number | 8         | 17            | 19        | 44    |

A section from each of the 44 lower uterine segment blocks used in the study was stained with haematoxylin and eosin prior to immunocytochemical staining. The sections that had endometrium were assessed as to phase of cycle and the normality of the tissue. Sections that did not have either the endometrium or serosa were not included in the study, as the phase of cycle and the three myometrial areas could not be determined. All sections cut for immunocytochemistry were picked up on adhesive slides to ensure that the sections did not come off during the immunocytochemical procedures.

Twenty three of the blocks were designated as normal tissue. These being myometria with no pathology affecting the muscle particularly an absence of leiomyomata. However, only 21 of the blocks had sections which contained both endometrium and serosa. Thus only these sections could be reliably used in the study. The age of these patients ranged between 13 and 60 years of age with a median of 40 years. The assessment of phase of the endometrial cycle and the number of patients in each phase of the cycle are shown in table 5.2. The specimens in the secretory group had the greatest age spread and were therefore used to ascertain if age had an effect on the staining of the myometrium.

Twenty one of the blocks were from myometria that contained one or more tumours and as such were designated as host myometria. However, 3 of these blocks when sectioned did not include the subendometrium and subserosa, so were not included in the study. The 18 host myometria used in the study were similar in age to the normal group, with a range between 20 and 54 with a median of 44 years. They were assessed

as to phase of the endometrial cycle and the number of patients examined in each phase of the cycle is shown in table 5.2.

**Table 5.2:** Phase of endometrial cycle and number of blocks in each phase examined for AC activity in the lower segment of normal and host myometrium.

|        | Proliferative | Secretory | Menstrual |
|--------|---------------|-----------|-----------|
| Normal | 7             | 10        | 4         |
| Host   | 6             | 8         | 4         |

Sections were cut from all 21 blocks of large or small tumours obtained from the 21 host uteri. The age of the patients ranged between 20 and 51 years of age with a median of 44 years. The endometrium of the tissue from which they were obtained was assessed as to phase of the endometrial cycle and the number of patients in each phase of the cycle (table 5.3). There were 9 small tumours (<3 cm but <3 mm diameter) and 12 large tumours (>3 cm diameter) spread throughout the cycle (table 5.3).

**Table 5.3:** Numbers of patients in each phase of the endometrial cycle from whom tumours were obtained including the ratio of large:small tumours in each group.

|                      | Proliferative | Secretory | Menstrual |
|----------------------|---------------|-----------|-----------|
| Number               | 8             | 9         | 4         |
| Ratio<br>Large:Small | 5:3           | 4:5       | 3:1       |

None of the patients selected for this study were known to have received exogenous hormone treatment prior to surgical removal of the uterus. Exogenous hormones used affect the natural cycle of the women using them and are sometimes used to reduce the size of leiomyomata prior to surgery (Azzopardi & Zayid, 1967). As a consequence it is highly likely that exogenous hormone treatment will affect the status of receptors and other normal cell activities in the uteri of those women using on such treatment.

## **5. Methods**

### **5.1 Immunocytochemistry**

A streptavidin- biotin peroxidase immunocytochemical method was used to stain the sections. The AC antibody was a polyclonal, raised in rabbit, against the AC isoform V's carboxy terminus, which is identical to the mouse/rat type VI carboxy terminus (Santa Cruz Biotech USA), here after referred to as the primary antibody. The secondary antibody was a horse anti rabbit which had been biotinylated to increase the sensitivity of the detection method (chapter 3). The reaction site was visualized using a streptavidin horseradish peroxidase-diaminobenzidine enhanced with nickel/cobalt.

Here, the method is described in brief with a fuller methodology given in appendix II and all the solutions used are provided in appendix I. The sections were dewaxed and following the quenching of endogenous peroxidase in hydrogen peroxide. Secondary antibody reactive sites on the sections were blocked with an incubation in normal horse serum for 30 minutes. The primary antibody was applied at a dilution of 2 µg/ml for one hour at room temperature (20-22°C). Following a wash in phosphate buffered saline the sections were incubated for 30 minutes in the biotinylated secondary at room temperature. The sections were washed again in phosphate buffered saline and incubated in the streptavidin peroxidase complex before the final incubation in a diaminobenzidine nickel/cobalt solution. The slides were then counter-stained in either haematoxylin or methyl green before being dehydrated and coverslipped to enhance the visualisation of the cell nuclei.

Both positive and negative controls were used to confirm the specificity of the reaction observed in the experimental sections. The positive control was an incubation of a section of known positive material (liver). The two negative controls used were 1) an incubation of the experimental tissue where the primary antibody was replaced by normal serum and 2) an incubation of the experimental tissue with pre-adsorbed primary antibody.

## **5.2 Data Collection**

For counting purposes the sections of myometrium, both normal and host, were subdivided into three equal regions: subendometrium, mid myometrium and subserosal. When counting the subendometrial region care was taken to avoid the transitional zone immediately below the endometrium. The total cell population of the 10 oil immersion fields ( $0.28 \text{ mm}^2$ ) was counted in each region per slide. As Richards *et al* (1994) have shown the total cell count per region of the myometrium varies in cellularity thus the percentage positive cells per region was used in the statistical analyses of the results. In the case of the tumour sections, ten random oil immersion fields per section were counted, irrespective of tumour size. A cell was deemed positive if any reaction product was visible on the cell regardless of the site within the cell (e.g. cytoplasmic or membrane).

To determine if there were differences in the location of the end product within the cell, the cellular staining pattern (cytoplasmic, membrane or both), of AC positive cells, one hundred cells from each area of each myometrial or tumour section were examined. The staining pattern was recorded for each cell and a percentage number of cells per area of myometrium or section of tumour was ascertained.

## **5.3 Statistical Analysis**

The counts (Appendix 3), either for positivity or staining pattern, were analysed using the following statistical tests to determine differences between areas within the myometrium, any changes through the cycle and if there were differences between normal, host and tumour tissue.

A Student's t-test, paired two sample for means, was used when the sample sizes were the same, such as between areas of myometrium. If the sample sizes were of unequal size, between phase of cycle and between host and normal, then a Student's t-test, two sample assuming unequal variance, was used. An analysis of variance was used to compare the differences in staining pattern within the myometrium and across the phases.

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## Chapter Six

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# **Adenylate Cyclase in Normal, Host and Leiomyomatous Tissue**

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### *Results*

#### **1 Myometrium - Normal**

Adenylate cyclase type V/VI was found in normal lower segment myometrium. The staining was observed in some but not all muscle fibres through out the bulk of the myometrium (Fig. 6.1). The cellular staining pattern was inconsistent, with some fibres showing a distinctive dark reaction product on the membranes while others stained only the cytoplasm or both the membrane and the cytoplasm (Fig 6.2). Mast cells and vascular smooth muscle cells were occasionally positive (Fig 6.3), as were nerves (not shown). The positive control sections of liver gave a similar result (Fig 6.4a) while the negative controls had no end product present in the cells (adsorbed and normal serum respectively) (Fig 6.4 b-c). These results confirmed that there was no non-specific staining of the myometrial cells.

The percentage positivity for each region of the myometrium through the endometrial cycle is shown in table 6.1 and figure 6.5. There was a highly significant increase in positivity ( $p < 0.001$ ) in the midmyometrial and subserosal portions of the myometrium in all phases of the cycle when compared to the subendometrial area (table 6.1). The subserosa had a significant decrease in positivity when compared to the midmyometrium ( $p < 0.05$ ) irrespective of the stage of the cycle.

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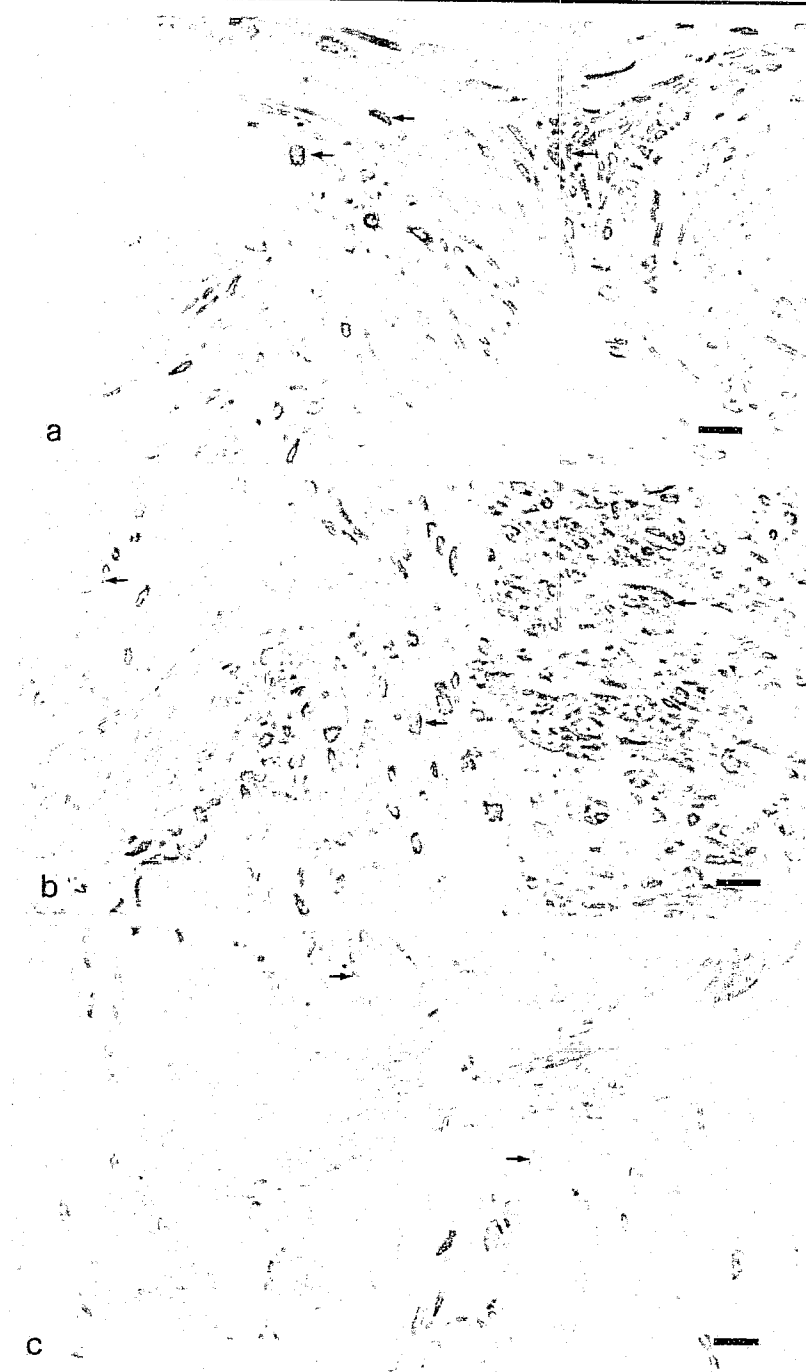


Figure 6.1: Light micrograph of AC positive staining cells in a section of normal myometrium a) subendometrium, b) midmyometrium and c) subserosa. Arrows indicate positive cells. Scale bar = 10  $\mu$ m.

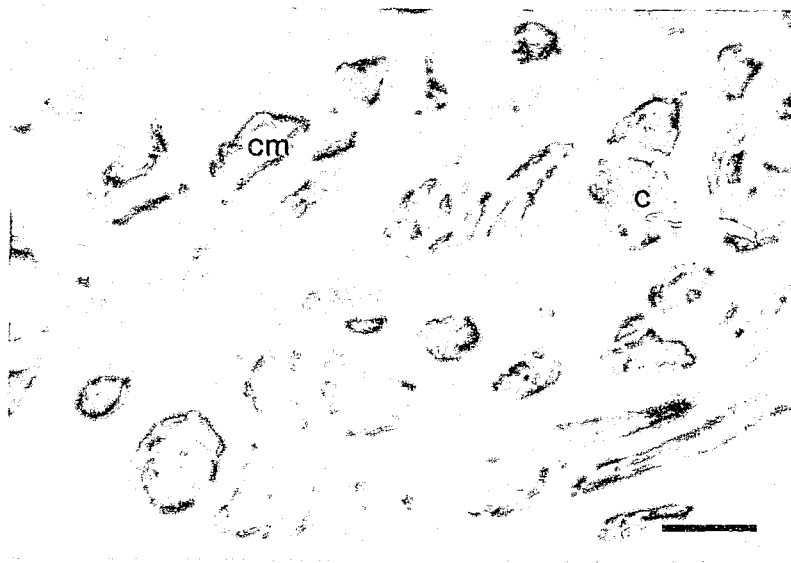


Figure 6.2: Light micrograph showing two of the different patterns of AC staining reaction in the cells of the myometrium. c - cytoplasm staining only cm - cytoplasm and membrane staining. Scale bar = 10  $\mu$ m.

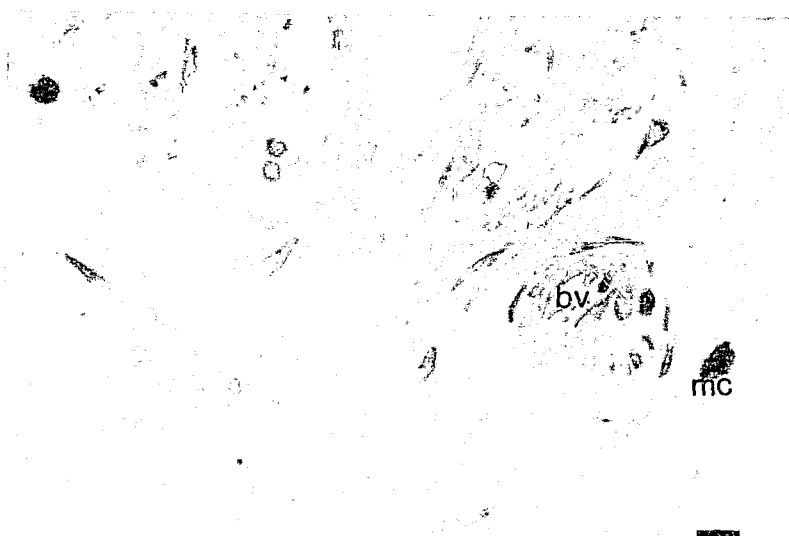


Figure 6.3: Light micrograph of an AC positive blood vessel (bv) and mast cell in the myometrium. Scale bar = 10  $\mu$ m.

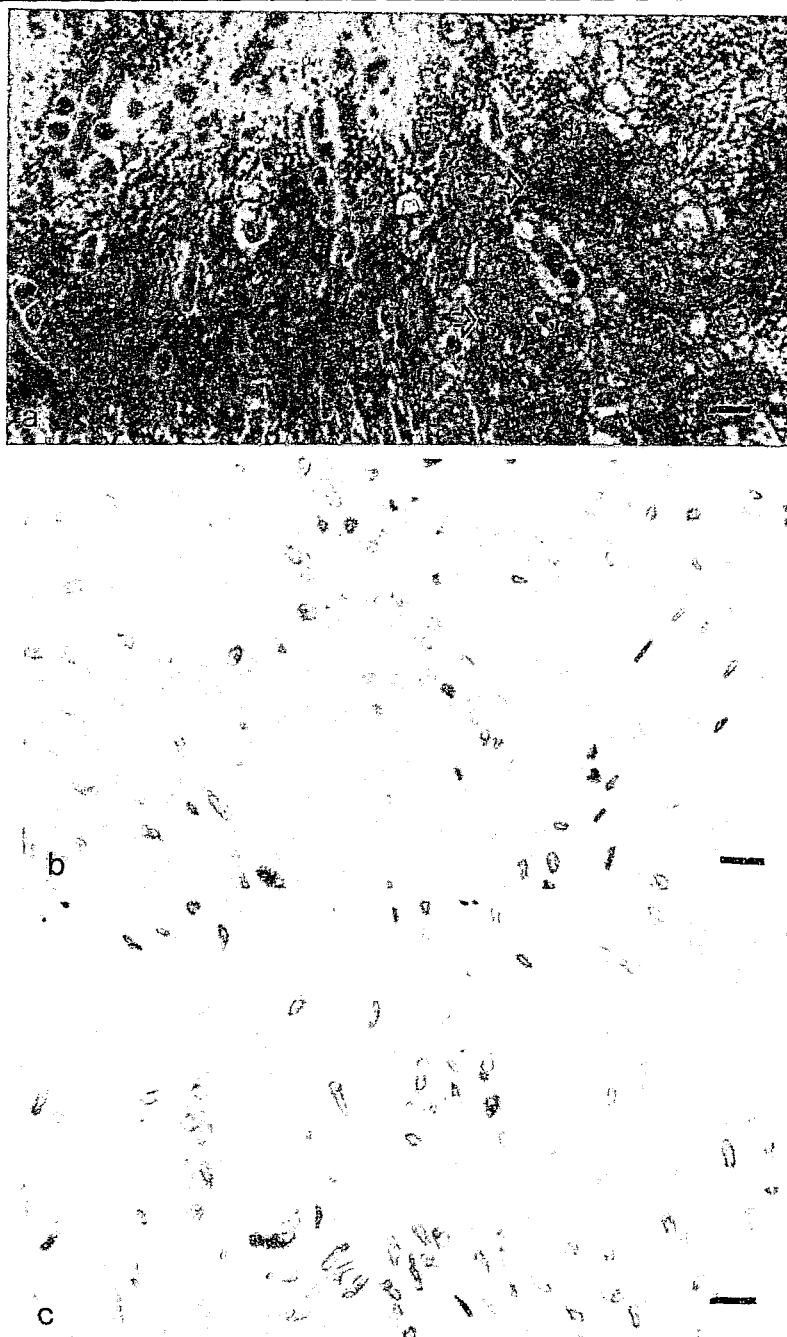


Figure 6.4: Light micrographs of control sections for AC staining. a) Liver, positive control tissue, b) adsorbed and c) normal serum negative controls, note the absence of positive reaction. Arrows indicate positive cells. Scale bar = 10  $\mu\text{m}$ .

**Table 6.1:** The average percentage positive cells counted per high power field (HPF) each region of normal myometrium in each stage of the endometrial cycle.

|                                | SE            |           | MM            |           | SS            |           |
|--------------------------------|---------------|-----------|---------------|-----------|---------------|-----------|
|                                | Mean<br>% +ve | Std. Dev. | Mean<br>% +ve | Std. Dev. | Mean<br>% +ve | Std. Dev. |
| <b>Menstrual</b><br>(n=40)     | 21.04         | 9.28      | 70.10         | 12.40     | 62.98         | 19.57     |
| <b>Proliferative</b><br>(n=70) | 24.52         | 8.57      | 80.52         | 10.20     | 73.93         | 15.35     |
| <b>Secretory</b><br>(n=100)    | 57.49         | 29.11     | 85.84         | 9.64      | 81.13         | 17.97     |

n = number of fields counted. SE - subendometrium, MM - midmyometrium, SS - subserosal

The subendometrial region showed no difference in positive staining between the menstrual and proliferative stages of the cycle (table 6.2) but the difference in positivity between the midmyometrium and subserosa regions of the myometrium in these two phases was significant (table 6.2).

