

STUDIES ON EPITHELIAL CELLS FROM THE HUMAN
UTERINE CERVIX IN MONOLAYER TISSUE CULTURE

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DEDICATION

To my parents

ACKNOWLEDGMENTS

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ABSTRACT

A project was designed to study a variety of aspects of cervical pathologies in an in vitro model, by light and scanning electron microscopy.

This thesis describes the use of human uterine ectocervical and endocervical cell cultures in monolayer tissue culture to study the growth of vaginal pathogens; the action of antifungal drugs on fungi infected cervical cultures, the growth of neoplastic cells in culture; the possible protective effect of zinc against the growth inhibiting effect of a concentration of copper found in the uterine secretions of women using copper containing contraceptive devices.

The cultures were inoculated with vaginal isolates of fungus. Candida albicans, Candida krusei, Candida tropicalis, Torulopsis glabrata and Saccharomyces cerevisiae and their growth on the cultures was described by a scanning electron microscopy study. Three stages of infection were described for Candida albicans. These were the attachment of yeast cells, the growth of germ tubes and the invasion of epithelial cells by hyphae.

The action of three azole antifungal drugs, clotrimazole, bifonazole and an oral agent viburazole were assessed on attachment of yeast cells to epithelial cells and the production of germ tubes. Attachment of yeasts was reduced by 80 per cent of the controls at the maximum concentration achieved in aqueous solution for clotrimazole at 0.15 µg per ml and for bifonazole at 0.28 µg/ml and for vibunazole at 400 µg/ml. Germ tube growth was reduced when treated with minimum concentrations of the drug at 5 ng per ml for clotrimazole and bifonazole and 5 µg per ml.

A scanning electron microscopy study of inclusion bodies of Chlamydia trachomatis in endocervical cells in cultures is described.

Cultures of cervical neoplastic cells were set up and three methods for the quantitative assessment of loss of contact inhibition were compared

Zinc at a concentration known not to affect ectocervical cell growth was tested for its possible protective effect over a concentration of copper known to cause reduction in cell growth.

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CHAPTER 1

INTRODUCTION

The position of the cervix at the top of the vagina leaves it exposed to the external environment and consequently it may be subjected to attack by chemicals, bacteria, viruses and fungi and, it has been suggested, by components in the male ejaculate. This may lead to cervical pathology including infections, abnormal growth of cells and even carcinoma of the cervix. The internal position of the cervix means that abnormalities and disease may not be noticed immediately and deterioration in the cervical tissues may be quite advanced before detection, particularly with carcinoma. In some cases the changes are only discovered during routine antenatal examinations. Increased awareness of occult cervical pathology led to the introduction of screening women regularly in an effort to detect signs of disease for early treatment. Screening and detection procedures have become high in priority in research concerned with the cervix, so that methods for the study of cervical tissue are continually being improved. Models discovered experimentally during the study of cervical pathology have added to knowledge of the cellular biology of the cervix. Techniques used are either derived from other research or have been invented for experiments on the cervix and have proved useful in other areas of research. One such technique is tissue culture.

Tissue culture techniques were described in the nineteenth century and are still expanding as a field of research in their own right, not just as tools in research. This thesis is based on a study of human uterine cervical epithelial cells in tissue culture in relation to growth of normal and abnormal cells; the development of bacterial, fungal and viral infections and antifungal treatment, and the action of heavy metal ions.

THE CERVIX

Embryological derivation

The gonads first appear in a human embryo four weeks after fertilisation. They are formed by proliferation of the coelomic epithelium and a condensation of underlying mesenchyme, which is derived from mesodermal cells, and lie as genital or gonadal ridges on each side of the midline between the mesonephros and the dorsal mesentery. The germ cells do not appear until six weeks after fertilisation and they arise in the wall of the yolk sac and migrate by amoeboid movement to the genital ridges. The coelomic epithelium of the genital ridge proliferates into the mesenchyme forming primitive sex cords which enclose the germ cells. This process occurs in both male and female embryos.

In the female the primitive sex cords are broken up into irregular cell clusters by invading mesenchyme. These cell clusters eventually disappear and are replaced by vascular stroma which forms the ovarian medulla. The surface epithelium of the female gonad remains thick and continues to proliferate, giving rise to a second generation of cords called the cortical cords which remain close to the surface epithelium. In the fourth month of fetal development these cords are also split into isolated cell clusters, each surrounding one or more primitive germ cells into follicular cells.

At six weeks after fertilisation the genital regions of both male and female embryos have two ducts; the Wolffian and mullerian ducts, which are also called the mesonephric and paramesonephric ducts respectively. The cranial end of the mullerian duct opens into the coelomic cavity and both ducts end on the

posterior wall of the urogenital sinus.

The mullerian ducts fuse at midline and are separated by a septum and present a small swelling on the inside of the sinus, the mullerian tubercle. The Wolffian ducts open into the urogenital sinus on either side of the mullerian tubercle. If the embryo is genetically female the Wolffian ducts disappear and the mullerian ducts develop into the oviducts and uterus (Figure 1).

At this stage three parts can be recognised in the mullerian duct. The genital ridge, which has rounded and developed into an oval shape and become the ovary, descends towards the caudal region to the embryo, pushes the upper part of the mullerian duct down and comes to lie transversely above the duct. The coelomic opening in the duct becomes the abdominal ostium of the fallopian tube and later develops into the fimbriae of the fallopian tube. The developing mullerian ducts fuse at the mid-line and above this a transverse pelvic fold formed from the lateral sides of the fused ducts becoming the broad ligament of the uterus, which holds the oviducts and ovaries in place, (Figure 2).

The best accepted explanation for the development of the vagina is the following. The solid caudal top of the fused mullerian ducts reach the posterior wall of the urogenital sinus at nine weeks post fertilisation. During this growth period the uterine septum separating the mullerian ducts disappears to form the lumen of the uterus. This fused part of the mullerian ducts gives rise to the corpus and the cervix of the uterus and they are surrounded by a layer of mesenchyme which thickens to form the muscular coat, the myometrium (Figure 3).

The vagina forms from two solid evaginations which grow out from the pelvic part of the urogenital sinus known as the sinovaginal bulbs. These proliferate strongly and form a solid plate known as the vaginal plate. The plate enfolds the solid end of the uterus and begins to form a lumen from its caudal end in embryos eleven weeks old. By five months the vaginal outgrowth is

Figure 1

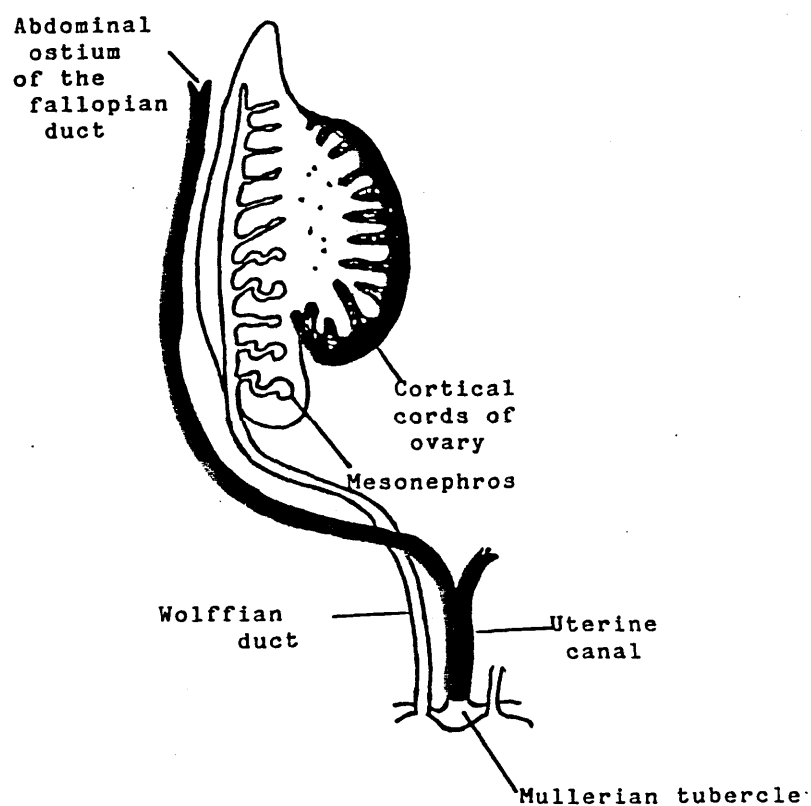


Diagram of the female genital ducts at eight weeks after fertilisation.
Drawn by hand from an illustration by J. Ireland
In: Medical Embryology, J Langman. 3rd. Ed.
Williams and Wilkins. Baltimore. 1975.

Figure 2

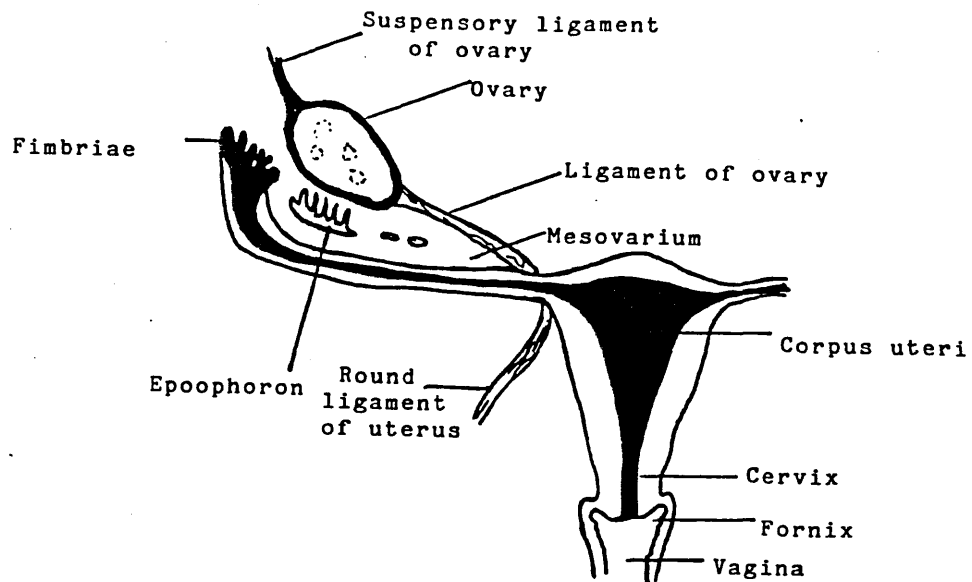


Diagram of the genital ducts after the descent of the ovary. The Wolffian duct has almost disappeared the remains being the epoophoron, and paraoophoron. The mullerian duct has become the Fallopian tube and uterus.