**Table 6.2:** The p values for the Student's t-test comparisons between the menstrual and proliferative phase of the cycle.

|                  | Proliferative |        |       |
|------------------|---------------|--------|-------|
|                  | SE            | MM     | SS    |
| <b>Menstrual</b> | >0.1          | <0.001 | <0.05 |

SE - subendometrium, MM - midmyometrium, SS - subserosal.

The percentage positivity in all regions of the myometrium in the secretory phase was significantly higher than the menstrual and proliferative phases (table 6.3).

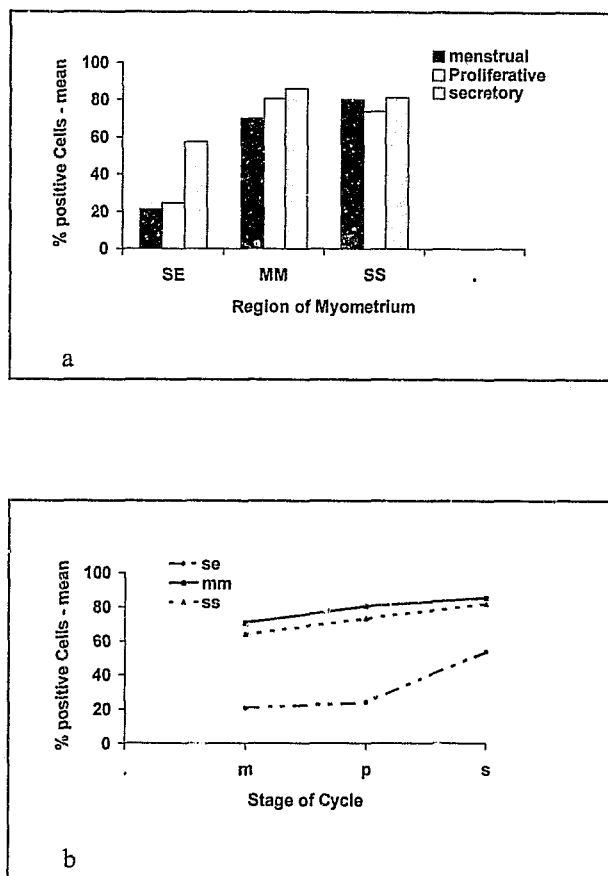


Figure 6.5: The percentage positive cells in normal myometrium a) across the myometrium and b) across the endometrial cycle. SE - subendometrium, MM - midmyometrium, SS - subserosal, m -menstrual, p -proliferative, s - secretory.

**Table 6.3:** The p values for the Student's t-test comparisons between the secretory and the other phases of the endometrial cycle.

|           | Menstrual |        |        | Proliferative |       |       |
|-----------|-----------|--------|--------|---------------|-------|-------|
|           | SE        | MM     | SS     | SE            | MM    | SS    |
| Secretory | <0.001    | <0.001 | <0.001 | <0.001        | <0.01 | <0.05 |

SE - subendometrium, MM - midmyometrium, SS - subserosal.

The reaction product could be localized in one of three combinations, a) cytoplasmic, b) cytoplasm and membrane or c) membrane only. Figure 6.6 a-c shows the variation of staining location within each region of the myometrium and how it varies during the cycle. The percentage number of cells for each of the three staining patterns in each region of the myometrium was compared across the cycle. For example, the subendometrium of the menstrual phase was compared to the subendometrium of the proliferative phase and the subendometrium of the secretory phase and so forth for each region, phase and staining pattern.

The cytoplasmic staining throughout the cycle is shown in table 6.4. The significance of these differences between the phases of the cycle and inter region of the myometrium are given in table 6.5.

**Table 6.4:** The average percentage cytoplasmic positive cells in each region of normal myometrium in each stage of the endometrial cycle.

|                     | SE            |           | MM            |           | SS            |           |
|---------------------|---------------|-----------|---------------|-----------|---------------|-----------|
|                     | Mean<br>% +ve | Std. Dev. | Mean<br>% +ve | Std. Dev. | Mean<br>% +ve | Std. Dev. |
| Menstrual (n=4)     | 28.00         | 14.93     | 19.33         | 13.05     | 43.67         | 16.65     |
| Proliferative (n=7) | 28.57         | 4.5       | 26.14         | 10.90     | 52.43         | 17.75     |
| Secretory (n=10)    | 41.27         | 11.44     | 42.36         | 7.04      | 49.64         | 9.2       |

n = number of slides counted. SE - subendometrium, MM - midmyometrium, SS - subserosal

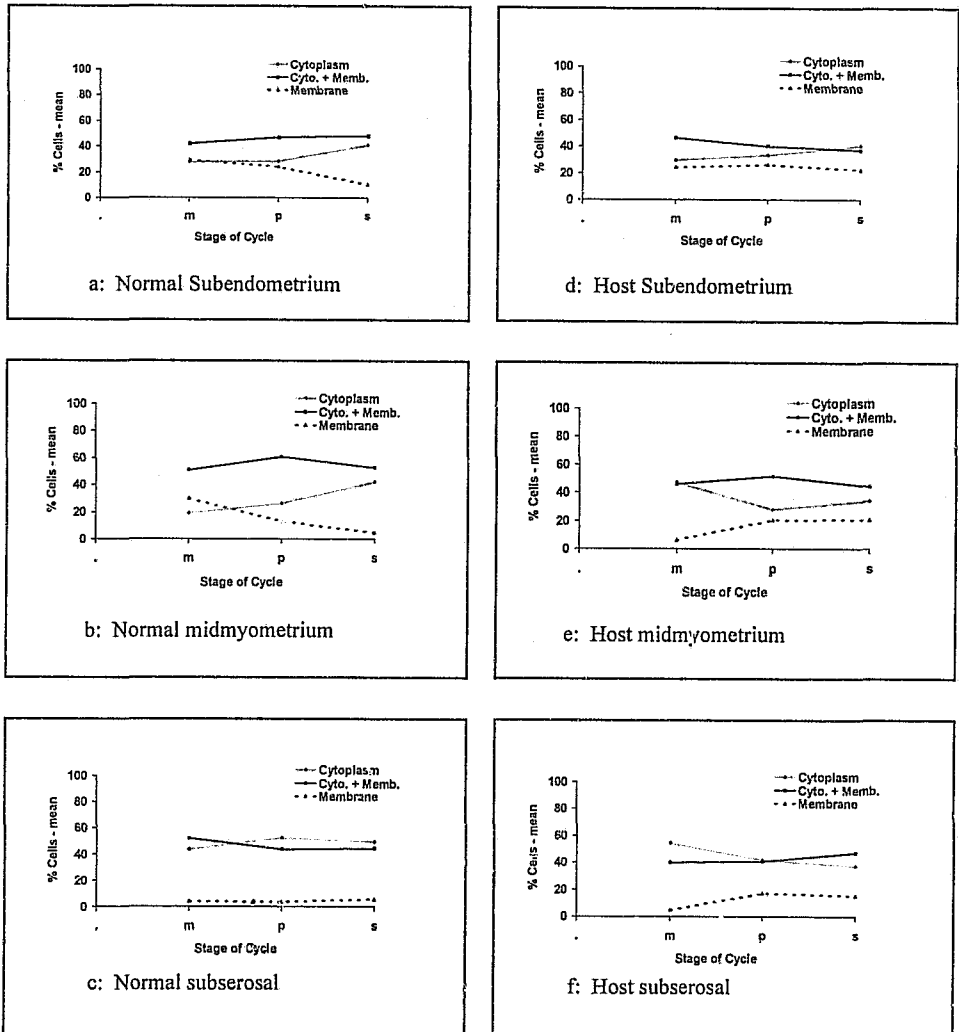


Figure 6.6: Patterns of staining across the wall of the myometrium in normal and host tissue. a-c) Normal myometrium. d-f) host myometrium. m - menstrual, p - proliferative, s - secretory.

**Table 6.5:** The p values for the Student's t-test comparisons in cytoplasmic staining in normal myometrium between the phases of the endometrial cycle.

|                      | Menstrual |       |      | Proliferative |       |      |
|----------------------|-----------|-------|------|---------------|-------|------|
|                      | SE        | MM    | SS   | SE            | MM    | SS   |
| <b>Proliferative</b> | >0.1      | >0.1  | >0.1 |               |       |      |
| <b>Secretory</b>     | >0.05     | <0.05 | >0.1 | <0.005        | <0.01 | >0.1 |

SE - subendometrium, MM - midmyometrium, SS - subserosal.

There are no significant differences across the myometrium or with phase of cycle in those cells that had the combined staining (Fig. 6.6 a-c; table 6.6).

**Table 6.6:** The average percentage cytoplasmic and membrane positive cells in each region of normal myometrium in each stage of the endometrial cycle.

|                            | SE            |           | MM            |           | SS            |           |
|----------------------------|---------------|-----------|---------------|-----------|---------------|-----------|
|                            | Mean<br>% +ve | Std. Dev. | Mean<br>% +ve | Std. Dev. | Mean<br>% +ve | Std. Dev. |
| <b>Menstrual (n=4)</b>     | 39.25         | 10.94     | 48.75         | 11.53     | 51.25         | 8.42      |
| <b>Proliferative (n=7)</b> | 47.14         | 13.50     | 60.71         | 5.12      | 43.57         | 16.13     |
| <b>Secretory (n=10)</b>    | 48.36         | 8.14      | 52.82         | 7.50      | 44.55         | 5.91      |

n = number of slides counted. SE - subendometrium, MM - midmyometrium, SS - subserosal

In the normal myometrium the number of membrane only staining cells falls as the endometrial cycle progresses towards the secretory phase in both the subendometrial and midmyometrial areas (Fig 6.6 a-b; table 6.7). There are significantly fewer membrane only staining cells in the secretory phase than the menstrual phase in these two regions of the myometrium (table 6.8). In the subserosal region membrane only staining cells are low in number throughout the cycle with no significant difference between the different phases for each staining pattern (Fig 6.6 c; table 6.8).

**Table 6.7:** The average percentage cytoplasmic and membrane positive cells in each region of normal myometrium in each stage of the endometrial cycle.

|                     | SE            |           | MM            |           | SS            |           |
|---------------------|---------------|-----------|---------------|-----------|---------------|-----------|
|                     | Mean<br>% +ve | Std. Dev. | Mean<br>% +ve | Std. Dev. | Mean<br>% +ve | Std. Dev. |
| Menstrual (n=4)     | 32.75         | 18.10     | 30.00         | 14.97     | 5.25          | 5.74      |
| Proliferative (n=7) | 24.29         | 11.46     | 13.14         | 6.06      | 4.00          | 3.77      |
| Secretory (n=10)    | 10.36         | 6.68      | 4.82          | 1.83      | 5.91          | 3.20      |

n = number of slides counted. SE - subendometrium, MM - midmyometrium, SS - subserosal

**Table 6.8:** The p values for the Student's t-test comparisons in membrane only staining between the phases of the endometrial cycle.

|               | Menstrual |       |      | Proliferative |        |      |
|---------------|-----------|-------|------|---------------|--------|------|
|               | SE        | MM    | SS   | SE            | MM     | SS   |
| Proliferative | >0.1      | >0.05 | >0.1 |               |        |      |
| Secretory     | >0.1      | <0.05 | >0.1 | <0.05         | <0.005 | >0.1 |

SE - subendometrium, MM - midmyometrium, SS - subserosal.

Within the normal secretory group there was an age range from less than 20 years of age to over 50 years of age. Though the numbers of specimens per group was small, each region of the myometrium, within each represented age group, was analysed to determine if age made a difference in the localization of AC in this tissue (table 6.9). The subendometrial area in each age group varied significantly with the subendometrium of the under 20 year age group ( $p < 0.005$ ) while all other areas were not significantly different from each other. There was no significant difference in any region of the myometrium of the 20-29 year age group when compared to the >50 year age group. There was a significant difference between the 20-29 year age group and all other groups in all regions of the myometrium ( $p < 0.005$ ). The 30-39 year age group and the 40-49 year age groups did not differ significantly in the subendometrial region. The 40-49 year age group had slightly more staining than the 30-39 year age group in the midmyometrium region ( $p < 0.05$ ). These two groups were significantly

different from the over 50 year age group in the subendometrium ( $p < 0.001$ ) but not in the other two regions.

**Table 6.9:** The mean and standard deviation for each age group across the myometrium in the secretory phase.

| Age Group    | SE    |          | MM    |          | SS    |          |
|--------------|-------|----------|-------|----------|-------|----------|
|              | Mean  | Std. Dev | Mean  | Std. Dev | Mean  | Std. Dev |
| <20 (n=20)   | 81.60 | 10.83    | 88.20 | 7.39     | 81.44 | 17.40    |
| 20-29 (n=10) | 26.84 | 13.16    | 79.26 | 14.64    | 88.63 | 8.30     |
| 30-39 (n=20) | 60.02 | 28.07    | 89.40 | 6.54     | 80.19 | 18.10    |
| 40-49 (n=40) | 59.78 | 28.91    | 84.60 | 10.33    | 79.24 | 20.21    |
| >50 (n=10)   | 25.59 | 6.21     | 85.57 | 7.00     | 82.45 | 19.26    |

SE - subendometrium, MM - midmyometrium, SS - subserosal. n = number of fields counted.

## 2 Myometrium - Host

The appearance of the AC V/VI staining was similar in the host myometria as was observed in the normal myometria and is shown in figure 6.7.

The percentage positive cells per region of myometrium (table 6.10) followed a similar trend to that seen in the normal myometrium (Fig 6.8). As with the normal myometrium there was a highly significant increase in positivity in midmyometrium and subserosal areas when compared to the subendometrium ( $p < 0.001$ ) across the cycle. The midmyometrium and subserosal was not significantly different in the menstrual and proliferative phases of the cycle but were significantly lower in the secretory phase ( $p < 0.05$ ).

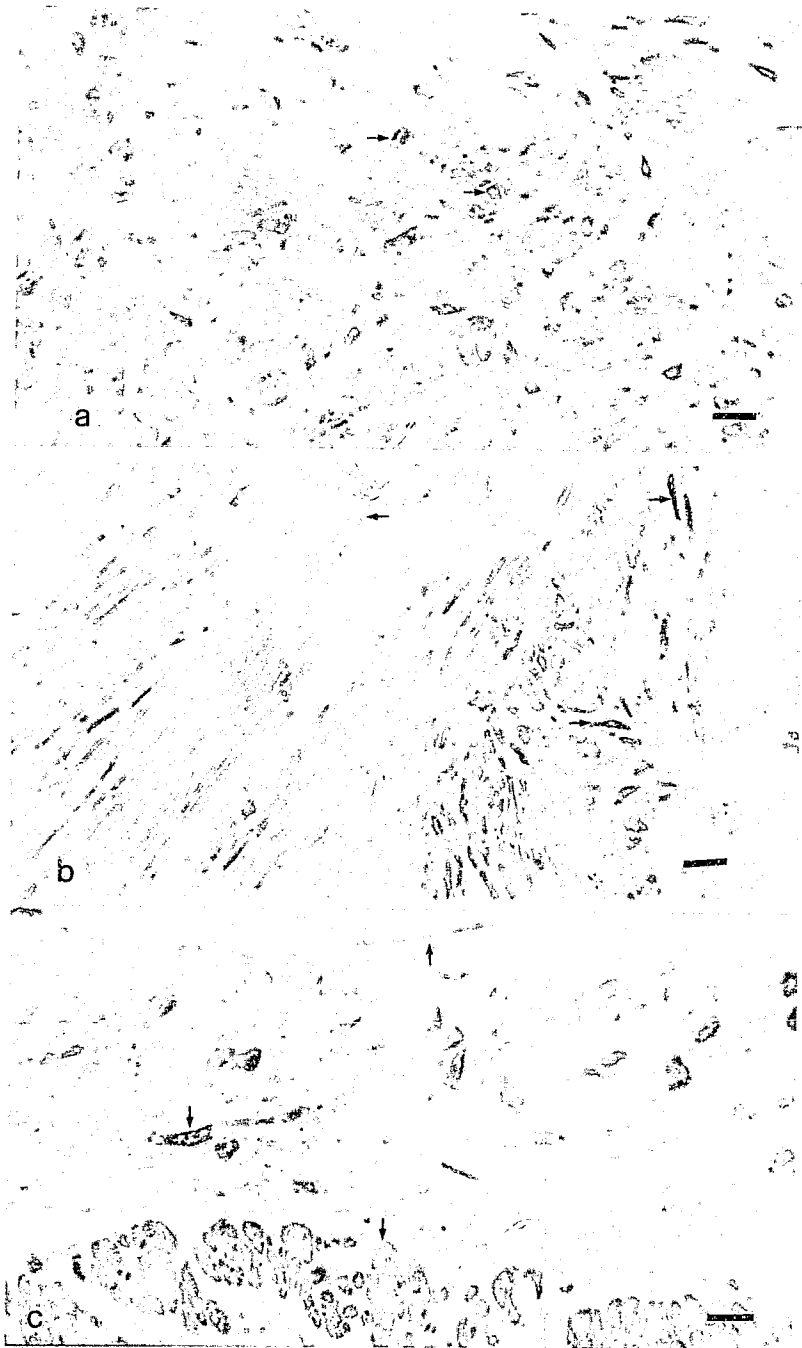


Figure 6.7: Light micrographs of AC positive cells in a section of host myometrium. a) subendometrium, b) midmyometrium and c) subserosa. Arrows denote positive cells. Scale bar = 10  $\mu\text{m}$ .

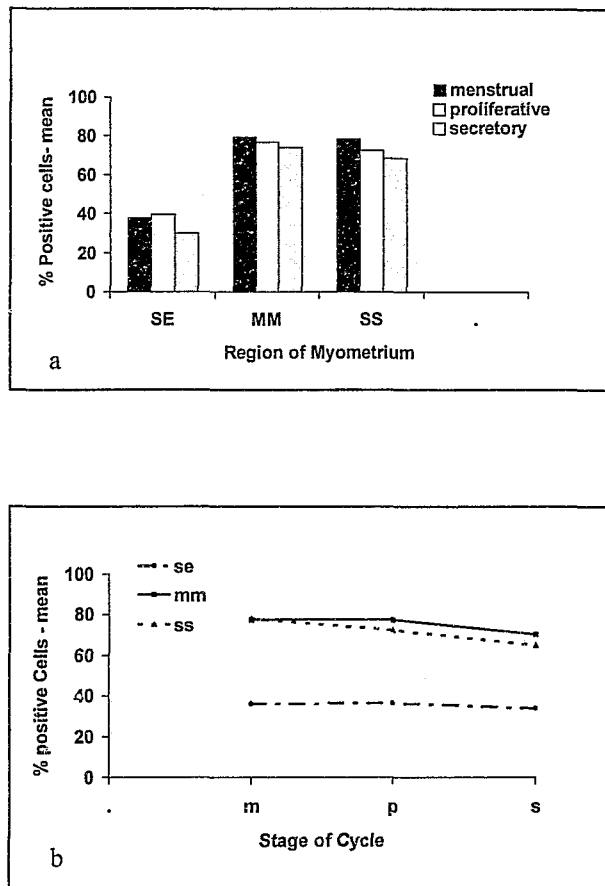


Figure 6.8: The percentage positive cells in host myometrium a) across the myometrium and b) across the endometrial cycle. SE - subendometrium, MM - midmyometrium, SS - subserosal, m - menstrual, p - proliferative, s - secretory.