Figure 3

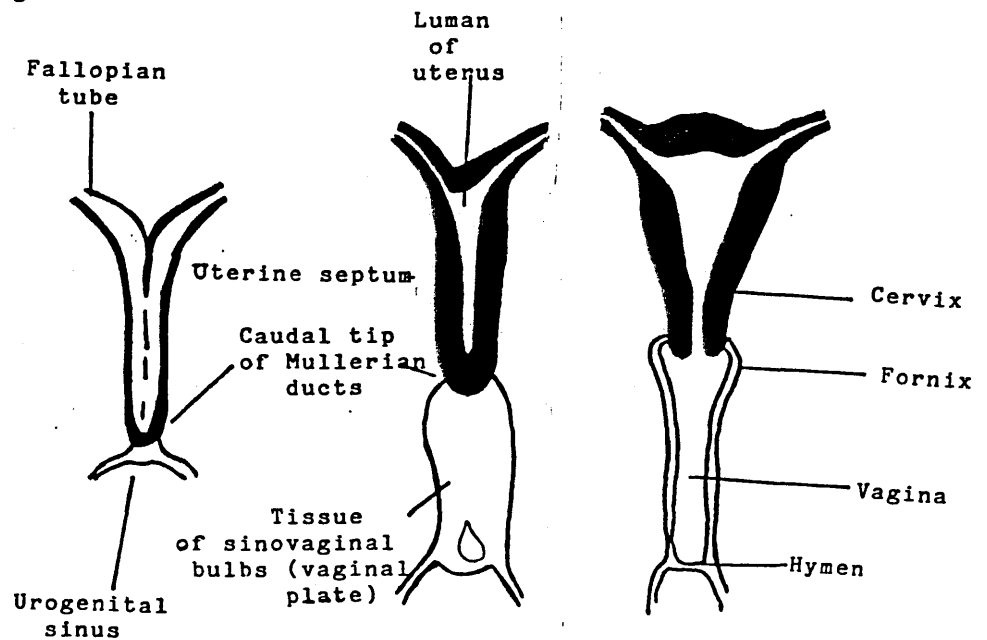


Diagram showing the formation of the uterus and vagina formed one week after the descent of the ovary.
 Drawn by hand from illustrations in; Medical Embryology.

longer and entirely canalised and the wing-like expansions of the plate around the end of the uterus form the vaginal fornices (Langman, 1973).

The mullerian epithelium in contact with the urogenital sinus is composed of pseudostratified columnar cells. In the medial walls of the Wolffian ducts the epithelium appears differentiated and extends into the dorsal wall of the urogenital sinus in the region of the mullerian tubercle. It is distinct from the typical sinus epithelium but the mullerian epithelium is in contact with this differentiated epithelium, a fact which may influence the exact derivation of cervical cells.

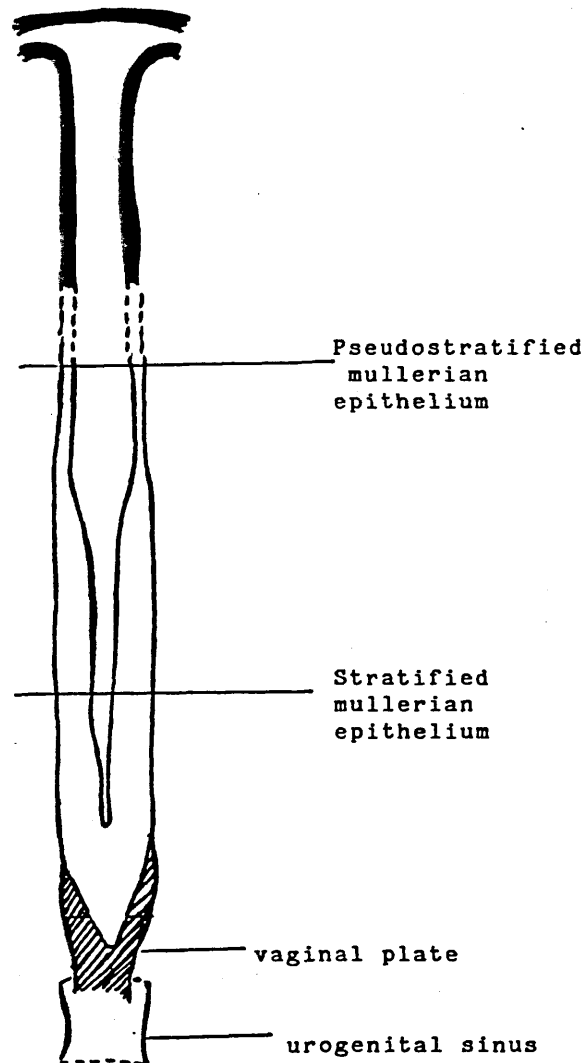
In the region of contact between the uterus and the urogenital sinus the uterine tissue changes transiently into stratified mullerian epithelium. Above this is pseudostratified mullerian epithelium and above this columnar. Between the dorsal wall of the urogenital sinus and the stratified mullerian epithelium a solid epithelial cell plate, the vaginal plate, appears. In the later stages of development the stratified epithelium of the uterus becomes reabsorbed until the vaginal plate cells border the columnar mullerian epithelium of the lowest part of the cervical canal. After this phase the border between the columnar mullerian epithelium and the vaginal plate epithelium becomes fixed at a point just inside the vaginal orifice. This is the squamo-columnar junction (Figure 4).