**Table 6.10:** The average percentage of positive cells counted per high power field (HPF) each region of host myometrium in each stage of the endometrial cycle.

|                      | SE            |           | MM            |           | SS            |           |
|----------------------|---------------|-----------|---------------|-----------|---------------|-----------|
|                      | Mean<br>% +ve | Std. Dev. | Mean<br>% +ve | Std. Dev. | Mean<br>% +ve | Std. Dev. |
| Menstrual (n=40)     | 37.96         | 18.66     | 79.36         | 7.17      | 78.62         | 21.07     |
| Proliferative (n=60) | 36.39         | 19.72     | 76.80         | 13.70     | 72.51         | 16.18     |
| Secretory (n=80)     | 30.02         | 10.99     | 74.21         | 10.74     | 68.60         | 15.77     |

n = number of fields counted. SE - subendometrium, MM - midmyometrium, SS - subserosal

There was no significant difference between the menstrual and proliferative phases across the myometrium (table 6.11).

**Table 6.11:** The p values for the Student's t-test comparisons between the menstrual and proliferative phase of the cycle in the host myometrium.

|           | Proliferative |      |      |
|-----------|---------------|------|------|
|           | SE            | MM   | SS   |
| Menstrual | >0.1          | >0.1 | >0.1 |

SE - subendometrium, MM - midmyometrium, SS - subserosal.

The secretory phase was also not significant in the subendometrial region in comparison to the other two phases of the cycle (table 6.12). However, there was a significant decrease in staining in the midmyometrial region when compared to the menstrual and proliferative phase of the cycle (table 6.12). This decrease in staining was also observed in the subserosal region (table 6.12).

**Table 6.12:** The p values for the Student's t-test comparisons between the secretory and the other phases of the endometrial cycle in the host myometrium.

|           | Menstrual |        |       | Proliferative |       |       |
|-----------|-----------|--------|-------|---------------|-------|-------|
|           | SE        | MM     | SS    | SE            | MM    | SS    |
| Secretory | >0.1      | <0.001 | <0.01 | >0.1          | <0.01 | <0.05 |

SE - subendometrium, MM - midmyometrium, SS - subserosal.

The differences in the location of the stain (cytoplasm; cytoplasm and membrane; membrane) across the cycle and in each region of the myometrium are shown in figure 6.6d-f. There was a progressive increase in cytoplasmic staining from the subendometrial region to the subserosal region of the menstrual phase (table 6.13) and the difference between the subendometrial region and the other two regions was significant ( $p < 0.001$ ). The midmyometrial region of the menstrual phase was significantly different from the same region in the proliferative phase (table 6.14). The subserosal region of the menstrual phase was significantly different from the same region in the other two phases (table 6.14). The myometrium of the proliferative and secretory phases showed no differences between them (tables 6.13-14) and the staining dropped in the midmyometrial region before rising again in the subserosal region (table 6.13).

**Table 6.13:** The average percentage cytoplasmic positive cells in each region of host myometrium in each stage of the endometrial cycle.

|                     | SE            |           | MM            |           | SS            |           |
|---------------------|---------------|-----------|---------------|-----------|---------------|-----------|
|                     | Mean<br>% +ve | Std. Dev. | Mean<br>% +ve | Std. Dev. | Mean<br>% +ve | Std. Dev. |
| Menstrual (n=4)     | 29.50         | 12.97     | 47.50         | 10.79     | 54.50         | 5.80      |
| Proliferative (n=6) | 33.50         | 12.11     | 28.00         | 8.15      | 42.00         | 7.35      |
| Secretory (n=10)    | 40.38         | 22.40     | 34.63         | 14.57     | 37.63         | 10.34     |

n = number of slides counted. SE - subendometrium, MM - midmyometrium, SS - subserosal

**Table 6.14:** The p values for the Student's t-test comparisons in cytoplasmic staining in host myometrium between the phases of the endometrial cycle.

|               | Menstrual |       |       | Proliferative |      |      |
|---------------|-----------|-------|-------|---------------|------|------|
|               | SE        | MM    | SS    | SE            | MM   | SS   |
| Proliferative | >0.1      | <0.05 | <0.05 |               |      |      |
| Secretory     | >0.1      | >0.1  | <0.05 | >0.1          | >0.1 | >0.1 |

SE - subendometrium, MM - midmyometrium, SS - subserosal.

The cells with combined staining were not significantly different throughout the cycle in the host (table 6.15).

**Table 6.15:** The average percentage cytoplasmic and membrane positive cells in each region of host myometrium in each stage of the endometrial cycle.

|                     | SE            |           | MM            |           | SS            |           |
|---------------------|---------------|-----------|---------------|-----------|---------------|-----------|
|                     | Mean<br>% +ve | Std. Dev. | Mean<br>% +ve | Std. Dev. | Mean<br>% +ve | Std. Dev. |
| Menstrual (n=4)     | 46.00         | 12.73     | 46.25         | 8.73      | 40.25         | 7.63      |
| Proliferative (n=7) | 40.17         | 12.35     | 51.50         | 12.03     | 40.83         | 7.86      |
| Secretory (n=10)    | 37.00         | 13.82     | 44.50         | 9.50      | 47.13         | 4.11      |

n = number of slides counted. SE - subendometrium, MM - midmyometrium, SS - subserosal

The average percentage membrane only staining cells in each region of the host myometrium in each stage of the endometrial cycle is shown in table 6.17-18. The only significant differences in this staining group was between the menstrual and proliferative cycle midmyometrium and subserosal regions (table 6.18).

**Table 6.17:** The average percentage membrane only positive cells in each region of host myometrium in each stage of the endometrial cycle.

|                     | SE            |           | MM            |           | SS            |           |
|---------------------|---------------|-----------|---------------|-----------|---------------|-----------|
|                     | Mean<br>% +ve | Std. Dev. | Mean<br>% +ve | Std. Dev. | Mean<br>% +ve | Std. Dev. |
| Menstrual (n=4)     | 24.50         | 19.47     | 6.25          | 6.50      | 4.75          | 4.03      |
| Proliferative (n=7) | 26.33         | 19.16     | 20.50         | 12.11     | 17.17         | 7.25      |
| Secretory (n=10)    | 22.63         | 8.91      | 20.88         | 5.48      | 15.25         | 7.63      |

n = number of slides counted. SE - subendometrium, MM - midmyometrium, SS - subserosal

**Table 6.18:** The p values for the Student's t-test comparisons in cytoplasmic staining in host myometrium between the phases of the endometrial cycle.

|               | Menstrual |       |       | Proliferative |      |      |
|---------------|-----------|-------|-------|---------------|------|------|
|               | SE        | MM    | SS    | SE            | MM   | SS   |
| Proliferative | >0.1      | <0.05 | <0.05 |               |      |      |
| Secretory     | >0.1      | >0.1  | >0.1  | >0.1          | >0.1 | >0.1 |

SE - subendometrium, MM - midmyometrium, SS - subserosal.

## 2.1 Normal : Host

The percentage staining in the host myometrium was significantly decreased when compared to the normal myometrium in all regions of the menstrual and secretory phases (table 6.19). In the proliferative phase there was no significant difference between the midmyometrium and subserosal regions but staining was significantly decreased in the subendometrium of the host myometrium (Fig. 6.9).

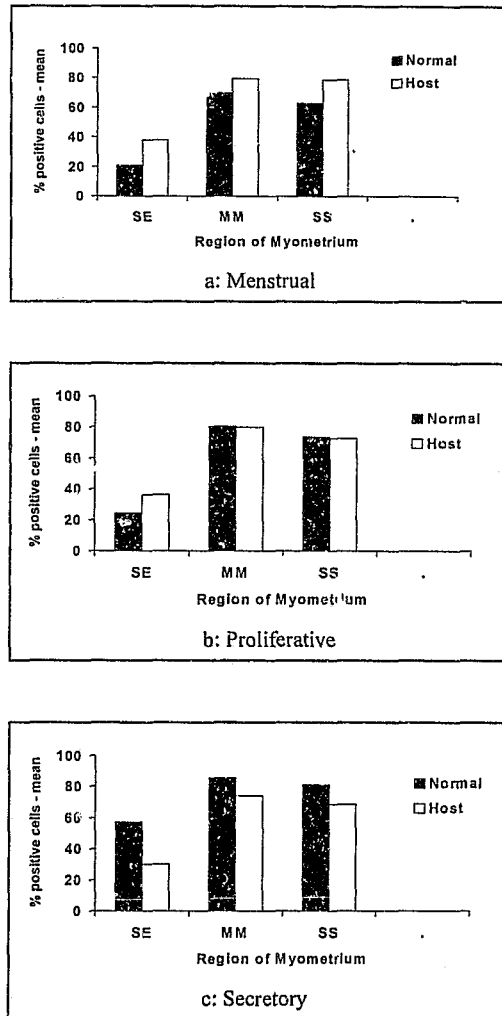


Figure 6.9: Comparison of AC staining in normal and host myometrium. a) menstrual, b) proliferative and c) secretory phases of the endometrial cycle. SE - subendometrium, MM - midmyometrium, SS - subserosal

**Table 6.19:** The p values for the Student's t-test comparing the different regions of the myometrium across the endometrial cycle between host and normal.

|               | Host Menstrual |       |       | Host Proliferative |      |      | Host Secretory |        |        |
|---------------|----------------|-------|-------|--------------------|------|------|----------------|--------|--------|
|               | SE             | MM    | SS    | SE                 | MM   | SS   | SE             | MM     | SS     |
| Normal        |                |       |       |                    |      |      |                |        |        |
| Menstrual     | <0.001         | <0.01 | <0.01 |                    |      |      |                |        |        |
| Proliferative |                |       |       | <0.001             | >0.1 | >0.1 |                |        |        |
| Secretory     |                |       |       |                    |      |      | <0.001         | <0.001 | <0.001 |

SE - subendometrium, MM - midmyometrium, SS - subserosal.

The comparison in the cytoplasmic staining pattern between normal and host myometrium is shown in figure 6.10. There are significant differences between the two groups in all phases and regions of the myometrium except the subendometrial region of the menstrual and proliferative phases (table 6.20). In the normal tissue there was a progressive increase in the number of cytoplasmic positive cells from the subendometrium to the subserosal region. In the proliferative and secretory phase of the host myometrium there is a drop in the percentage cells in the midmyometrial region.

**Table 6.20:** The p values for the Student's t-test comparing the cytoplasmic staining in the different regions of the myometrium across the endometrial cycle between host and normal.

|               | Host Menstrual |        |        | Host Proliferative |        |        | Host Secretory |        |        |
|---------------|----------------|--------|--------|--------------------|--------|--------|----------------|--------|--------|
|               | SE             | MM     | SS     | SE                 | MM     | SS     | SE             | MM     | SS     |
| Normal        |                |        |        |                    |        |        |                |        |        |
| Menstrual     | >0.1           | <0.001 | <0.001 |                    |        |        |                |        |        |
| Proliferative |                |        |        | >0.1               | <0.001 | <0.001 |                |        |        |
| Secretory     |                |        |        |                    |        |        | <0.005         | <0.005 | <0.005 |

SE - subendometrium, MM - midmyometrium, SS - subserosal.

There was no difference between normal and host myometria when the cytoplasmic and membrane staining pattern was compared.

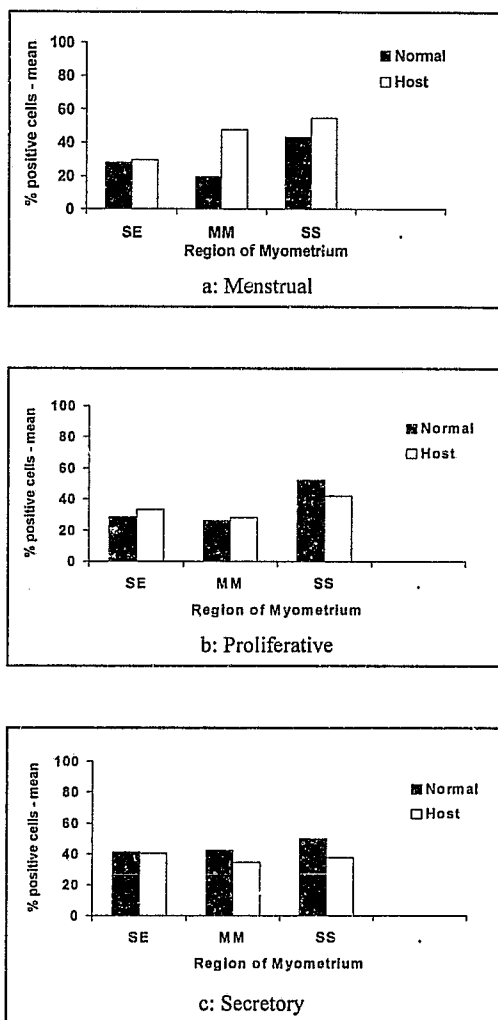


Figure 6.10: Comparison of AC cytoplasmic staining in normal and host myometrium. a) menstrual, b) proliferative and c) secretory phases of the endometrial cycle. SE - subendometrium, MM - midmyometrium, SS - subserosal

Figure 6.11 shows the pattern of membrane only staining in the normal tissue compared to host. In the menstrual phase the membrane only staining cells were significantly lowered in the midmyometrial and subserosal regions (table 6.21) of the host tissue (Fig 6.11a). The proliferative phase had a significantly higher percentage of membrane staining cells in the midmyometrial and subserosal regions (table 6.21) of the host myometrium but the subendometrial region of the host was not different from normal (Fig 6.11b). In the secretory phase the host myometrium was significantly increased above normal in all regions of the myometrium (table 6.21; Fig. 6.11c).

**Table 6.21:** The p values for the Student's t-test comparing the membrane staining in the different regions of the myometrium across the endometrial cycle between host and normal.

| Normal        | Host Menstrual |        |        | Host Proliferative |        |        | Host Secretory |        |        |
|---------------|----------------|--------|--------|--------------------|--------|--------|----------------|--------|--------|
|               | SE             | MM     | SS     | SE                 | MM     | SS     | SE             | MM     | SS     |
| Menstrual     | >0.1           | <0.001 | <0.001 |                    |        |        |                |        |        |
| Proliferative |                |        |        | >0.1               | <0.001 | <0.001 |                |        |        |
| Secretory     |                |        |        |                    |        |        | <0.005         | <0.005 | <0.005 |

SE - subendometrium, MM - midmyometrium, SS - subserosal.

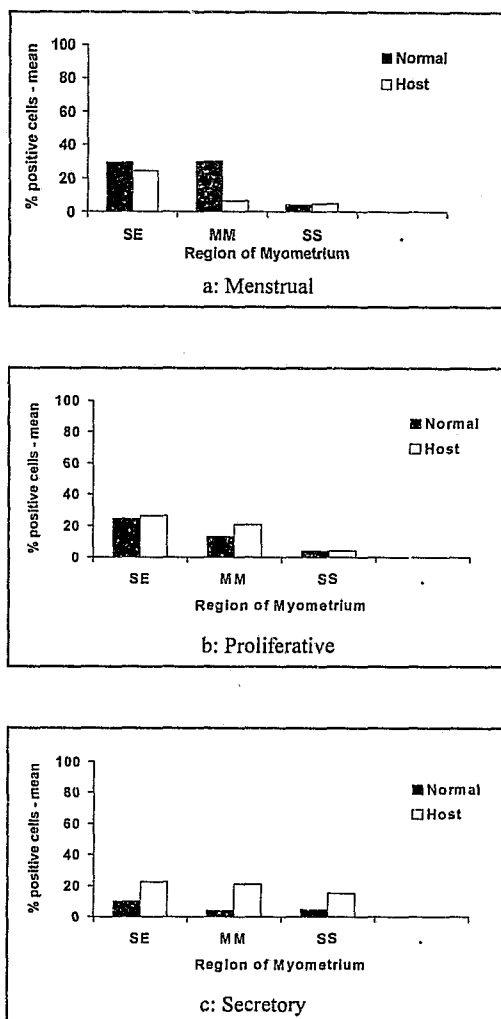


Figure 6.11: Comparison of AC membrane staining in normal and host myometrium. a) menstrual, b) proliferative and c) secretory phases of the endometrial cycle. SE - subendometrium, MM - midmyometrium, SS - subserosal

### 3 Leiomyomata

Adenylate cyclase type V/VI was found in all tumours with a similar staining pattern to that seen within the myometrium (Fig 6.12). The staining of the muscle cells fell into the same three categories as previously detailed; cytoplasm, cytoplasm and membrane and membrane only cells. The size of the tumour did not affect the percentage of positive cells in the tumour sections. The tumours' level of AC staining varied when compared to the myometrium of the host in the corresponding phase of cycle (Table 6.22-23; Fig 6.13).

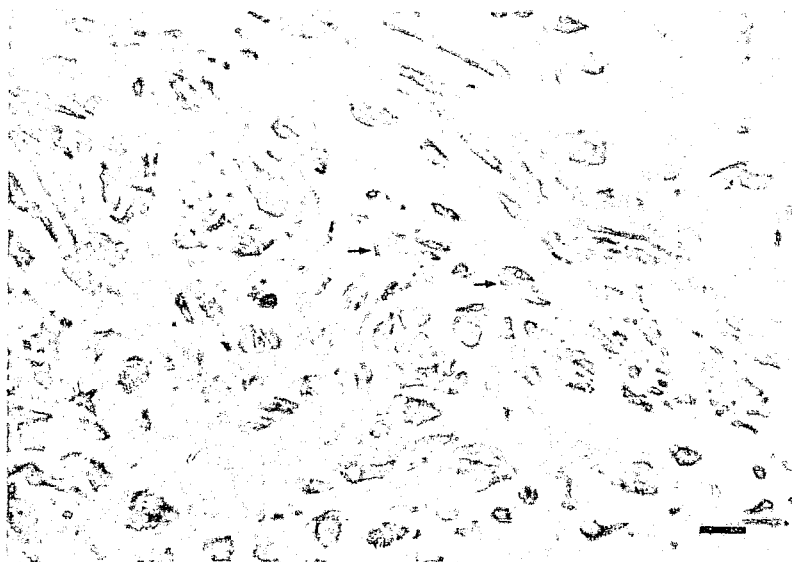


Figure 6.12: Light micrograph of AC positive cells in a section of leiomyomata. Arrows indicate positive cells. Scale bar = 10  $\mu$ m.

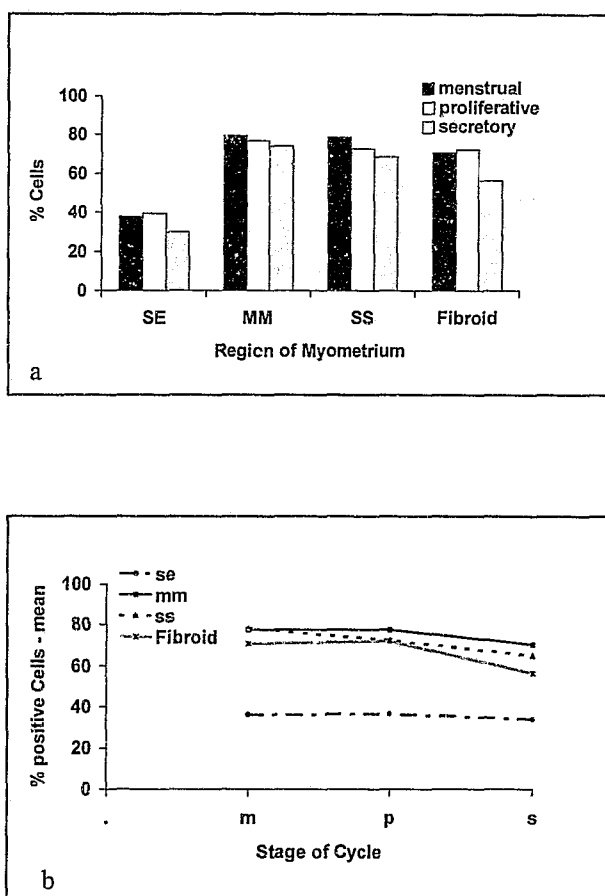


Figure 6.13: The percentage positive cells in the leiomyomata compared to host a) across the myometrium and b) across the endometrial cycle. SE - subendometrium, MM - midmyometrium, SS - subserosal, m - menstrual, p - proliferative, s - secretory.

**Table 6.22:** The average percentage of positive cells counted per high power field (HPF) of the leiomyomata in each stage of the endometrial cycle.

|                      | Mean<br>% +ve | Std. Dev. |
|----------------------|---------------|-----------|
| Menstrual (n=40)     | 70.78         | 19.45     |
| Proliferative (n=80) | 72.31         | 19.82     |
| Secretory (n=90)     | 56.66         | 16.73     |

n = number of fields counted.

The tumour had significantly higher levels of staining when compared to the subendometrial region of the myometrium irrespective of stage of cycle (table 6.23). The leiomyomata had significantly less staining than the host midmyometrium in both the menstrual and secretory phases of the cycle but was not significantly different to the proliferative phase in this region (table 6.23). There was also less staining for AC in the subserosal region of the secretory phase (table 6.23). There was no difference between the tumour and the subserosal region during the other phases of the endometrial cycle (table 6.23).

**Table 6.23:** The p-values for the comparison between the tumours from each phase of the cycle and areas of the host myometrium from the corresponding phase of the cycle.

|        | Host Menstrual |       |      | Host Proliferative |      |      | Host Secretory |        |       |
|--------|----------------|-------|------|--------------------|------|------|----------------|--------|-------|
|        | SE             | MM    | SS   | SE                 | MM   | SS   | SE             | MM     | SS    |
| Tumour | <0.001         | <0.05 | >0.1 | <0.001             | >0.1 | >0.1 | <0.001         | <0.001 | <0.01 |

SE - subendometrium, MM - midmyometrium, SS - subserosal.

The cytoplasmic staining of the cells was significantly less in the tumour tissue when compared to the host irrespective of the region of the myometrium or the phase of the endometrial cycle ( $p < 0.001$ ) (table 6.24). There was no significant difference between the tumour tissue and host tissue with regards the number of cells with both cytoplasmic and membrane staining again irrespective of myometrial region and phase of cycle. The membrane only staining cells in the tumour tissue was significantly

**Table 6.22:** The average percentage of positive cells counted per high power field (HPF) of the leiomyomata in each stage of the endometrial cycle.

|                      | Mean<br>% +ve | Std. Dev. |
|----------------------|---------------|-----------|
| Menstrual (n=40)     | 70.78         | 19.45     |
| Proliferative (n=80) | 72.31         | 19.82     |
| Secretory (n=90)     | 56.66         | 16.73     |

n = number of fields counted.

The tumour had significantly higher levels of staining when compared to the subendometrial region of the myometrium irrespective of stage of cycle (table 6.23). The leiomyomata had significantly less staining than the host midmyometrium in both the menstrual and secretory phases of the cycle but was not significantly different to the proliferative phase in this region (table 6.23). There was also less staining for AC in the subserosal region of the secretory phase (table 6.23). There was no difference between the tumour and the subserosal region during the other phases of the endometrial cycle (table 6.23).

**Table 6.23:** The p-values for the comparison between the tumours from each phase of the cycle and areas of the host myometrium from the corresponding phase of the cycle.

|        | Host Menstrual |       |      | Host Proliferative |      |      | Host Secretory |        |       |
|--------|----------------|-------|------|--------------------|------|------|----------------|--------|-------|
|        | SE             | MM    | SS   | SE                 | MM   | SS   | SE             | MM     | SS    |
| Tumour | <0.001         | <0.05 | >0.1 | <0.001             | >0.1 | >0.1 | <0.001         | <0.001 | <0.01 |

SE - subendometrium, MM - midmyometrium, SS - subserosal.

The cytoplasmic staining of the cells was significantly less in the tumour tissue when compared to the host irrespective of the region of the myometrium or the phase of the endometrial cycle ( $p < 0.001$ ) (table 6.24). There was no significant difference between the tumour tissue and host tissue with regards the number of cells with both cytoplasmic and membrane staining again irrespective of myometrial region and phase of cycle. The membrane only staining cells in the tumour tissue was significantly

increased over the host tissue throughout the cycle and the myometrium ( $p < 0.001$ ) (table 6.24).

**Table 6.24:** Mean percentage number of cells in the host and leiomyomata tissue that had cytoplasmic, cytoplasmic and membrane and membrane only staining.

|        | Cytoplasmic |          | Cytoplasmic +<br>Membrane |          | Membrane |          |
|--------|-------------|----------|---------------------------|----------|----------|----------|
|        | Mean %      | Std. Dev | Mean %                    | Std. Dev | Mean %   | Std. Dev |
| Host   | 37.93       | 14.04    | 43.59                     | 11.53    | 18.44    | 12.25    |
| Tumour | 15.10       | 12.22    | 44.95                     | 16.64    | 39.90    | 19.93    |

### **Summary of Results**

1. In the normal and host tissue the subendometrium had a lower percentage of AC staining cells than the midmyometrium and the subserosa.
2. The highest percentage AC staining in both normal and host tissues was found in the midmyometrium.
3. The secretory phase had the highest percentage of AC positive cells in normal myometrium and the lowest in host myometrium.
4. In the leiomyomata AC staining was not affected by tumour size.
5. AC staining in leiomyomata had the greatest similarity to the midmyometrial and subserosal regions of the host myometrium.
6. The cytoplasmic staining was the most varied of the staining patterns between normal and host tissue. In leiomyomata this pattern had the lowest percentage cells stained.
7. The combined staining showed no change with cycle or region in either normal, host or tumour tissue.
8. The membrane stained cells were the lowest percentage staining cells in normal and host tissue. The tumour had an increased number of membrane only stained cells compared to either normal or host.

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# **Discussion and Conclusion**

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## **1. Discussion**

### **1.1 AC Isoform**

The positive reaction obtained with the AC V/VI antibody, indicates the presence of either or both these isoforms in the lower segment of the normal and host myometrium and is in agreement with Richards *et al.* (1998) who determined the staining of AC V/VI in the fundus of the normal myometrium. As the two isoforms share a 93% homology in the C-terminal amino acids (Watson & Arkinstall, 1994) it is not possible to determine which isoform is predominant. The genetic message for both these isoforms has been found in a large number of tissues to a greater or lesser extent (Iyengar, 1993). As the most abundant source for type VI is cardiac muscle and that for the other isoforms, including type V, is neural (Cooper *et al.*, 1995) the muscle reaction in the myometrium is likely to be type VI while the neural tissue reaction may well be type V.

### **1.2 Trends in Staining**

The percentage positive cells are, in the majority of comparisons in the present study, significantly different from each other when using the student t-test. The comparatively low standard deviations would suggest that the counting technique used was accurate and that the results are, as a consequence, valid. There are clear trends in the staining of cells for AC across the myometrial wall and within the cycle. These trends are not affected by the individual differences, albeit significant, that the experiments have shown. Thus the general trends are discussed below rather than each individual difference.

### 1.3 AC and the Contraction Cycle

#### 1.3.1 Normal myometrium

Adenylate cyclase's pivotal role in smooth muscle relaxation is well documented (Carsten & Miller, 1987). The activation of AC by  $\beta$ -adrenergic agonists increases cAMP levels in the cell. The increase in cAMP activates protein kinase A and cyclic guanosine monophosphate kinase, which in their turn activate the  $\text{Ca}^{2+}$  ATPase pumps (Lincoln & Cornwell, 1991), thus effectively lowering the intracellular calcium concentration ( $[\text{Ca}^{2+}]_i$ ). The lowering of the  $[\text{Ca}^{2+}]_i$  activates a period of relaxation in those muscle cells that have contracted as a result of a  $\text{Ca}^{2+}$  ion influx at an earlier moment (Somolyo & Somolyo, 1994). An increase in AC may indicate that the myometrium is becoming increasingly relaxed and the opposite, a decrease in AC, indicating a contractile state. Full relaxation is thought to require only a fraction of the available  $\beta$ -adrenergic receptors and AC activity (Daftary *et al.*, 1996). Thus any partial decrease in AC activity is likely to lead to increases in muscle activity.

The distribution of AC positive cells in the lower segment of normal myometria is similar to that described for the fundal region of the normal myometrium (Richards *et al.*, 1998), such that the midmyometrium and subserosal regions have a higher positivity than the subendometrium. This is in accordance with the suggestion that the midmyometrial region has the greatest contractile potential in the non-pregnant state (Richards *et al.*, 1998). The number of AC positive cells within the myometrium, through the endometrial cycle, suggests that the muscle bulk is relaxing as the uterus moves from day one of the cycle (the menstrual phase) to the secretory phase. However, contractions are said to increase rather than relax in the late secretory period (Bell *et al.*, 1968), which would suggest that a drop in AC positivity would be expected in the pre-menstrual phase, as is implicated in the present study by the significant drop in staining from secretory to menstrual phases.

Cheng and Farquhar (1976) suggested that the presence of AC in cytoplasmic elements reflected pre-cursor sites of the enzyme. The cytoplasmic staining of AC in this study may denote the cells which have pre-cursor activity in the myometrium or

where AC is being retained as part of a pool prior to insertion in the membrane in a similar manner to receptor or channel turn around (Els & Butterworth, 1997). Krupinski *et al* (1989) suggested a channel type role for AC when they elucidated the structure of the type I isoform. Although this has since been disproved (Tang & Gilman, 1992), the 'channel' like structure may allow the enzyme to utilise a similar type of turn around mechanism described by Els and Butterworth (1997). In the cytoplasmic staining cells, the antibody recognises an amino acid sequence but the positivity does not indicate whether the isoform is complete or incomplete. Even though there is a high percentage of AC positive cells in a given area the AC is probably unavailable. A cytoplasmic and membrane staining cell would then represent an intermediate stage between full activation and no activation. The cells in this group may be at the start of a relaxation cycle with the cells beginning to insert AC into the membrane and thus becoming active. This group of cells stays constant throughout the cycle, reflecting the constant contraction:relaxation cycle this tissue undergoes. As the active form of AC is predominantly localized to the plasma membrane (see chapter 3), the cells with a membrane only localization would thus represent a group of cells that have a high AC activity and be in a fully relaxed state. The three stage staining pattern may be a useful model for predicting the contractile state of the muscle at the time of biopsy or hysterectomy. Thus a majority of cytoplasmic staining cells are indicative of a hyper-contractile state, while a majority of membrane staining cells are indicative of a highly relaxed state.

If this hypothesis of AC activity status, as determined from the staining patterns, is correct then the results from the differential localization of AC suggests that AC activity (ie membrane positive AC) in normal myometrium is decreasing as it enters the secretory phase (Fig. 6.5), with a consequential increase in muscle activity. Such an increase in contractile activity as the myometrium enters late secretory has been well documented (Bell *et al.*, 1968). In this study the secretory group was not divided into early or late secretory. A larger group that can be divided into two or three day intervals may help to determine whether there is a decrease through out this phase.

Analysis of tissue taken from the myometrium of pregnant uteri suggests that the stimulatory  $G_{sa}$  is expressed at higher levels than in the non-pregnant uterus (Europe-

Finner *et al.*, 1994). This G-protein increases AC activity, thus sustaining relaxation during pregnancy. However, Europe-Finner and colleagues (1994) note that the level of forskolin stimulated AC does not vary from non-pregnant through to labouring tissue and thus the number of AC stained cells will probably remain the same as reported here and by Richards *et al* (1998). In the pregnant uterus the number of membrane staining cells may increase in the midmyometrial and subserosal areas as a result of the increased  $G_{s\alpha}$  levels. At the end of the third stage of labour  $G_{s\alpha}$  levels are down regulated (Europe-Finner *et al.*, 1994), triggering labour. The sustained contractions would then be maintained by the inhibition of AC following the sudden increase of  $[Ca^{2+}]_i$  that is achieved by the influx of  $Ca^{2+}$  through L type channels (Yu *et al.*, 1993). An additional mechanism by which AC may be maintained in an inactive state is through the presence of prostaglandins at this stage in the pregnancy. The prostaglandins activate the inositol phospholipid and diacylglycerol pathways through the stimulation of  $G_i$  (Hepler & Gilman, 1992). These pathways activate smooth muscle contraction but at the same time release 'G' protein  $\beta$  and  $\gamma$  subunits. These will tend to inactivate AC as the local  $\beta\gamma$  pool increases (see chapter 2). Thus there would be a down regulation of AC just prior to parturition.

### **1.3.2 Host myometrium**

The contractile ability of the muscle in host myometria has not been shown to differ from normal. However, it is common for patients with fibromyomata to present with a symptom of dysmenorrhoea (Morton, 1958), which is thought to be a result of hyper-contraction within the myometrium. In the host myometrium the staining trend, with regards to the cellular staining pattern, appears to be opposite to that of normal. Thus the number of cytoplasmic staining cells decreases through the cycle with a corresponding increase in the number of the membrane staining group of cells, especially in the subendometrial and subserosal regions of the myometrium. If the hypothesis stated above is correct (that the staining pattern reflects activity status) this implies that there is an increase in the number of cells that have decreased contractile ability, in these areas, rather than an increase in contractility suggested by dysmenorrhoea. However, in the midmyometrial area of the menstrual host myometrium there is a significantly lower number of membrane staining cells than are

found in the normal myometrium. There is a corresponding increase in the number of cytoplasmic staining cells in this tissue. Thus, during the menstrual period of the endometrial cycle, the muscle in the midmyometrium is in an increased contractile state, which would account for a symptom of dysmenorrhoea in these patients.

### **1.3.3 Leiomyomata**

Tumours are often associated with the abnormal contraction of the uterus (Coutinho & Maia, 1971), it being hypothesized that the interruption to the contractile ability being a result of their architecture (Richards, 1995). However, the reversal of the normal endometrial cycle trend in the tumour (decreased cytoplasmic and cytoplasmic + membrane staining; increased membrane staining) suggests that this tissue is more relaxed, in comparison to the host myometrium, as the cycle progresses. This would impose focally relaxed areas of muscle within the rhythmically contracting muscle bulk of the host myometrium. This bolus of relaxed muscle would mechanically disrupt the normal contraction process which is one of the proposed mechanisms by which leiomyomata cause infertility (Coutinho & Maia, 1971). A mechanical obstruction of this type may contribute towards the occurrence of dysmenorrhoea within these patients. Thus as the myometrium contracts it tries to compress the tumour causing localized pain.

## **1.4 AC and Age**

Richards *et al.* (1998) have stated that there may be an age effect on the distribution of AC in the fundal region of the myometrium but they lacked specimens to ascertain this to any degree of certainty. In this study, even though the numbers in each age group was low, a statistical analysis of the different age groups suggests that age influences the distribution of AC within the subendometrial region of the lower segment myometrium. This is seen as an increase in activity probably up to the climateric after which it falls off. This would be expected as the myometrium loses bulk and tone following the climateric as a result of the drop off in oestrogen (Llwelllyn-Jones, 1982) thus producing a fall in AC positive cells which increases with age. The high levels of AC in the under 20 year age group followed by a drop is

difficult to explain and may be due to the low numbers used in this portion of the study.

## **1.5 AC and Tumourigenesis**

Richards (1995) has shown that the oestrogen receptor levels in the fibromyomata are similar to those found in the subendometrium of normal myometrium and on a par with the raised levels of oestrogen receptor in the midmyometrial portion of the host myometrium. As Richards (1995) stated, such levels are to be expected as the greater proportion of fibromyomata found are intramural tumours and would thus be expected to have the characteristics of the midmyometrial region, being of unicellular origin (Townsend *et al.*, 1970). Thus, as shown in this study, adenylate cyclase has a high percentage distribution in the fibromyomata that is similar to the midmyometrial levels of the normal and host myometria. An increase in oestrogen receptors in this tissue implies heightened sensitivity to oestrogen. If this is the case than the increased oestrogen activity may be having a synergistic effect on the increase in AC, as suggested by Cho and Katzenellenbogen (1993), thus increasing the downstream effects of the enzyme in this tissue.

As previously mentioned, the heightened AC reflects a relaxed state for the muscle fibres, which in turn implies a reduced  $[Ca^{2+}]_i$ . The concentration of  $Ca^{2+}$  ions in a tissue has an inverse relationship to the amount of insulin-like growth factor I (IGF-I) mRNA (Flovis *et al.*, 1993). This relationship is such that if  $[Ca^{2+}]_i$  increases then the amount of IGF-I decreases and vice versa where  $[Ca^{2+}]_i$  decreases IGF-I mRNA increases. It would then be expected that in the leiomyomata there would be an increase in IGF-I mRNA as the tissue is in a relaxed low  $[Ca^{2+}]_i$  situation. However, studies looking at IGF-I in this tissue have demonstrated no increase in IGF-I mRNA over host tissue (Vollenhoven *et al.*, 1993). However, it has been established that the position at which the sample of non-neoplastic host myometrium is taken in the tissue is extremely important (Richards & Tiltman, 1995) As Vollenhoven and colleagues (1993) do not give any indication as to region of sampling, it is possible that similar results could be obtained when investigating tissue from within the host myometrium and leiomyomata.