Histological staining for enzymes has revealed that there were greater similarities between Wolffian and vaginal plate epithelium than between vaginal plate and sinus epithelium. (Forsberg, 1963).

Anatomy of the cervix

The cervix is the lower part of the uterus. It is divided from the upper part by a fibromuscular junction, the internal os. It projects into the vagina through the apex wall and is surrounded by vaginal skin which forms the vaginal fornices. The cervix is cylindrical in shape, about 3 cm in length and

Figure 4



Diagrammatic representation of the regions of different epithelial cells in the human fetal uterivaginal canal.

Drawn by hand from a diagram in The Cervix. (1976) Ed. J. Jorden and A. Singer.

Figure 6

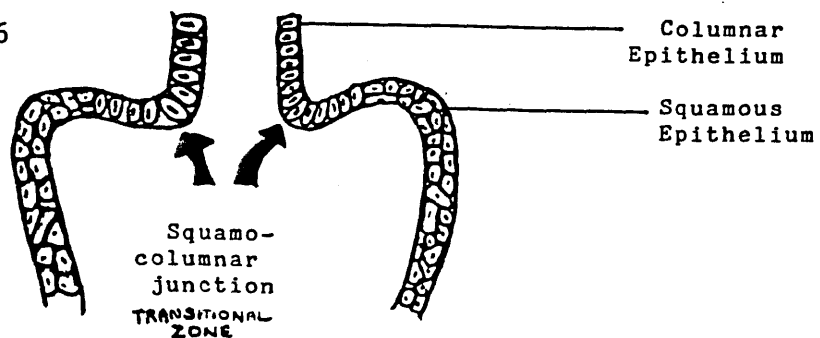


Diagram of the region of the transitional zone in the human cervix.

Drawn by hand from a diagram in The Cervix (1976) Ed. J. Jorden and A. Singer.

2.5 cm in diameter in the nulliparous woman and larger, usually more bulbous, with a horizontal external os in the multiparous woman. (Figure 5)

The main tissue constituent of the cervix is fibrous connective tissue containing isolated smooth muscle fibres and smooth muscle bands. The cervix, including the ducts and crypts of the cervical glands, is lined with mucin secreting columnar epithelium. The ectocervix is covered with stratified squamous epithelium which blends peripherally into the epithelium of the vagina. These two areas of cells are divided by a region of variable width; the squamo-columnar junction. (Figure 6). At certain times in the life of the female cervix: late fetal life, adolescence and pregnancy, alterations occur within the original columnar epithelium which result in formation of metaplastic squamous epithelium.

Ultrastructure of squamous epithelium of the ectocervix

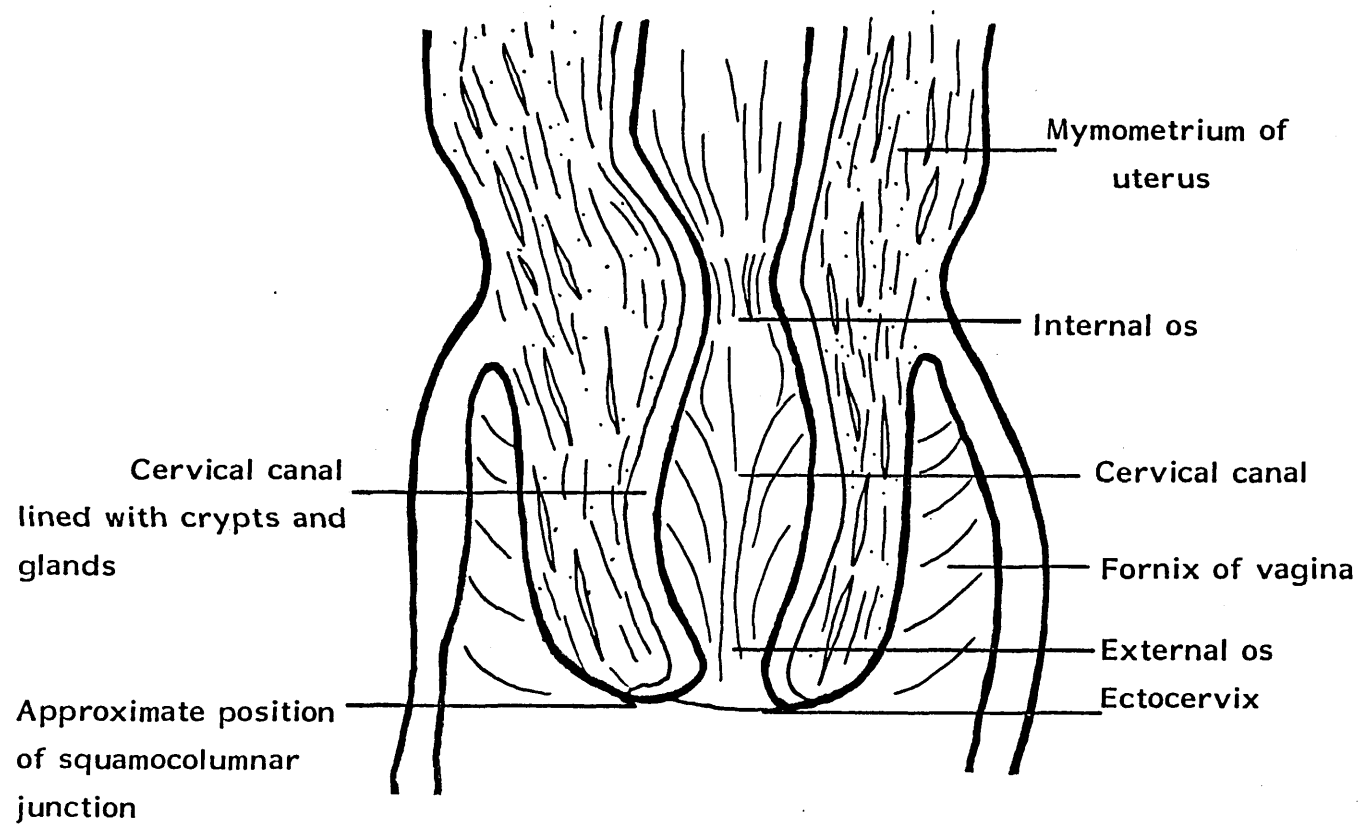
Under the light microscope the squamous epithelium has five zones or layers:-

The stratum cylindricum is in contact with the basement membrane. It consists of a single row of neatly arranged cells with a large nuclei. The cells may be either tightly packed or have intercellular spaces. The bases are convoluted.

The stratum spinosum profundum also called the parabasal cell layer (Papanicolaou, 1954; Frost 1961) and the prickle cell layer (Friedell et al., 1960). The cells in this layer are larger and have wider intercellular spaces. These polyhedral cells also have large nuclei and numerous cytoplasmic processes called intercellular bridges. Cells can be found in mitosis in this layer.

Figure 5

The cervix of the human uterus.



The stratum spinosum superficial cells are flatter, have smaller intercellular spaces and short cytoplasmic bridges. The cells have a glycogen-rich cytoplasm which often has large vacuoles. This is also called the intermediate navicular or clear layer.

The stratum granulosum, also called the intra-epithelial zone or the condensation zone, consists of a number of closely packed polyhedral cells which have keratohyaline granules (Hackman *et al.*, 1968).

The stratum corneum, or functionalis cells are elongated and flat. The nuclei are small and the cell wall is thickened. They are most plentiful at times of high oestrogen levels and they show a degree of keratinisation.

Ultra-structure of columnar epithelium

Columnar epithelium lines the endocervical canal and occasionally extends on to the ectocervix. These tall cells are sometimes ciliated. They are arranged in rows and actively secrete mucins. They stain deep magenta red with periodic Schiff reagent (periodic-acid leukofuchsin). It is because they are arranged in single rows that resemble a picket fence that they have been called picket cells. (Novak and Novak, 1958).

The nuclei are round or oval and are generally situated in the lower third of the cell although during active secretion as in pregnancy or at mid-cycle ovulation, they are found in the middle of the cell.

The epithelium exists in two general forms. On the lip of the cervix it appears as two or three rounds or cushions which are called rugae, disposed radially. In the endocervical canal these rounds are further developed into longitudinal folds of which smaller folds point towards the internal os. These

folds are called the 'arbor vitae', palmate folds or plicae palmatae. The second grouping of folds are small and pendulous and they look like bunches of grapes.

The basic subunit of this epithelium is the villus, each of which is separated by an intervillous crypt. The glands of the cervix are not true glands, but more a system of clefts which sometimes becomes a pathological situation when the glands become pinched off and then lack continuity with the lumen of the cervical canal, forming a Naboth's follicle.

Views differ as to whether or not the cells of the endocervix are subject to cyclical morphological changes in response to oestrogens and progesterone or chorionic gonadotrophin stimulation - the Arias-Stella reaction. This reaction occurs in the endometrium of the uterus in 10 per cent of pregnant women, in women with ectopic pregnancy and in women on exogenous hormones, such as the contraceptive pill. The Arias-Stella changes include an increase in nuclear chromatinism so that the nucleus is strongly eosinophilic and nuclear pleomorphism, the cytoplasm of the cells becoming clear. The reaction is sometimes mistaken for adenocarcinoma (Hendrickson and Kempson, 1980).