The action of insulin-like growth factor has recently been linked to the AC-cAMP pathway (Pertseva *et al.*, 1996) and has been demonstrated to effect the production of progesterone receptors (Cho *et al.*, 1994). In the leiomyomatous tissue where AC activity may be increased the effects of IGF-I on this tissue will also be increased, enhancing cell proliferation. This explanation would help to explain the high growth rates of some tumours and the regrowth of the leiomyomata following GnRH therapy (Friedman *et al.*, 1987). As progesterone receptors are also affected by increases in insulin-like growth factor I (Cho *et al.*, 1994), the reported effects of progestins on leiomyoma (Tiltman, 1985; Harrison-Woolrych & Robinson, 1995), such as an increase in growth, may be a result of the increased AC and hence sensitivity of the tissue to these agents.

The effects of IGF-I are not the only growth promotive effects of the cAMP cascade. Adenylate cyclase stimulated cAMP activates the mitogen activated protein kinase cascade which enhances the induction of differentiation (Frodin *et al.*, 1994). Increased AC activity has also been associated with uterine morphogenesis (Stewart and Webster, 1983) and with the initiation of cell growth induced by oestradiol (Nagibneva *et al.*, 1985). The increased presence of many other receptors and cDNAs of growth promoting substances that have been demonstrated in tumours (Harrison-Woolrych *et al.*, 1994) may also be linked to this alteration in AC activity and the increased second messenger activity. Thus the synergistic effects of the increased oestrogen on the AC/cAMP have increased the probability of myometrial morphogenesis and growth in those areas of the myometrium that contain cells with increased AC activity. These suggestions are in accordance with results obtained from other tumour tissue that have increased AC activity (Hunt & Martin, 1979). It is thus possible that the elevated levels of AC in the tissue may be promoting or enhancing tumourigenesis.

GnRH agonists are used prior to surgical intervention to reduce some leiomyoma (Schriock, 1989). It has been demonstrated that GnRH agonists work through a multitude of 'G' proteins some of which enhance AC and cAMP (Hawes *et al.*, 1993). The increased activity of AC in the leiomyomata could be an influencing factor in the

rapid reduction of the tumour's size. Some tumours do not respond to GnRH activity (Shriock, 1989) and this could be explainable by a decrease of AC activity in these tumours possibly through the stimulation of  $G_i$  proteins which may be expressed differentially in some tumours.

## **2. Conclusion**

Answers to the questions posed at the begining of the study can be extrapolated from the results and foregoing discussion.

### **2.1 Question One**

***Where is AC localized in the normal lower segment myometrium?***

Adenylate cyclase has an unequal distribution within the normal myometrium. In the lower segment there is low AC in the subendometrial level which is mainly associated with a combined (cytoplasm and membrane) staining of the muscle cells. The amount of staining rises towards the midmyometrial and subserosal regions. The staining of the muscle cells in these regions tends to have an increased cytoplasmic component. This pattern is affected by the endometrial cycle with fluctuations occurring in the subendometrial and midmyometrial areas. However, the subserosal region maintains a low number of cells displaying the active form throughout the cycle. The staining is also affected by the age of the patient such that AC expression peaks in the thirty and forty year age group before decreasing to below the levels that are present at the onset of menstruation.

### **2.2 Question Two**

***Is there a difference in localization between normal, host myometrium and leiomyomata?***

The proliferative and secretory phase of the host myometrium shows a significant drop in the percentage of cells that show cytoplasmic staining when compared to the normal myometrium. The general trend in membrane only staining cells is increased in the host myometrium. This is particularly the case in the secretory phase of the

endometrial cycle. The increase in membrane only staining cells may be at the expense of the number of cytoplasmic staining cells as the combined staining pattern (cytoplasm and membrane) shows no difference when the host and normal tissue is compared. As this change in staining pattern may reflect changes in muscle contractility it is possible that the differences in AC staining helps to explain some of the symptoms, such as dysmenorrhoea, that afflict patients with leiomyoma.

### **2.3 Question three**

***If the distribution is different between these tissues, does AC have a role in the aetiology of the tumour or the symptoms associated with their presence?***

The distribution of AC that has been noted in the tumour and the host myometrium may have a bearing on the symptoms noted when leiomyomata are present. The tumour may be forming a relaxed bolus within the contracting myometrium and thus causing pain when contractions occur around it. The increase in oestrogen receptor in the tumour and the host myometrium (Richards, 1995; Richards & Tiltman, 1996) may be having a synergistic effect on the AC system. This in turn could be enhancing the effects of growth hormones such as IGF-I and be promoting the morphogenic effects of AC and cAMP in those cells that have an increased AC activity. Although these effects are unlikely to be the final aetiological factor they would promote the growth once initiation of tumour formation has occurred or promote tumourigenesis, if certain cells are more sensitive to external hormonal factors. Further, the effects of the GnRH agonists on this tissue are explainable in terms of the AC system either through  $G_i$  inhibition or through AC enhancement.

### **2.4 Future Considerations**

This study has shown that AC and the second messenger system may have an effect on the aetiology and/or events post initiation of leiomyomas as a consequence of the synergistic effects of oestrogen. Future studies need to be undertaken to determine the activity levels of AC in this tissue to confirm the hypothesis drawn from the observed staining patterns observed. Although this study only examined the variation in the AC

staining the other components of the system,  $G_s$ ,  $G_i$  and cAMP may also be effected and have consequential downstream effects on the cell. Other cellular factors that effect the second messenger system and oestrogen, such as the heat shock proteins during proliferation and morphogenesis (Devaja *et al.*, 1997), may also play a role in the initiation of tumourigenesis but require further investigation.

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## Appendix 1

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# **Solutions**

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### Phosphate Buffered Saline (PBS)

#### Constituents:

- \* 8 gr Sodium Chloride (NaCl)
- \* 1.25 gr Di-Sodium Phosphate ( $\text{Na}_2\text{HPO}_4$ )
- \* 0.2 gr Potassium Chloride (KCl)
- \* 0.2 gr Potassium di-Hydrogen Phosphate ( $\text{KH}_2\text{PO}_4$ )
- \* 1000 mls water

#### Method:

Weigh out required quantities and place in a flask. Add water and stir until dissolved on a magnetic stirrer. Adjust the pH of the solution to 7.2.

### Immunocytochemistry Buffer (ICC Buffer)

#### Constituents:

- \* 500 mls PBS
- \* 2.5 gr Bovine Serum Albumin (BSA)

#### Method:

Weigh out required amount of BSA and add to the correct volume of PBS. Stir with a magnetic stirrer until completely dissolved. The solution can be kept in the fridge and used for 48 hours.

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3% Hydrogen Peroxidase ( $H_2O_2$ )

Constituents:

- \* 3 mls  $H_2O_2$  (%)
- \* 97 mls Distilled Water

Method:

Measure out water and add the  $H_2O_2$  just prior to use.

5% Normal Horse Serum (NHS)

Constituents:

- \* 5 mls NHS (stock)
- \* 95 mls ICC buffer

Method:

Measure out ICC buffer into a cylinder and add NHS. Cover with parafilm and mix well. Can be stored for 1 week at 4°C.

Primary Antibody

Constituents:

- \* 10  $\mu$ l stock adenylyl cyclase type V/VI antibody (Santa Cruz, USA)
- \* 490  $\mu$ l ICC buffer.

Method:

Using a Gilson® pipette measure out primary antibody into a test tube and add the ICC buffer. Mix well before use.

**Biotinylated Secondary Antibody (horse anti rabbit)**

Constituents:

- \* 10  $\mu$ l secondary antibody stock solution
- \* 2 mls ICC buffer.

Method:

Measure out desired quantity of secondary antibody using a Gilson<sup>®</sup> pipette into a test tube. Add the ICC buffer and mix well. Diluted solution can be kept frozen until required.

**Streptavidin Peroxidase**

Constituents:

- \* ABC Kit (Dako, USA)
- \* 5 mls ICC buffer

Method:

Mix ingredients as directed 30 minutes before use. One drop solution A and 1 drop solution B added to 5 mls ICC buffer. Mix well. Solution can be kept at 4°C for 24 hrs.

### Diaminobenzidine (DAB)

#### Constituents:

- \* 0.025 gr DAB (Sigma, USA)
- \* 25 mls ICC buffer
- \* 25 mls distilled water
- \* 3 drops 33% H<sub>2</sub>O<sub>2</sub>
- \* 0.03 gr nickel

#### Method:

Ten minutes before use, weigh out DAB in a flask and add ICC buffer. Mix until dissolved. Dissolve nickel chloride in water and just prior to use add H<sub>2</sub>O<sub>2</sub> to the solution. Mix DAB and nickel solution before applying to sections.

### Haematoxylin

#### Constituents:

- \* 1 gr Haematoxylin
- \* 1000 mls Distilled water
- \* 50 gr Potassium alum
- \* 1 gr Citric acid
- \* 50 gr Chloral hydrate
- \* 0.2 gr Sodium iodate

#### Method:

Dissolve the haematoxylin, potassium alum and sodium iodate in the distilled water over a gentle heat while continuously stirring. Add chloral hydrate and citric acid and bring to the boil. Boil for 5 minutes before cooling the solution and filtering prior to use.

*Methyl Green*

Constituents:

- \* 0.5 gr Methyl green
- \* 100 mls distilled water
- \* 0.1 M Sodium acetate
- \* 1 M Acetic acid

Method:

Dissolve methyl green in water. Adjust pH to 4.0 using sodium acetate and acetic acid.

## Appendix 2

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### **Methods used in the Study**

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#### *Methods*

#### **Preparation of Adhesive Slides**

##### Chemicals:

- Decon 90 (Contrad)
- Aminopropylethoxysilane
- Acetone (100%)

##### Method:

- 1) Place a box of slides in racks and immerse in a pre-warmed (60°C) solution of 2% Contrad in distilled water to clean them.
  - 2) Rinse slides thoroughly in distilled water followed by acetone. Allow the slides to air dry.
  - 3) Immerse the slides in a solution of 2% aminopropylethoxysilane in acetone for 30-45 minutes.
  - 4) Rinse the slides in acetone and then clean distilled water.
  - 5) Air dry the slides overnight at 37°C.
  - 6) Store in fridge at 4°C until required.
-

### **Adenylate cyclase immunocytochemistry**

#### Chemicals:

- Xylene
- Alcohols (50-100%)
- Hydrogen Peroxide
- ICC buffer
- Normal horse serum
- Primary Antibody
- Biotinylated secondary antibody
- Streptavidin-peroxidase kit
- DAB solution
- Haematoxylin or Methyl green.
- Scott's Water
- DPX mounting medium

#### Method:

- 1) Dewax sections. Place slides in xylene and a descending series of alcohol. Leave in each solution for 10 minutes.
- 2) Wash in distilled water for 10 minutes.
- 3) Place slides in a coplin jar and pour in 3% hydrogen peroxide. Leave for 30 minutes.
- 4) Wash in 3 changes of ICC buffer. Five minutes each change.
- 5) Place slides in moist chamber.
- 6) Apply 5% normal horse serum made up in ICC buffer to each section. Place a cover slip over each section and leave for 45 minutes.
- 7) Remove coverslips and wipe excess serum from around each section. DO NOT WASH or DRY.
- 8) Apply 50 µl of primary antibody at a dilution of 1:50 to each section. Coverslip and leave for 60 minutes.
- 9) Remove coverslips and wash in 3 changes of ICC buffer for 5 minutes each change.
- 10) Apply 50 µl of secondary antibody at a dilution of 1:200 to each section. Coverslip and leave for 30 minutes.
- 11) Remove coverslips and wash in 3 changes of ICC buffer for 5 minutes each change.
- 12) Apply 50 µl streptavidin peroxidase from kit to each section. Coverslip and leave for 45 minutes.

- 13) Remove coverslips and wash in 3 changes of ICC buffer for 5 minutes each change.
- 14) Place slides in a coplin jar and add DAB solution. Leave for 5 minutes.
- 15) Pour of DAB solution into a flask and wash slides in running tap water for 10 minutes.
- 16) Transfer slides to a carrier and place in haematoxylin solution for 60 seconds.
- 17) Wash in running tap water for 2 minutes.
- 18) Blue in Scott's water.
- 19) Wash in running tap water for 2 minutes.
- 20) Dehydrate in an ascending alcohol series to Xylene. Mount and coverslip using DPX.

### Appendix 3

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## **Data**

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### *Raw data*

The following tables show the counts obtained per slide for AC positivity and the cellular distribution of the stain. The tables are in order of the endometrial cycle (menstrual phase, proliferative phase, secretory phase). The results for normal are first followed by host and then tumour. Each table is headed as to the phase and normality of the tissue.

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## Normal - Menstrual

| Specimen | Subendometrium |                |            | Midmyometrium |                |            | Subserosal  |                |            |
|----------|----------------|----------------|------------|---------------|----------------|------------|-------------|----------------|------------|
|          | Total Cells    | Positive Cells | % Positive | Total Cells   | Positive Cells | % Positive | Total Cells | Positive Cells | % Positive |
| A        | 141            | 7              | 4.96       | 92            | 52             | 56.52      | 19          | 11             | 57.89      |
|          | 117            | 10             | 8.55       | 85            | 34             | 40.00      | 23          | 14             | 60.87      |
|          | 135            | 11             | 8.15       | 69            | 33             | 47.83      | 32          | 6              | 18.75      |
|          | 108            | 22             | 20.37      | 102           | 64             | 62.75      | 36          | 17             | 47.22      |
|          | 109            | 16             | 14.68      | 98            | 57             | 58.16      | 47          | 39             | 82.98      |
|          | 104            | 41             | 39.42      | 109           | 58             | 53.21      | 43          | 35             | 81.40      |
|          | 105            | 9              | 8.57       | 54            | 33             | 61.11      | 26          | 7              | 26.92      |
|          | 101            | 30             | 29.70      | 90            | 55             | 61.11      | 11          | 5              | 45.45      |
|          | 89             | 9              | 10.11      | 88            | 49             | 55.68      | 20          | 4              | 20.00      |
|          | 89             | 20             | 22.47      | 73            | 41             | 56.16      | 12          | 5              | 41.67      |
| B        | 107            | 25             | 23.36      | 94            | 63             | 67.02      | 10          | 10             | 100.00     |
|          | 81             | 11             | 13.58      | 89            | 54             | 60.67      | 21          | 8              | 38.10      |
|          | 127            | 39             | 30.71      | 66            | 45             | 68.18      | 14          | 9              | 64.29      |
|          | 79             | 24             | 30.38      | 82            | 57             | 69.51      | 42          | 27             | 64.29      |
|          | 117            | 35             | 29.91      | 110           | 86             | 78.18      | 21          | 16             | 76.19      |
|          | 113            | 50             | 44.25      | 91            | 84             | 92.31      | 25          | 16             | 64.00      |
|          | 108            | 17             | 15.74      | 107           | 102            | 95.33      | 22          | 8              | 36.36      |
|          | 130            | 25             | 19.23      | 50            | 38             | 76.00      | 60          | 36             | 60.00      |
|          | 81             | 29             | 35.80      | 101           | 93             | 92.08      | 35          | 25             | 71.43      |
|          | 82             | 31             | 37.80      | 79            | 57             | 72.15      | 28          | 20             | 71.43      |
| C        | 102            | 18             | 17.65      | 88            | 56             | 63.64      | 27          | 22             | 81.48      |
|          | 88             | 21             | 23.86      | 81            | 48             | 59.26      | 43          | 24             | 55.81      |
|          | 101            | 23             | 22.77      | 94            | 69             | 73.40      | 37          | 29             | 78.38      |
|          | 98             | 16             | 16.33      | 52            | 43             | 82.69      | 28          | 22             | 78.57      |
|          | 77             | 16             | 20.78      | 61            | 41             | 67.21      | 18          | 18             | 100.00     |
|          | 94             | 23             | 24.47      | 83            | 63             | 75.90      | 16          | 9              | 56.25      |
|          | 75             | 15             | 20.00      | 99            | 77             | 77.78      | 20          | 16             | 80.00      |
|          | 76             | 13             | 17.11      | 77            | 56             | 72.73      | 24          | 15             | 62.50      |
|          | 91             | 18             | 19.78      | 91            | 74             | 81.32      | 4           | 4              | 100.00     |
|          | 84             | 21             | 25.00      | 94            | 81             | 86.17      | 20          | 14             | 70.00      |
| D        | 63             | 21             | 33.33      | 69            | 57             | 82.61      | 21          | 10             | 47.62      |
|          | 75             | 12             | 16.00      | 80            | 58             | 72.50      | 13          | 11             | 84.62      |
|          | 76             | 8              | 10.53      | 50            | 36             | 72.00      | 17          | 9              | 52.94      |
|          | 99             | 15             | 15.15      | 74            | 52             | 70.27      | 11          | 7              | 63.64      |
|          | 69             | 13             | 18.84      | 37            | 25             | 67.57      | 54          | 32             | 59.26      |
|          | 77             | 23             | 29.87      | 84            | 70             | 83.33      | 43          | 27             | 62.79      |
|          | 86             | 17             | 19.77      | 70            | 61             | 87.14      | 21          | 12             | 57.14      |
|          | 83             | 15             | 18.07      | 83            | 51             | 61.45      | 24          | 18             | 75.00      |
|          | 90             | 12             | 13.33      | 67            | 47             | 70.15      | 29          | 17             | 58.62      |
|          | 80             | 9              | 11.25      | 74            | 54             | 72.97      | 23          | 15             | 65.22      |
| Total    | 3807           | 790            |            | 3237          | 2274           |            | 1040        | 649            |            |
| Average  | 95.18          | 19.75          | 21.04      | 80.93         | 56.85          | 70.10      | 26.00       | 16.23          | 62.98      |
| Std Dev  | 18.73          | 9.66           | 9.28       | 17.32         | 17.04          | 12.40      | 12.51       | 9.35           | 19.57      |

## Normal - Proliferative

| Specimen | Subendometrium |                |            | Midmyometrium |                |            | Subserosal  |                |            |
|----------|----------------|----------------|------------|---------------|----------------|------------|-------------|----------------|------------|
|          | Total Cells    | Positive Cells | % Positive | Total Cells   | Positive Cells | % Positive | Total Cells | Positive Cells | % Positive |
| A        | 95             | 22             | 23.16      | 97            | 60             | 61.86      | 20          | 12             | 60.00      |
|          | 90             | 25             | 27.78      | 78            | 55             | 70.51      | 8           | 5              | 62.50      |
|          | 85             | 17             | 20.00      | 70            | 56             | 80.00      | 36          | 26             | 72.22      |
|          | 93             | 14             | 15.05      | 75            | 61             | 81.33      | 21          | 14             | 66.67      |
|          | 80             | 16             | 20.00      | 88            | 69             | 78.41      | 2           | 2              | 100.00     |
|          | 74             | 12             | 16.22      | 65            | 53             | 81.54      | 15          | 13             | 86.67      |
|          | 97             | 19             | 19.59      | 61            | 39             | 63.93      | 16          | 10             | 62.50      |
|          | 76             | 22             | 28.95      | 78            | 54             | 69.23      | 11          | 7              | 63.64      |
|          | 76             | 35             | 46.05      | 74            | 61             | 82.43      | 19          | 15             | 78.95      |
|          | 65             | 24             | 36.92      | 79            | 75             | 94.94      | 33          | 20             | 60.61      |
| B        | 101            | 24             | 23.76      | 90            | 77             | 85.56      | 15          | 13             | 86.67      |
|          | 97             | 25             | 25.77      | 105           | 90             | 85.71      | 34          | 29             | 85.29      |
|          | 93             | 27             | 29.03      | 80            | 75             | 93.75      | 18          | 13             | 72.22      |
|          | 85             | 14             | 16.47      | 71            | 55             | 77.46      | 12          | 12             | 100.00     |
|          | 76             | 24             | 31.58      | 60            | 54             | 90.00      | 34          | 15             | 44.12      |
|          | 87             | 19             | 21.84      | 74            | 60             | 81.08      | 42          | 36             | 85.71      |
|          | 99             | 25             | 25.25      | 48            | 40             | 83.33      | 19          | 10             | 52.63      |
|          | 96             | 23             | 23.96      | 85            | 85             | 100.00     | 27          | 19             | 70.37      |
|          | 93             | 11             | 11.83      | 78            | 57             | 73.08      | 20          | 15             | 75.00      |
|          | 94             | 17             | 18.09      | 80            | 60             | 75.00      | 23          | 19             | 82.61      |
| C        | 107            | 41             | 38.32      | 78            | 68             | 87.18      | 41          | 41             | 100.00     |
|          | 100            | 31             | 31.00      | 40            | 38             | 95.00      | 14          | 14             | 100.00     |
|          | 99             | 35             | 35.35      | 76            | 36             | 46.15      | 20          | 18             | 90.00      |
|          | 94             | 31             | 32.98      | 72            | 52             | 72.22      | 24          | 20             | 83.33      |
|          | 96             | 35             | 36.46      | 62            | 46             | 74.19      | 33          | 22             | 66.67      |
|          | 77             | 30             | 38.96      | 79            | 67             | 84.81      | 35          | 21             | 60.00      |
|          | 115            | 26             | 22.61      | 61            | 46             | 75.41      | 18          | 16             | 88.89      |
|          | 98             | 18             | 18.37      | 97            | 65             | 67.01      | 25          | 19             | 76.00      |
|          | 57             | 13             | 22.81      | 79            | 58             | 73.42      | 42          | 33             | 78.57      |
|          | 81             | 18             | 22.22      | 75            | 52             | 69.33      | 30          | 18             | 60.00      |
| D        | 96             | 20             | 20.83      | 82            | 64             | 78.05      | 14          | 12             | 85.71      |
|          | 103            | 12             | 11.65      | 87            | 70             | 80.46      | 12          | 7              | 58.33      |
|          | 96             | 25             | 26.04      | 96            | 75             | 78.13      | 25          | 19             | 76.00      |
|          | 96             | 14             | 14.58      | 109           | 78             | 71.56      | 27          | 16             | 59.26      |
|          | 99             | 20             | 20.20      | 96            | 84             | 87.50      | 18          | 10             | 55.56      |
|          | 97             | 25             | 25.77      | 97            | 64             | 65.98      | 27          | 18             | 66.67      |
|          | 97             | 33             | 34.02      | 72            | 60             | 83.33      | 27          | 25             | 92.59      |
|          | 98             | 19             | 19.39      | 64            | 37             | 57.81      | 19          | 13             | 68.42      |
|          | 107            | 21             | 19.63      | 74            | 66             | 89.19      | 28          | 20             | 71.43      |
|          | 112            | 16             | 14.29      | 86            | 64             | 74.42      | 52          | 36             | 69.23      |
| E        | 85             | 20             | 23.53      | 92            | 77             | 83.70      | 7           | 7              | 100.00     |
|          | 97             | 12             | 12.37      | 72            | 58             | 80.56      | 18          | 13             | 72.22      |
|          | 107            | 21             | 19.63      | 82            | 69             | 84.15      | 15          | 10             | 66.67      |
|          | 112            | 11             | 9.82       | 74            | 53             | 71.62      | 19          | 17             | 89.47      |
|          | 101            | 33             | 32.67      | 70            | 64             | 91.43      | 11          | 11             | 100.00     |
|          | 97             | 32             | 32.99      | 31            | 27             | 87.10      | 7           | 4              | 57.14      |
|          | 103            | 19             | 18.45      | 54            | 42             | 77.78      | 26          | 19             | 73.08      |
|          | 109            | 43             | 39.45      | 48            | 31             | 64.58      | 11          | 3              | 27.27      |
|          | 85             | 8              | 9.41       | 54            | 39             | 72.22      | 13          | 4              | 30.77      |
|          | 99             | 32             | 32.32      | 64            | 52             | 81.25      | 27          | 19             | 70.37      |
| F        | 85             | 22             | 25.88      | 81            | 77             | 95.06      | 12          | 11             | 91.67      |
|          | 83             | 29             | 34.94      | 34            | 31             | 91.18      | 24          | 16             | 66.67      |
|          | 80             | 26             | 32.50      | 82            | 71             | 86.59      | 11          | 8              | 72.73      |
|          | 83             | 20             | 24.10      | 69            | 59             | 85.51      | 17          | 12             | 70.59      |
|          | 70             | 17             | 24.29      | 50            | 48             | 96.00      | 14          | 9              | 64.29      |
|          | 96             | 14             | 14.58      | 69            | 61             | 88.41      | 8           | 7              | 87.50      |
|          | 75             | 15             | 20.00      | 75            | 72             | 96.00      | 17          | 15             | 88.24      |
|          | 62             | 10             | 16.13      | 72            | 72             | 100.00     | 25          | 20             | 80.00      |
|          | 81             | 21             | 25.93      | 65            | 61             | 93.85      | 40          | 31             | 77.50      |
|          | 62             | 19             | 30.65      | 65            | 58             | 89.23      | 14          | 10             | 71.43      |
| G        | 102            | 35             | 34.31      | 64            | 52             | 81.25      | 32          | 24             | 75.00      |
|          | 67             | 17             | 25.37      | 50            | 39             | 78.00      | 22          | 14             | 63.64      |
|          | 85             | 18             | 21.18      | 53            | 41             | 77.36      | 28          | 22             | 71.43      |
|          | 57             | 13             | 22.81      | 66            | 50             | 75.76      | 25          | 19             | 76.00      |
|          | 88             | 12             | 13.64      | 66            | 53             | 80.30      | 18          | 12             | 66.67      |
|          | 66             | 15             | 22.73      | 63            | 49             | 77.78      | 45          | 25             | 55.56      |
|          | 72             | 8              | 11.11      | 41            | 36             | 87.80      | 30          | 29             | 96.67      |
|          | 46             | 22             | 47.83      | 66            | 59             | 89.39      | 24          | 21             | 87.50      |
|          | 66             | 14             | 21.21      | 62            | 46             | 74.19      | 21          | 14             | 66.67      |
|          | 77             | 23             | 29.87      | 37            | 29             | 78.38      | 24          | 20             | 83.33      |
| Total    | 6175           | 1499           |            | 4991          | 4002           |            | 1561        | 1147           |            |
| Average  | 88.21          | 21.41          | 24.52      | 71.30         | 57.17          | 80.52      | 22.30       | 16.39          | 73.93      |
| Std dev  | 14.85          | 7.92           | 8.57       | 16.38         | 14.32          | 10.20      | 10.09       | 8.02           | 15.35      |

Normal - Secretary

| Specimen | Subendometrium |                |            | Midmyometrium |                |            | Subserosal  |                |            |
|----------|----------------|----------------|------------|---------------|----------------|------------|-------------|----------------|------------|
|          | Total Cells    | Positive Cells | % Positive | Total Cells   | Positive Cells | % Positive | Total Cells | Positive Cells | % Positive |
| A        | 99             | 75             | 75         | 100           | 75             | 75         | 22          | 15             | 68         |
|          | 96             | 89             | 93         | 73            | 66             | 90         | 12          | 12             | 100        |
|          | 78             | 74             | 95         | 73            | 63             | 85         | 14          | 14             | 100        |
|          | 98             | 91             | 93         | 54            | 43             | 80         | 22          | 20             | 91         |
|          | 83             | 60             | 72         | 65            | 71             | 84         | 15          | 12             | 80         |
|          | 73             | 44             | 60         | 38            | 33             | 87         | 34          | 29             | 85         |
|          | 68             | 43             | 63         | 80            | 77             | 96         | 24          | 20             | 83         |
|          | 103            | 98             | 95         | 53            | 50             | 94         | 36          | 35             | 97         |
|          | 81             | 68             | 85         | 85            | 84             | 99         | 16          | 14             | 88         |
|          | 86             | 82             | 95         | 68            | 52             | 76         | 18          | 10             | 56         |
|          | 103            | 88             | 85         | 65            | 55             | 85         | 19          | 19             | 100        |
|          | 77             | 63             | 82         | 62            | 52             | 84         | 6           | 6              | 100        |
| B        | 71             | 57             | 80         | 54            | 40             | 74         | 29          | 20             | 69         |
|          | 91             | 75             | 82         | 64            | 60             | 94         | 6           | 2              | 33         |
|          | 89             | 65             | 73         | 58            | 53             | 91         | 15          | 10             | 67         |
|          | 100            | 80             | 80         | 73            | 70             | 96         | 23          | 19             | 83         |
|          | 66             | 42             | 64         | 65            | 60             | 92         | 44          | 30             | 68         |
|          | 76             | 63             | 83         | 64            | 51             | 80         | 25          | 19             | 76         |
|          | 70             | 57             | 81         | 76            | 69             | 91         | 25          | 20             | 80         |
|          | 77             | 60             | 78         | 71            | 60             | 85         | 15          | 11             | 73         |
|          | 85             | 22             | 26         | 66            | 77             | 90         | 32          | 28             | 88         |
|          | 93             | 14             | 17         | 68            | 63             | 93         | 30          | 30             | 100        |
|          | 91             | 18             | 20         | 62            | 47             | 76         | 25          | 16             | 64         |
|          | 88             | 24             | 27         | 57            | 47             | 82         | 21          | 21             | 100        |
| C        | 94             | 18             | 19         | 69            | 15             | 15         | 10          | 10             | 97         |
|          | 99             | 35             | 36         | 91            | 90             | 99         | 18          | 8              | 50         |
|          | 72             | 22             | 31         | 66            | 59             | 89         | 25          | 24             | 96         |
|          | 90             | 25             | 28         | 59            | 48             | 81         | 20          | 20             | 100        |
|          | 122            | 35             | 29         | 81            | 66             | 81         | 22          | 15             | 68         |
|          | 68             | 16             | 24         | 70            | 54             | 77         | 38          | 35             | 92         |
|          | 71             | 62             | 87         | 56            | 53             | 95         | 10          | 10             | 100        |
|          | 94             | 89             | 95         | 90            | 80             | 100        | 12          | 10             | 100        |
|          | 79             | 71             | 90         | 47            | 35             | 77         | 13          | 13             | 100        |
|          | 69             | 55             | 80         | 84            | 80             | 95         | 7           | 8              | 88         |
|          | 84             | 65             | 77         | 52            | 41             | 79         | 12          | 12             | 100        |
|          | 94             | 86             | 91         | 58            | 50             | 85         | 27          | 23             | 85         |
| D        | 86             | 75             | 87         | 68            | 66             | 97         | 16          | 15             | 94         |
|          | 75             | 58             | 77         | 58            | 55             | 95         | 19          | 15             | 79         |
|          | 75             | 62             | 83         | 100           | 99             | 99         | 9           | 5              | 56         |
|          | 76             | 71             | 93         | 58            | 54             | 93         | 5           | 2              | 40         |
|          | 96             | 79             | 82         | 48            | 39             | 81         | 17          | 11             | 65         |
|          | 80             | 47             | 59         | 63            | 40             | 63         | 14          | 14             | 100        |
|          | 87             | 54             | 62         | 75            | 59             | 92         | 31          | 10             | 32         |
|          | 82             | 64             | 78         | 84            | 56             | 88         | 7           | 7              | 100        |
|          | 105            | 94             | 90         | 80            | 79             | 99         | 17          | 13             | 76         |
|          | 92             | 78             | 85         | 65            | 61             | 94         | 15          | 11             | 73         |
|          | 116            | 104            | 90         | 73            | 41             | 56         | 27          | 22             | 81         |
|          | 85             | 82             | 96         | 71            | 56             | 79         | 12          | 12             | 100        |
|          | 78             | 62             | 79         | 67            | 57             | 85         | 46          | 34             | 74         |
| E        | 111            | 103            | 93         | 85            | 75             | 89         | 21          | 15             | 76         |
|          | 82             | 58             | 71         | 83            | 70             | 80         | 13          | 8              | 62         |
|          | 83             | 59             | 71         | 95            | 55             | 59         | 19          | 15             | 79         |
|          | 109            | 71             | 65         | 57            | 50             | 88         | 18          | 10             | 56         |
|          | 88             | 59             | 67         | 92            | 57             | 62         | 20          | 13             | 65         |
|          | 90             | 33             | 37         | 54            | 48             | 89         | 16          | 13             | 81         |
|          | 134            | 37             | 28         | 108           | 102            | 94         | 19          | 27             | 142        |
|          | 108            | 42             | 39         | 60            | 47             | 78         | 11          | 7              | 64         |
|          | 103            | 37             | 36         | 53            | 44             | 83         | 9           | 7              | 78         |
|          | 113            | 50             | 44         | 60            | 44             | 67         | 21          | 17             | 81         |
|          | 95             | 65             | 68         | 56            | 49             | 88         | 20          | 11             | 55         |
|          | 54             | 50             | 93         | 78            | 74             | 95         | 10          | 6              | 60         |
| G        | 57             | 54             | 95         | 43            | 38             | 88         | 21          | 20             | 95         |
|          | 82             | 70             | 85         | 69            | 60             | 87         | 17          | 13             | 76         |
|          | 94             | 59             | 63         | 43            | 40             | 93         | 15          | 10             | 67         |
|          | 36             | 34             | 40         | 61            | 58             | 95         | 32          | 28             | 88         |
|          | 80             | 59             | 74         | 57            | 54             | 95         | 23          | 18             | 78         |
|          | 106            | 15             | 14         | 49            | 36             | 73         | 19          | 14             | 74         |
|          | 77             | 13             | 17         | 55            | 50             | 91         | 18          | 12             | 67         |
|          | 79             | 10             | 13         | 49            | 41             | 84         | 24          | 24             | 100        |
|          | 76             | 6              | 8          | 71            | 67             | 94         | 6           | 6              | 100        |
|          | 78             | 13             | 17         | 67            | 53             | 79         | 20          | 14             | 70         |
|          | 76             | 17             | 22         | 102           | 81             | 79         | 23          | 15             | 65         |
|          | 104            | 20             | 19         | 48            | 35             | 73         | 40          | 40             | 100        |
| H        | 87             | 13             | 15         | 70            | 62             | 89         | 22          | 18             | 82         |
|          | 97             | 20             | 21         | 53            | 47             | 89         | 15          | 10             | 67         |
|          | 98             | 22             | 22         | 63            | 54             | 86         | 16          | 13             | 81         |
|          | 108            | 20             | 19         | 52            | 44             | 85         | 35          | 35             | 100        |
|          | 96             | 14             | 15         | 64            | 53             | 83         | 23          | 18             | 78         |
|          | 103            | 20             | 19         | 67            | 55             | 82         | 22          | 13             | 59         |
|          | 98             | 21             | 21         | 94            | 74             | 79         | 29          | 25             | 86         |
|          | 97             | 61             | 64         | 60            | 54             | 90         | 27          | 25             | 93         |
|          | 82             | 61             | 74         | 66            | 62             | 94         | 16          | 6              | 38         |
|          | 79             | 53             | 67         | 40            | 37             | 93         | 43          | 37             | 86         |
|          | 108            | 85             | 78         | 78            | 73             | 94         | 17          | 10             | 59         |
|          | 114            | 81             | 71         | 72            | 52             | 72         | 25          | 25             | 100        |
| I        | 44             | 30             | 68         | 45            | 40             | 89         | 17          | 17             | 100        |
|          | 85             | 73             | 86         | 45            | 43             | 96         | 17          | 12             | 71         |
|          | 106            | 45             | 42         | 41            | 37             | 90         | 10          | 6              | 60         |
|          | 101            | 56             | 55         | 54            | 48             | 89         | 32          | 30             | 94         |
|          | 109            | 80             | 73         | 68            | 59             | 87         | 9           | 9              | 100        |
|          | 83             | 23             | 28         | 50            | 45             | 90         | 9           | 9              | 100        |
|          | 97             | 20             | 21         | 48            | 36             | 75         | 12          | 10             | 83         |
|          | 86             | 24             | 28         | 64            | 52             | 81         | 11          | 11             | 100        |
|          | 93             | 21             | 23         | 70            | 63             | 90         | 33          | 33             | 100        |
|          | 84             | 7              | 8          | 83            | 56             | 89         | 26          | 24             | 92         |
|          | 92             | 34             | 37         | 51            | 42             | 82         | 13          | 10             | 77         |
|          | 96             | 29             | 30         | 59            | 38             | 76         | 27          | 22             | 81         |
|          | 107            | 25             | 23         | 66            | 27             | 41         | 29          | 24             | 83         |
| J        | 119            | 17             | 14         | 63            | 56             | 89         | 40          | 34             | 85         |
|          | 78             | 44             | 56         | 58            | 46             | 79         | 42          | 41             | 98         |
|          |                |                |            |               |                |            |             |                |            |
| Total    | 6334           | 3411           |            | 4481          | 3833           |            | 1394        | 1139           |            |
| Average  | 89.11          | 90.32          | 57.49      | 65.36         | 66.28          | 85.84      | 20.58       | 18.9           | 81.13      |
| Std Dev  | 15.01          | 26.21          | 29.11      | 14.49         | 93.66          | 9.64       | 34.3        | 28.07          | 17.07      |