Transformation zone

During late fetal life, in adolescence, and mainly in a first pregnancy the columnar epithelium nearest the transformation zone changes into squamous epithelium by squamous metaplasia. It has been suggested that this change occurs due to exposure of the columnar epithelium to acid pH in the vagina, and due to the weight of a pregnancy pushing some of the endocervical canal out into contact with vaginal fluids (Singer, 1975).

CERVICAL INTRAEPITHELIAL NEOPLASIA

If the cervix displays any atypical metaplastic epithelium it is generally accepted that it has the potential to develop into a neoplastic lesion. There are two forms of abnormal cervical epithelium; atypical metaplastic epithelium and carcinoma in situ; the two are histologically different. The third kind of abnormal cervical epithelium is frank invasive carcinoma, a condition which is irreversible.

Atypical metaplastic epithelium

Three distinctive epithelial patterns have been described: abnormal epithelium, basal cell hyperplasia and dysplasia.

Abnormal epithelium was a term first described by Glatthaar (1950) as a lesion in which the prickle cell layer increased in width in association with epithelial rete-like pegs.

Basal cell hyperplasia was originally described by Galvin et al., (1955). In this state there is an increased mitotic rate and hyperchromatism leading to marked proliferation in the basal epithelial layer.

Dysplasia was a term introduced by Papanicolaou (1954) to describe a condition in which inadequate maturation has occurred in the epithelial cells. It is associated with an increase in nuclear pleomorphism, hyperchromatism and the loss of cellular differentiation. The abnormal and basal cell hyperplasias are conditions which are regularly found in sexually active young girls (Singer, 1976) but are not generally thought to have malign significance. Dysplasia, has variable neoplastic potential.

Carcinoma in situ is generally thought of as an obligate precursor of invasive cancer but this is debatable. Carcinoma in situ is a lesion where there is no invasion through the basement membrane, but the whole thickness of

the surface epithelial lining contains

abnormal cells, where little differentiation takes place.

Invasive carcinoma is defined as a lesion where epithelial formations invade the stroma underlying the basement membrane by infiltration or destruction of normal tissues. Dysplasia, carcinoma in situ and invasive carcinoma have been divided into grades to achieve a system of classification useful for both cytology and histology.

Classification of cervical intra-epithelial neoplasia

CIN I Cervical intra-epithelial neoplasia grade 1; a borderline lesion with mild dysplastic changes.

CIN II Cervical intra-epithelial neoplasia grade II; also a borderline lesion with moderate dysplastic changes.

CIN III Cervical intra-epithelial neoplasias grade III; severe dysplasia and carcinoma in situ. Carcinoma in situ may be defined as where the whole thickness of the epithelial layer is involved in the lesion.

Invasive carcinoma occurs when the underlying stroma is infiltrated with epithelial cells. It is termed micro-invasive if the depth of invasion is less than 5 mm from the surface of the cervix.

The latest diagnostic procedures for the study of cervical pathology involve the use of electron microscopy and tissue culture.

TISSUES IN CULTURE WITH PARTICULAR REFERENCE TO HUMAN

UTERINE CERVICAL CELLS

In 1885 Rous first observed that a chick embryo could be kept alive for several days in warm saline, and this was the start of the study of tissues in culture. Harrison in 1907 (Harrison 1910) demonstrated that nerve cells in culture could produce long axons, discrediting the theory that nerve fibres were distinct from nerve cells. Rous in 1910 induced a tumour by using filtered extract of chicken tumour which were later found to contain the RNA virus known as the Rous sarcoma virus.

It was probably Alexis Carrel in 1913 who made the first systematic studies on tissue culture. In Carrel's publication: 'A method for the physiological study of tissues in vitro.' (Carrell 1923), In 1923 he recorded the types of apparatus suitable for tissue culture, the ways in which they should be prepared and the constituents for tissue culture growth media. He was also the first to make detailed measurements of the growth obtained from fibroblasts, epithelial cells and leucocytes, the likely causes of death in the cultures, and what factors could cause an increase in cell growth. Carrel stipulated the necessity for sterile apparatus and aseptic procedures when handling the tissue cultures so that risk of bacterial contamination was minimised; and the need to monitor the hydrogen ion concentration.

Unco, who in 1933 is reported to have been the first to grow cells from the human uterine cervix from invasive squamous cell carcinoma, described the growth from an explant as an epithelial-like sheet of cells which continued to grow for 11 days. Glatthaar(1948) was the first to report growth from cells from intra-epithelial carcinoma using the Maximow double coverslip. This technique involved using a glass slide with a depression in the middle. A few

explants of tissue were stuck with a plasma clot onto a glass coverslip which was placed centrally over the depression and the tissue culture medium added. The chamber was then sealed by placing a mica coverslip over the top and fixing the edges with paraffin wax (Figure 7). The whole apparatus was then inverted during incubation.

Another popular method of tissue culture at this time was the roller tube (Figure 8). This consisted of a test-tube in which explants were stuck down the side of the tube. Medium was constantly washed over the explants by placing the tube on a rotor apparatus which turned at 7 revolutions per hour. Gey (1954,1955) used this method to set up the famous Hela cell line from a squamous carcinoma of the cervix. The cells are still used in experiments.