**Menstrual- Normal**

| Specimen | Subendometrium |       |       | Midmyometrium |       |       | Subserosal |       |      |
|----------|----------------|-------|-------|---------------|-------|-------|------------|-------|------|
|          | C              | C + M | M     | C             | C + M | M     | C          | C + M | M    |
| A        | 11             | 41    | 48    | 7             | 59    | 34    | 25         | 63    | 12   |
| B        | 39             | 54    | 7     | 33            | 58    | 10    | 49         | 50    | 1    |
| C        | 34             | 32    | 34    | 18            | 36    | 46    | 57         | 43    | 0    |
| D        | 22             | 42    | 30    | 25            | 42    | 30    | 27         | 49    | 8    |
| Total    | 106            | 169   | 119   | 83            | 195   | 120   | 158        | 205   | 21   |
| Average  | 26.50          | 42.25 | 29.75 | 20.75         | 48.75 | 30.00 | 39.50      | 51.25 | 5.25 |
| Std Dev  | 12.56          | 9.03  | 17.02 | 11.03         | 11.53 | 14.97 | 15.95      | 8.42  | 5.74 |

**Proliferative - Normal**

| Specimen | Subendometrium |       |       | Midmyometrium |       |       | Subserosal |       |      |
|----------|----------------|-------|-------|---------------|-------|-------|------------|-------|------|
|          | C              | C + M | M     | C             | C + M | M     | C          | C + M | M    |
| A        | 31             | 31    | 38    | 25            | 64    | 11    | 60         | 31    | 9    |
| B        | 32             | 54    | 14    | 10            | 72    | 18    | 46         | 51    | 3    |
| C        | 36             | 42    | 22    | 27            | 62    | 11    | 51         | 48    | 1    |
| D        | 31             | 34    | 35    | 28            | 56    | 16    | 54         | 45    | 1    |
| E        | 25             | 50    | 25    | 46            | 50    | 4     | 66         | 34    | 0    |
| F        | 20             | 52    | 28    | 21            | 62    | 17    | 27         | 66    | 7    |
| G        | 25             | 67    | 8     | 26            | 59    | 15    | 63         | 30    | 7    |
| Total    | 200            | 330   | 170   | 183           | 425   | 92    | 367        | 305   | 28   |
| Average  | 28.57          | 47.14 | 24.29 | 26.14         | 60.71 | 13.14 | 52.43      | 43.57 | 4.00 |
| Std Dev  | 4.50           | 13.50 | 11.46 | 10.90         | 5.12  | 6.06  | 17.75      | 16.13 | 3.77 |

**Secretory - Normal**

| Specimen | Subendometrium |       |       | Midmyometrium |       |      | Subserosal |       |      |
|----------|----------------|-------|-------|---------------|-------|------|------------|-------|------|
|          | C              | C + M | M     | C             | C + M | M    | C          | C + M | M    |
| A        | 42             | 49    | 9     | 44            | 54    | 2    | 55         | 42    | 4    |
| B        | 57             | 36    | 7     | 50            | 45    | 5    | 43         | 53    | 4    |
| C        | 38             | 53    | 9     | 59            | 34    | 7    | 48         | 42    | 10   |
| D        | 36             | 52    | 12    | 54            | 35    | 10   | 43         | 43    | 14   |
| E        | 54             | 34    | 12    | 23            | 72    | 5    | 26         | 64    | 10   |
| F        | 42             | 52    | 6     | 47            | 51    | 2    | 49         | 51    | 0    |
| G        | 48             | 41    | 11    | 58            | 36    | 6    | 66         | 30    | 4    |
| H        | 30             | 63    | 7     | 31            | 67    | 2    | 63         | 31    | 6    |
| I        | 50             | 44    | 6     | 43            | 52    | 5    | 42         | 52    | 6    |
| J        | 34             | 51    | 15    | 30            | 67    | 3    | 52         | 48    | 0    |
| K        | 23             | 57    | 20    | 27            | 67    | 6    | 59         | 34    | 7    |
| Total    | 454            | 532   | 114   | 466           | 581   | 53   | 546        | 490   | 65   |
| Average  | 41.27          | 48.36 | 10.36 | 42.36         | 52.82 | 4.82 | 49.64      | 44.55 | 5.91 |
| Std Dev  | 11.44          | 8.14  | 6.68  | 7.04          | 7.50  | 1.83 | 9.20       | 10.31 | 3.20 |

## Host- Menstrual

| Specimen | Subendometrium |                |            | Midmyometrium |                |            | Subserosal  |                |            |
|----------|----------------|----------------|------------|---------------|----------------|------------|-------------|----------------|------------|
|          | Total Cells    | Positive Cells | % Positive | Total Cells   | Positive Cells | % Positive | Total Cells | Positive Cells | % Positive |
| A        | 106            | 91             | 85.85      | 90            | 74             | 82.22      | 32          | 28             | 87.50      |
|          | 82             | 41             | 50.00      | 53            | 43             | 81.13      | 20          | 13             | 65.00      |
|          | 76             | 28             | 36.84      | 59            | 52             | 88.14      | 3           | 3              | 100.00     |
|          | 70             | 20             | 28.57      | 89            | 81             | 91.01      | 30          | 30             | 100.00     |
|          | 67             | 42             | 62.69      | 84            | 66             | 78.57      | 40          | 36             | 90.00      |
|          | 69             | 18             | 26.09      | 107           | 98             | 91.59      | 36          | 23             | 63.89      |
|          | 100            | 76             | 78.00      | 38            | 32             | 84.21      | 24          | 22             | 91.67      |
|          | 89             | 9              | 10.23      | 72            | 59             | 81.94      | 14          | 14             | 100.00     |
|          | 70             | 15             | 21.43      | 27            | 23             | 85.19      | 49          | 29             | 59.18      |
|          | 98             | 38             | 38.78      | 76            | 61             | 80.26      | 54          | 39             | 72.22      |
| B        | 95             | 33             | 34.74      | 68            | 56             | 82.35      | 18          | 3              | 16.67      |
|          | 97             | 30             | 30.93      | 81            | 65             | 80.25      | 29          | 16             | 55.17      |
|          | 128            | 53             | 41.41      | 72            | 52             | 72.22      | 19          | 13             | 68.42      |
|          | 114            | 18             | 15.79      | 98            | 77             | 78.57      | 28          | 22             | 78.57      |
|          | 104            | 22             | 21.15      | 76            | 62             | 81.58      | 32          | 25             | 78.13      |
|          | 93             | 23             | 24.73      | 68            | 50             | 73.53      | 20          | 11             | 55.00      |
|          | 96             | 30             | 31.25      | 47            | 30             | 63.83      | 29          | 10             | 34.48      |
|          | 91             | 32             | 35.16      | 77            | 53             | 68.83      | 10          | 7              | 70.00      |
|          | 96             | 30             | 31.25      | 44            | 32             | 72.73      | 23          | 14             | 60.87      |
|          | 118            | 40             | 33.90      | 64            | 47             | 73.44      | 13          | 7              | 53.85      |
| C        | 98             | 84             | 85.71      | 80            | 64             | 80.00      | 23          | 23             | 100.00     |
|          | 106            | 45             | 42.45      | 74            | 58             | 78.38      | 25          | 25             | 100.00     |
|          | 95             | 80             | 84.21      | 52            | 39             | 75.00      | 33          | 31             | 93.94      |
|          | 118            | 96             | 81.36      | 41            | 35             | 85.37      | 25          | 21             | 84.00      |
|          | 102            | 48             | 47.06      | 59            | 51             | 86.44      | 60          | 58             | 96.67      |
|          | 105            | 35             | 33.33      | 60            | 50             | 83.33      | 25          | 25             | 100.00     |
|          | 85             | 22             | 25.88      | 69            | 55             | 79.71      | 31          | 29             | 93.55      |
|          | 90             | 28             | 31.11      | 56            | 40             | 71.43      | 32          | 28             | 87.50      |
|          | 91             | 19             | 20.88      | 68            | 55             | 80.88      | 41          | 37             | 90.24      |
|          | 87             | 21             | 24.14      | 78            | 52             | 66.67      | 19          | 19             | 100.00     |
| D        | 114            | 20             | 17.54      | 54            | 38             | 70.37      | 19          | 16             | 84.21      |
|          | 82             | 36             | 43.90      | 69            | 52             | 75.36      | 26          | 18             | 69.23      |
|          | 98             | 33             | 33.67      | 31            | 31             | 100.00     | 16          | 16             | 100.00     |
|          | 98             | 42             | 42.86      | 72            | 56             | 77.78      | 32          | 29             | 90.63      |
|          | 111            | 42             | 37.84      | 77            | 62             | 80.52      | 26          | 21             | 80.77      |
|          | 76             | 19             | 25.00      | 88            | 66             | 75.00      | 11          | 11             | 100.00     |
|          | 99             | 32             | 32.32      | 75            | 55             | 73.33      | 28          | 10             | 35.71      |
|          | 86             | 32             | 37.21      | 75            | 61             | 81.33      | 39          | 31             | 79.49      |
|          | 82             | 21             | 25.61      | 85            | 63             | 74.12      | 3           | 3              | 100.00     |
|          | 72             | 20             | 27.78      | 83            | 73             | 87.95      | 24          | 14             | 58.33      |
| Total    | 2927           | 1066           |            | 2041          | 1580           |            | 759         | 593            |            |
| Average  | 93.83          | 36.15          | 37.96      | 68.40         | 54.23          | 79.36      | 26.53       | 20.75          | 78.62      |
| Std Dev  | 14.72          | 20.41          | 18.66      | 17.65         | 15.29          | 7.17       | 12.06       | 11.29          | 21.07      |

## Host - Proliferative

| Specimen | Subendometrium |                |            | Midmyometrium |                |            | Subserosal  |                |            |
|----------|----------------|----------------|------------|---------------|----------------|------------|-------------|----------------|------------|
|          | Total Cells    | Positive Cells | % Positive | Total Cells   | Positive Cells | % Positive | Total Cells | Positive Cells | % Positive |
| A        | 90             | 12             | 13.33      | 77            | 65             | 85.71      | 30          | 20             | 66.67      |
|          | 131            | 45             | 34.35      | 65            | 57             | 87.69      | 23          | 18             | 78.26      |
|          | 88             | 29             | 32.95      | 82            | 69             | 84.15      | 49          | 36             | 77.55      |
|          | 92             | 20             | 21.74      | 127           | 105            | 82.69      | 44          | 41             | 93.18      |
|          | 107            | 35             | 32.71      | 61            | 52             | 85.25      | 61          | 56             | 91.80      |
|          | 79             | 34             | 43.04      | 77            | 49             | 63.64      | 45          | 33             | 73.33      |
|          | 104            | 15             | 14.42      | 95            | 73             | 76.84      | 47          | 36             | 76.60      |
|          | 114            | 30             | 26.32      | 40            | 21             | 52.50      | 74          | 74             | 100.00     |
|          | 86             | 22             | 25.58      | 75            | 60             | 80.00      | 42          | 37             | 88.10      |
|          | 120            | 25             | 20.83      | 82            | 65             | 79.27      | 26          | 25             | 96.15      |
| B        | 87             | 23             | 34.33      | 120           | 98             | 81.67      | 39          | 35             | 89.74      |
|          | 116            | 15             | 12.93      | 114           | 99             | 86.84      | 33          | 31             | 93.94      |
|          | 66             | 11             | 16.67      | 104           | 59             | 55.77      | 31          | 24             | 77.42      |
|          | 105            | 37             | 35.24      | 77            | 55             | 71.43      | 29          | 25             | 86.21      |
|          | 90             | 26             | 28.89      | 67            | 52             | 77.61      | 62          | 54             | 87.10      |
|          | 89             | 24             | 26.97      | 79            | 65             | 82.28      | 20          | 18             | 90.00      |
|          | 103            | 30             | 29.13      | 89            | 76             | 85.39      | 68          | 63             | 92.65      |
|          | 81             | 20             | 24.69      | 86            | 65             | 75.58      | 18          | 17             | 94.44      |
|          | 76             | 54             | 71.05      | 81            | 68             | 83.95      | 38          | 32             | 84.21      |
|          | 92             | 75             | 81.52      | 75            | 36             | 48.00      | 46          | 40             | 86.96      |
| C        | 99             | 81             | 81.82      | 90            | 69             | 76.67      | 33          | 26             | 78.79      |
|          | 79             | 30             | 37.97      | 96            | 74             | 77.08      | 49          | 26             | 53.06      |
|          | 75             | 23             | 30.67      | 76            | 65             | 85.53      | 47          | 24             | 51.06      |
|          | 106            | 23             | 21.70      | 59            | 54             | 91.53      | 57          | 24             | 41.93      |
|          | 106            | 17             | 16.04      | 77            | 64             | 83.12      | 29          | 21             | 72.41      |
|          | 91             | 12             | 13.19      | 75            | 62             | 82.67      | 83          | 56             | 67.47      |
|          | 97             | 26             | 26.80      | 72            | 65             | 90.28      | 45          | 35             | 77.78      |
|          | 56             | 13             | 23.21      | 81            | 67             | 82.72      | 34          | 22             | 64.71      |
|          | 103            | 17             | 16.50      | 87            | 72             | 82.76      | 35          | 21             | 60.00      |
|          | 92             | 41             | 44.57      | 122           | 89             | 72.95      | 37          | 29             | 78.38      |
| D        | 93             | 25             | 26.88      | 120           | 106            | 88.33      | 39          | 19             | 48.72      |
|          | 84             | 15             | 17.86      | 55            | 44             | 80.00      | 22          | 14             | 63.64      |
|          | 94             | 26             | 27.66      | 87            | 68             | 78.16      | 43          | 16             | 41.86      |
|          | 112            | 38             | 33.93      | 77            | 61             | 79.22      | 39          | 27             | 69.23      |
|          | 101            | 36             | 35.64      | 105           | 78             | 74.29      | 44          | 16             | 36.36      |
|          | 123            | 45             | 36.59      | 80            | 61             | 76.25      | 28          | 21             | 75.00      |
|          | 111            | 38             | 34.23      | 109           | 82             | 75.23      | 38          | 29             | 76.32      |
|          | 77             | 19             | 24.68      | 41            | 29             | 70.73      | 19          | 13             | 68.42      |
|          | 115            | 70             | 60.87      | 82            | 63             | 76.83      | 12          | 9              | 75.00      |
|          | 86             | 23             | 26.74      | 59            | 43             | 72.88      | 32          | 22             | 68.75      |
| E        | 77             | 23             | 29.87      | 96            | 78             | 81.25      | 32          | 15             | 46.88      |
|          | 104            | 48             | 46.15      | 80            | 66             | 82.50      | 28          | 20             | 71.43      |
|          | 92             | 20             | 21.74      | 69            | 56             | 81.16      | 30          | 12             | 40.00      |
|          | 117            | 23             | 19.66      | 89            | 80             | 89.89      | 31          | 11             | 35.48      |
|          | 76             | 19             | 25.00      | 58            | 44             | 75.86      | 42          | 33             | 78.57      |
|          | 108            | 25             | 23.15      | 38            | 27             | 71.05      | 42          | 26             | 61.90      |
|          | 131            | 24             | 18.32      | 72            | 61             | 84.72      | 20          | 13             | 65.00      |
|          | 116            | 41             | 35.34      | 71            | 2              | 2.82       | 44          | 26             | 59.09      |
|          | 74             | 23             | 31.08      | 78            | 63             | 80.77      | 53          | 42             | 79.25      |
|          | 122            | 65             | 53.28      | 54            | 36             | 66.67      | 59          | 33             | 55.93      |
| F        | 123            | 97             | 78.86      | 34            | 20             | 58.82      | 46          | 38             | 82.61      |
|          | 95             | 67             | 70.53      | 104           | 61             | 58.65      | 65          | 39             | 60.00      |
|          | 97             | 48             | 49.48      | 79            | 67             | 84.81      | 23          | 10             | 43.48      |
|          | 77             | 34             | 44.16      | 96            | 69             | 71.88      | 31          | 22             | 70.97      |
|          | 86             | 66             | 76.74      | 79            | 57             | 72.15      | 6           | 5              | 83.33      |
|          | 106            | 95             | 89.62      | 60            | 58             | 96.67      | 36          | 31             | 86.11      |
|          | 109            | 74             | 67.89      | 97            | 92             | 94.85      | 8           | 8              | 100.00     |
|          | 89             | 50             | 56.18      | 106           | 100            | 94.34      | 36          | 23             | 63.89      |
|          | 90             | 53             | 58.89      | 81            | 56             | 69.14      | 41          | 30             | 73.17      |
|          | 87             | 20             | 22.99      | 85            | 64             | 75.29      | 44          | 34             | 77.27      |
| Total    | 5772           | 2115           | 36.65      | 4855          | 3762           | 77.49      | 2287        | 1660           | 72.63      |
| Average  | 96.20          | 35.25          | 36.65      | 80.92         | 62.70          | 76.80      | 38.12       | 27.67          | 72.51      |
| Std Dev  | 16.72          | 20.90          | 19.72      | 20.33         | 20.17          | 13.70      | 14.98       | 13.60          | 16.18      |