There were advantages and disadvantages to these methods; the Maximow double coverslip allowed for more accurate control of fluid and gas exchange, but observation with high powered microscopy was difficult without special equipment. The roller-tube method of Gey has the advantage of being simple in terms of the container, a test-tube, but disadvantageous in that it requires a rotor to keep the explants constantly covered by medium. In both these methods a plasma clot was used to hold the explants and there was a problem with the plasma clot liquifying when holding the explants of carcinoma. This was thought to be because of the high rate of cell division in the carcinoma cells. Evens and Earle (1947) introduced a method of utilising cellophane as a solid substrate for the migration of cells.

A compromise between the roller tube and double coverslip method was by culturing normal as well as abnormal tissue on the roller-slide (Southam and Goettler 1953). This involved placing the explants in plasma clots on to a slide in a test-tube and then incubating at 37°C on a rotor.

The first controlled study was carried out by Moore (1952). One half of the tissue biopsy taken for culture was sent for histological examination and

Figure 7

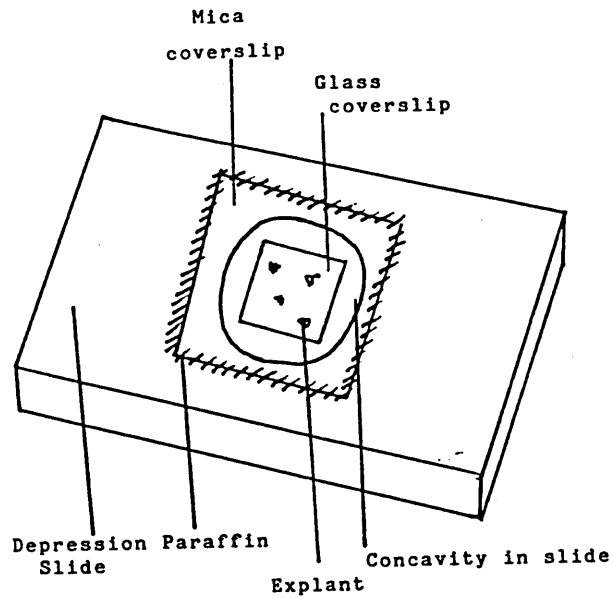


Diagram of the Maximow double coverslip.

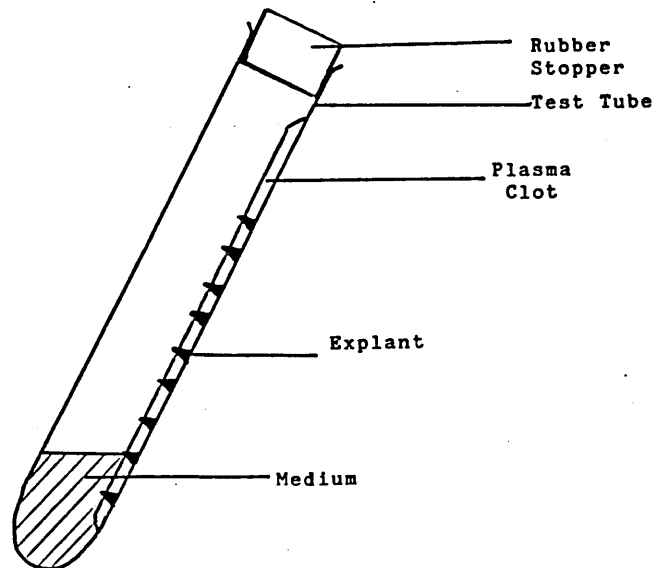


Figure 8
Diagram of the Roller tube.

diagnosis of the pathological state of the cervix. He found normal tissue from the cervix was difficult to grow, obtaining only about 45 per cent growth from normal tissue biopsies. He was not sure if it was growth or just migration of the cells out of the explant. Tissue taken from carcinoma of the cervix grew vigorously. Between 24 and 48 hours after the cultures were set up fifteen out of seventeen explants had distinct areas of growth.

In a systematic study of cultured fragments from cytologically different cervixes, the least amount of growth was obtained in tissue from normal cervix; only 10 per cent of the fragments grew after 10 days. The next category studied was fragments taken from a cervix where the cytology smears showed chronic inflammation. Growth success was about 25 per cent. In tissue fragments from precancerous cervix better growth was obtained than with normal or inflammatory tissue. Sixty-five per cent of fragments showed epithelial cell outgrowths after only 3 days in culture. This rate and quantity of growth improved with carcinoma-in-situ. Cells appeared in approximately 75 per cent of the cultures and required only 2 days. There was variation in nuclear shape and size. Some cells had several nuclei and some were surrounded by haloes of denser cytoplasm. The pavement-type arrangement of cell growth was still predominant although there were areas of non-cohesiveness between neighbouring cells. Overlapping and cells piling up on each other were also noted so that the outgrowth was not strictly a monolayer. The nuclei and cytoplasm stained deeply basophilic. Cultures from clinical cancer showed all of these characteristics, but they grew faster. Within 24 hours of incubation 95 per cent of the fragments had started growth. During mitosis, division was multipolar and the cells were in almost continuous division (Grand and Ayre 1954).