## Host - Secretary

| Specimen | Subendometrium |                |            | Midmyometrium |                |            | Subsarcosal |                |            |
|----------|----------------|----------------|------------|---------------|----------------|------------|-------------|----------------|------------|
|          | Total Cells    | Positive Cells | % Positive | Total Cells   | Positive Cells | % Positive | Total Cells | Positive Cells | % Positive |
| A        | 121            | 35             | 28.93      | 114           | 61             | 71.05      | 25          | 19             | 76.00      |
|          | 186            | 60             | 32.26      | 50            | 28             | 56.00      | 34          | 12             | 35.29      |
|          | 161            | 93             | 57.76      | 106           | 76             | 71.70      | 29          | 15             | 51.72      |
|          | 156            | 105            | 67.31      | 86            | 58             | 67.44      | 83          | 53             | 63.85      |
|          | 97             | 43             | 44.33      | 47            | 26             | 55.32      | 69          | 41             | 59.42      |
|          | 129            | 73             | 56.59      | 57            | 25             | 43.86      | 54          | 18             | 33.33      |
|          | 141            | 45             | 31.91      | 98            | 65             | 66.33      | 92          | 70             | 76.09      |
|          | 251            | 81             | 32.27      | 45            | 30             | 66.67      | 34          | 9              | 26.47      |
|          | 52             | 27             | 51.92      | 52            | 34             | 65.38      | 78          | 46             | 58.97      |
|          | 155            | 104            | 67.10      | 138           | 84             | 60.87      | 88          | 57             | 64.77      |
| B        | 114            | 72             | 63.16      | 67            | 49             | 73.13      | 91          | 85             | 93.41      |
|          | 82             | 34             | 41.45      | 98            | 75             | 76.53      | 58          | 34             | 58.62      |
|          | 68             | 27             | 39.71      | 105           | 53             | 50.48      | 34          | 14             | 41.18      |
|          | 103            | 31             | 30.10      | 56            | 31             | 55.36      | 38          | 26             | 68.42      |
|          | 85             | 22             | 25.88      | 67            | 45             | 67.16      | 36          | 11             | 30.56      |
|          | 97             | 33             | 34.02      | 76            | 43             | 56.58      | 30          | 12             | 40.00      |
|          | 84             | 32             | 38.10      | 85            | 58             | 68.24      | 59          | 24             | 40.68      |
|          | 127            | 36             | 28.35      | 45            | 28             | 62.22      | 63          | 53             | 84.13      |
|          | 108            | 44             | 40.74      | 92            | 70             | 76.08      | 31          | 22             | 70.97      |
|          | 103            | 50             | 48.54      | 54            | 31             | 57.41      | 92          | 50             | 54.35      |
| C        | 98             | 38             | 38.78      | 77            | 60             | 77.92      | 34          | 26             | 76.47      |
|          | 124            | 42             | 33.87      | 123           | 93             | 75.61      | 38          | 24             | 63.16      |
|          | 119            | 39             | 32.77      | 64            | 45             | 70.31      | 77          | 45             | 58.44      |
|          | 173            | 45             | 26.01      | 99            | 74             | 74.75      | 62          | 44             | 70.97      |
|          | 157            | 29             | 18.47      | 50            | 34             | 68.00      | 83          | 33             | 39.76      |
|          | 184            | 84             | 45.65      | 128           | 85             | 66.41      | 49          | 29             | 59.18      |
|          | 96             | 51             | 53.13      | 91            | 61             | 67.03      | 16          | 10             | 62.50      |
|          | 106            | 46             | 43.40      | 95            | 67             | 70.53      | 50          | 32             | 64.00      |
|          | 80             | 29             | 36.25      | 94            | 62             | 65.96      | 45          | 13             | 28.89      |
|          | 67             | 26             | 38.81      | 46            | 32             | 69.57      | 74          | 48             | 64.86      |
| D        | 69             | 27             | 39.13      | 46            | 32             | 69.57      | 31          | 26             | 83.87      |
|          | 83             | 21             | 25.30      | 90            | 75             | 83.33      | 35          | 23             | 65.71      |
|          | 67             | 15             | 22.39      | 72            | 65             | 90.28      | 46          | 34             | 73.91      |
|          | 128            | 42             | 32.81      | 51            | 32             | 62.75      | 39          | 34             | 87.18      |
|          | 107            | 44             | 41.12      | 65            | 45             | 69.23      | 50          | 35             | 70.00      |
|          | 100            | 48             | 48.00      | 101           | 84             | 83.17      | 25          | 10             | 40.00      |
|          | 63             | 15             | 23.81      | 81            | 70             | 86.42      | 61          | 41             | 67.21      |
|          | 96             | 16             | 16.67      | 76            | 55             | 72.37      | 61          | 51             | 83.61      |
|          | 56             | 21             | 37.50      | 71            | 48             | 67.61      | 25          | 15             | 60.00      |
|          | 133            | 25             | 18.80      | 55            | 55             | 100.00     | 29          | 17             | 58.62      |
| E        | 89             | 24             | 26.97      | 61            | 38             | 62.30      | 68          | 45             | 66.18      |
|          | 94             | 21             | 22.34      | 52            | 31             | 59.62      | 35          | 23             | 65.71      |
|          | 130            | 38             | 29.23      | 65            | 49             | 75.38      | 37          | 15             | 40.54      |
|          | 93             | 25             | 26.88      | 91            | 64             | 70.33      | 57          | 37             | 64.91      |
|          | 127            | 31             | 24.41      | 83            | 61             | 73.49      | 49          | 27             | 55.10      |
|          | 114            | 22             | 19.30      | 91            | 63             | 69.23      | 59          | 30             | 50.85      |
|          | 90             | 5              | 5.56       | 107           | 74             | 69.16      | 29          | 20             | 68.97      |
|          | 129            | 32             | 24.81      | 96            | 78             | 81.25      | 26          | 26             | 100.00     |
|          | 112            | 17             | 15.18      | 88            | 61             | 69.32      | 44          | 31             | 70.45      |
|          | 94             | 26             | 27.66      | 54            | 30             | 55.56      | 33          | 33             | 100.00     |
| F        | 64             | 22             | 34.38      | 71            | 51             | 71.83      | 42          | 32             | 76.19      |
|          | 69             | 23             | 33.33      | 67            | 61             | 91.04      | 25          | 21             | 84.00      |
|          | 91             | 48             | 52.75      | 86            | 81             | 94.19      | 31          | 13             | 41.94      |
|          | 109            | 42             | 38.53      | 78            | 69             | 88.46      | 53          | 40             | 75.47      |
|          | 105            | 52             | 49.52      | 46            | 37             | 80.43      | 31          | 18             | 58.06      |
|          | 91             | 32             | 35.16      | 87            | 72             | 82.76      | 39          | 32             | 82.05      |
|          | 78             | 24             | 30.77      | 78            | 59             | 75.64      | 50          | 31             | 62.00      |
|          | 93             | 39             | 41.94      | 79            | 58             | 73.42      | 24          | 18             | 75.00      |
|          | 81             | 21             | 25.93      | 84            | 67             | 79.76      | 51          | 35             | 68.63      |
|          | 67             | 24             | 35.82      | 80            | 47             | 58.75      | 41          | 27             | 65.85      |
| G        | 117            | 53             | 45.30      | 108           | 87             | 80.56      | 29          | 19             | 65.52      |
|          | 112            | 22             | 19.64      | 74            | 44             | 59.46      | 30          | 22             | 73.33      |
|          | 83             | 22             | 26.51      | 87            | 41             | 47.13      | 10          | 8              | 80.00      |
|          | 94             | 14             | 14.89      | 75            | 55             | 73.33      | 39          | 25             | 64.10      |
|          | 97             | 32             | 32.99      | 107           | 77             | 71.96      | 32          | 30             | 93.75      |
|          | 45             | 13             | 28.89      | 99            | 64             | 64.65      | 31          | 17             | 54.84      |
|          | 95             | 26             | 27.37      | 104           | 82             | 78.85      | 13          | 13             | 100.00     |
|          | 82             | 15             | 18.29      | 37            | 24             | 64.86      | 43          | 34             | 79.07      |
|          | 132            | 35             | 26.52      | 72            | 46             | 63.89      | 75          | 60             | 80.00      |
|          | 66             | 14             | 21.21      | 78            | 50             | 64.10      | 77          | 57             | 74.03      |
| H        | 85             | 18             | 21.18      | 84            | 68             | 80.95      | 22          | 16             | 72.73      |
|          | 96             | 34             | 35.42      | 38            | 28             | 73.68      | 19          | 12             | 63.16      |
|          | 105            | 25             | 23.81      | 81            | 58             | 71.60      | 64          | 50             | 78.13      |
|          | 92             | 22             | 23.91      | 99            | 71             | 71.72      | 31          | 22             | 70.97      |
|          | 110            | 32             | 29.09      | 97            | 59             | 60.82      | 17          | 11             | 64.71      |
|          | 46             | 15             | 32.61      | 62            | 50             | 80.65      | 19          | 10             | 52.63      |
|          | 59             | 20             | 33.90      | 48            | 33             | 68.75      | 30          | 28             | 93.33      |
|          | 88             | 27             | 30.68      | 108           | 81             | 75.00      | 53          | 33             | 62.26      |
|          | 103            | 30             | 29.13      | 30            | 27             | 90.00      | 26          | 20             | 76.92      |
|          | 88             | 16             | 18.18      | 85            | 47             | 55.29      | 29          | 21             | 72.41      |
| Total    | 8241           | 2803           | 34.01      | 6261          | 4415           | 70.52      | 3581        | 2323           | 64.87      |
| Average  | 96.00          | 28.40          | 30.02      | 77.10         | 57.55          | 74.21      | 41.20       | 27.70          | 66.60      |
| Std Dev  | 19.65          | 11.09          | 10.99      | 15.74         | 15.00          | 10.74      | 12.44       | 8.52           | 15.77      |

## Tumour - Menstrual

| Specimen | Fibroids    |               |            |
|----------|-------------|---------------|------------|
|          | Total Cells | Positive Cell | % Positive |
| AFL      | 63          | 63            | 100.00     |
|          | 45          | 44            | 97.78      |
|          | 51          | 30            | 58.82      |
|          | 45          | 26            | 57.78      |
|          | 49          | 20            | 40.82      |
|          | 44          | 24            | 54.55      |
|          | 82          | 48            | 58.54      |
|          | 58          | 36            | 62.07      |
|          | 52          | 18            | 34.62      |
|          | 57          | 32            | 56.14      |
| BFL      | 51          | 43            | 84.31      |
|          | 82          | 78            | 92.59      |
|          | 54          | 50            | 92.59      |
|          | 59          | 44            | 74.58      |
|          | 65          | 49            | 75.28      |
|          | 57          | 55            | 96.25      |
|          | 39          | 30            | 76.92      |
|          | 53          | 36            | 67.92      |
|          | 51          | 40            | 78.43      |
|          | 60          | 28            | 46.67      |
| CFL      | 27          | 23            | 85.19      |
|          | 15          | 15            | 100.00     |
|          | 59          | 51            | 86.44      |
|          | 54          | 38            | 66.67      |
|          | 90          | 82            | 91.11      |
|          | 59          | 28            | 47.46      |
|          | 78          | 54            | 69.23      |
|          | 90          | 66            | 73.33      |
|          | 80          | 43            | 53.75      |
|          | 63          | 20            | 31.75      |
| DFL      | 35          | 34            | 97.14      |
|          | 53          | 51            | 96.23      |
|          | 49          | 31            | 63.27      |
|          | 40          | 22            | 55.00      |
|          | 23          | 14            | 60.87      |
|          | 50          | 33            | 66.00      |
|          | 33          | 22            | 66.67      |
|          | 38          | 35            | 92.11      |
|          | 34          | 27            | 79.41      |
|          | 32          | 13            | 40.63      |
| Total    | 1573        | 1152          |            |
| Average  | 52.98       | 37.33         | 70.76      |
| Std Dev  | 17.34       | 16.51         | 19.45      |

## Tumour - Proliferative

| Specimen | Fibroids Small |               |            |
|----------|----------------|---------------|------------|
|          | Total Cells    | Positive Cell | % Positive |
| AFS      | 67             | 52            | 77.61      |
|          | 31             | 26            | 83.87      |
|          | 30             | 27            | 90.00      |
|          | 35             | 32            | 91.43      |
|          | 27             | 24            | 88.89      |
|          | 49             | 36            | 73.47      |
|          | 51             | 29            | 56.86      |
|          | 32             | 28            | 87.50      |
|          | 27             | 17            | 62.96      |
|          | 66             | 41            | 62.12      |
| BFS      | 81             | 66            | 81.48      |
|          | 64             | 47            | 73.44      |
|          | 70             | 50            | 71.43      |
|          | 29             | 25            | 86.21      |
|          | 80             | 57            | 71.25      |
|          | 59             | 49            | 83.05      |
|          | 58             | 45            | 77.59      |
|          | 33             | 27            | 81.82      |
|          | 27             | 25            | 92.59      |
|          | 12             | 8             | 66.67      |
| CFS      | 39             | 22            | 56.41      |
|          | 91             | 58            | 63.74      |
|          | 80             | 47            | 58.75      |
|          | 37             | 20            | 54.05      |
|          | 50             | 34            | 68.00      |
|          | 26             | 26            | 100.00     |
|          | 62             | 52            | 83.87      |
|          | 77             | 53            | 68.83      |
|          | 59             | 42            | 71.19      |
|          | 66             | 66            | 100.00     |
| DFS      | 40             | 34            | 85.00      |
|          | 73             | 61            | 83.56      |
|          | 64             | 48            | 75.00      |
|          | 73             | 67            | 91.78      |
|          | 71             | 56            | 78.87      |
|          | 51             | 50            | 98.04      |
|          | 98             | 72            | 73.47      |
|          | 51             | 41            | 80.39      |
|          | 87             | 27            | 31.03      |
|          | 38             | 37            | 97.37      |
| AFL      | 71             | 65            | 91.55      |
|          | 27             | 19            | 70.37      |
|          | 42             | 40            | 95.24      |
|          | 55             | 55            | 100.00     |
|          | 75             | 75            | 100.00     |
|          | 62             | 39            | 62.90      |
|          | 71             | 70            | 98.59      |
|          | 80             | 70            | 87.50      |
|          | 70             | 67            | 95.71      |
|          | 71             | 71            | 100.00     |
| BFL      | 63             | 50            | 79.37      |
|          | 49             | 42            | 85.71      |
|          | 54             | 42            | 77.78      |
|          | 58             | 40            | 68.97      |
|          | 41             | 35            | 85.37      |
|          | 48             | 32            | 66.67      |
|          | 84             | 34            | 40.48      |
|          | 56             | 38            | 67.86      |
|          | 50             | 12            | 24.00      |
|          | 41             | 12            | 29.27      |
| CFL      | 44             | 44            | 100.00     |
|          | 26             | 16            | 61.54      |
|          | 36             | 32            | 88.89      |
|          | 82             | 59            | 71.95      |
|          | 74             | 58            | 78.38      |
|          | 40             | 39            | 97.50      |
|          | 56             | 42            | 75.00      |
|          | 66             | 58            | 87.88      |
|          | 35             | 33            | 94.29      |
|          | 69             | 69            | 100.00     |
| DFL      | 71             | 29            | 40.85      |
|          | 47             | 18            | 38.30      |
|          | 46             | 25            | 54.35      |
|          | 19             | 4             | 21.05      |
|          | 51             | 28            | 54.90      |
|          | 60             | 44            | 73.33      |
|          | 68             | 36            | 52.94      |
|          | 39             | 18            | 46.15      |
|          | 57             | 19            | 33.33      |
|          | 25             | 12            | 48.00      |
| EFL      | 72             | 47            | 65.28      |
|          | 122            | 71            | 58.20      |
|          | 72             | 41            | 56.94      |
|          | 75             | 24            | 32.00      |
|          | 26             | 20            | 76.92      |
|          | 56             | 40            | 71.43      |
|          | 81             | 52            | 64.20      |
|          | 83             | 36            | 43.37      |
|          | 69             | 25            | 36.23      |
|          | 69             | 47            | 68.12      |
| Total    | 5025           | 3616          |            |
| Average  | 55.83          | 40.18         | 72.31      |
| Std Dev  | 19.93          | 17.18         | 19.82      |

## Tumour - Secretory

| Specimen | Fibroids    |               |            |
|----------|-------------|---------------|------------|
|          | Total Cells | Positive Cell | % Positive |
| AFL      | 59          | 42            | 71.19      |
|          | 96          | 60            | 62.50      |
|          | 106         | 41            | 38.68      |
|          | 90          | 27            | 30.00      |
|          | 42          | 26            | 61.90      |
|          | 106         | 56            | 52.83      |
|          | 102         | 55            | 53.92      |
|          | 102         | 48            | 47.06      |
|          | 43          | 30            | 69.77      |
|          | 67          | 44            | 65.67      |
| BFL      | 71          | 40            | 56.34      |
|          | 85          | 35            | 41.18      |
|          | 105         | 50            | 47.62      |
|          | 55          | 23            | 41.82      |
|          | 49          | 35            | 71.43      |
|          | 84          | 25            | 29.76      |
|          | 61          | 32            | 52.46      |
|          | 72          | 52            | 72.22      |
|          | 37          | 19            | 51.35      |
|          | 70          | 42            | 60.00      |
| AFS      | 25          | 13            | 52.00      |
|          | 40          | 30            | 75.00      |
|          | 36          | 30            | 83.33      |
|          | 60          | 45            | 75.00      |
|          | 33          | 18            | 54.55      |
|          | 57          | 26            | 45.61      |
|          | 30          | 19            | 63.33      |
|          | 46          | 35            | 76.09      |
|          | 42          | 20            | 47.62      |
|          | 34          | 26            | 76.47      |
| CFL      | 111         | 49            | 44.14      |
|          | 70          | 23            | 32.86      |
|          | 116         | 55            | 47.41      |
|          | 93          | 25            | 26.88      |
|          | 53          | 26            | 49.06      |
|          | 71          | 20            | 28.17      |
|          | 61          | 23            | 37.70      |
|          | 113         | 60            | 53.10      |
|          | 60          | 38            | 63.33      |
|          | 87          | 67            | 77.01      |
| BFS      | 53          | 17            | 32.08      |
|          | 39          | 27            | 69.23      |
|          | 41          | 14            | 34.15      |
|          | 31          | 22            | 70.97      |
|          | 56          | 30            | 53.57      |
|          | 68          | 38            | 55.88      |
|          | 61          | 35            | 57.38      |
|          | 56          | 49            | 87.50      |
|          | 42          | 26            | 61.90      |
|          | 26          | 22            | 84.62      |
| CFS      | 36          | 23            | 63.89      |
|          | 80          | 33            | 41.25      |
|          | 48          | 39            | 81.25      |
|          | 61          | 33            | 54.10      |
|          | 72          | 39            | 54.17      |
|          | 46          | 34            | 73.91      |
|          | 48          | 39            | 81.25      |
|          | 49          | 29            | 59.18      |
|          | 56          | 42            | 75.00      |
|          | 66          | 39            | 59.09      |
| DFS      | 70          | 28            | 40.00      |
|          | 111         | 79            | 71.17      |
|          | 55          | 26            | 47.27      |
|          | 81          | 46            | 56.79      |
|          | 79          | 33            | 41.77      |
|          | 131         | 88            | 67.18      |
|          | 26          | 12            | 46.15      |
|          | 34          | 14            | 41.18      |
|          | 33          | 12            | 36.36      |
|          | 47          | 12            | 25.53      |
| EFS      | 23          | 14            | 60.87      |
|          | 24          | 15            | 79.17      |
|          | 40          | 7             | 17.50      |
|          | 59          | 18            | 30.51      |
|          | 36          | 23            | 63.89      |
|          | 82          | 51            | 62.20      |
|          | 116         | 84            | 72.41      |
|          | 127         | 101           | 79.53      |
|          | 55          | 19            | 34.55      |
|          | 91          | 40            | 43.96      |
| DFL      | 52          | 31            | 59.62      |
|          | 67          | 53            | 78.10      |
|          | 51          | 31            | 60.78      |
|          | 72          | 47            | 65.28      |
|          | 42          | 32            | 76.19      |
|          | 58          | 44            | 75.00      |
|          | 85          | 56            | 65.88      |
|          | 23          | 18            | 78.26      |
|          | 33          | 31            | 93.94      |
|          | 59          | 51            | 86.44      |
| Total    | 4824        | 2755          |            |
| Average  | 61.45       | 35.30         | 56.66      |
| Std Dev  | 26.19       | 19.49         | 16.73      |

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