Moore (1955) over a period of two years grew tissue from the cervix of 27 patients with cervical carcinoma, 8 from patients with normal cervix, 8 during pregnancy and 8 with possibly malignant lesions of the cervix. He found that tissue from carcinoma in situ grew as rapidly and floridly as tissue from invasive carcinoma and that tissue from the normal cervix was least successful in culture. He pointed out that punch biopsy for diagnosis of invasive carcinoma may give false negatives because although serial sections may be examined, inflammation or 'reserve cell hyperplasia' can be mistaken for invasive carcinoma. True invasive carcinoma may be missed completely because of non-specific sampling. If tissue culture is used in conjunction with punch or cone biopsy the rapid growth of the culture can indicate carcinoma in situ or invasive carcinoma. Tissue culture is a time-consuming method and is also subject to sampling error, but it was thought that it may have a place in determining the degree of malignancy in ambiguous cases and thereby prevent unnecessary cone biopsies.

Biopsies of normal tissue from the histologically malignant cervix grow even faster in culture than the tissue from the malignant area. This may have been due to severe necrosis in the malignant tissue (Moore and Brandkamp, 1956).

A study of normal, inflammatory, precancer, carcinoma-in-situ and clinical cancer and normal tissue taken from an inflamed cervix showed more prolific growth than normal tissue from a normal cervix (Grand 1956). This suggests that the rapid growth achieved in the Moore and Brandkamp (1956) study was due to the samples being taken from an inflamed cervix with clinical cancer. Growth patterns correlated well with cytological smears of the same tissue, but in a single cervix cell types may vary depending on the biopsy site. One

woman's cervical cells varied from normal to neoplastic (Grand 1956).

Zinser (1962) used the tissue culture method of Glatthaar to try to resolve the question of whether or not carcinoma in situ was the penultimate stage in the progress of invasive carcinoma. The results of his study did not answer his questions because it is not possible to see invasion of the basement membrane in tissue cultures and there were no other obvious indications in the cultures he grew that carcinoma in situ would continue to grow into invasive cancer. He concluded that there was a difference in growth patterns between cells taken from carcinoma in situ and cells from invasive cancer. It was not possible to refute that carcinoma in situ, although having an abnormal growth pattern in vitro would not revert to normal cells in vivo.

Richart (1964) claimed to have produced the most carefully controlled tissue culture study of the cervix, in which the cervix in women attending colposcopy clinics was stained with toluidine blue dye and then photographed. When the women attended their next appointment the photograph of the dyed cervix was used to obtain two biopsies from specific sites, where there were uniform cell types. One biopsy was sent for histology and the other used for tissue culture. The stroma was carefully dissected away from the epithelial cell layer and in this way Richart claims to have reduced the amount of fibroblast contamination in his cultures. The cultures from the normal cervix had the pattern of growth described by many other tissue culturists and there were areas of cells in the monolayers which changed in appearance after one week in culture to that of the mature squamous cells seen on the surface of the cervix in vivo.

The exact procedure for tissue culture Richart adopted was to place the biopsy of cervical tissue in a test-tube containing a transparent solution of glucose, potassium and sodium with the antibiotics penicillin and streptomycin. The biopsy was then washed and placed in a watch glass. Using a dissecting microscope the sample was split longitudinally using forceps and a scalpel.

One half of this biopsy was sent for histology, the other half was transferred in Eagle's minimal essential medium with 15 per cent fetal calf serum. The epithelium was carefully separated from the stroma, which was also retained for histology. The stripped epithelium was minced into fine fragments which were then aspirated with a bent-tipped Pasteur pipette and transferred to microglass circular tissue culture chambers. Precut pieces of perforated cellophane were moistened in minimal essential medium and placed over the minced fragments and the chamber was put in a humidified water-jacketed incubator at 37°C with 5 per cent carbon dioxide and air as the gas phase. The cultures were left for 3 days initially and then the medium was changed three times a week. When growth was achieved the cellophane was removed with non-viable fragments.

The cells were subcultured by removing the medium and replacing glucose, potassium and sodium solution for 5 minutes, then this was removed and replaced with a similar solution containing 0.25 per cent trypsin and 0.2 per cent sodium versenate, (the disodium salt of ethylenediaminetetraacetic acid). The progress of cell detachment in the trypsin/versene mixture was monitored using an inverted "Plankton" microscope and when the cells were just starting to lift off, the glass was scraped with a siliconised rubber pipette bulb. The cells were harvested by centrifugation at 3000g. The supernatant was discarded and the cells were resuspended in fresh medium and replated. The same method of tissue culture was used for normal and abnormal tissue. The normal cell cultures could be frozen, thawed and subcultured quite successfully, but areas of mature cells were not seen in the subcultures.

Richart (1964) claimed that abnormal tissue showed characteristic growth patterns related to the donors from particular cultures had come. He did not find the time differences mentioned by Grand for growing various types of normal and abnormal tissue. He credited his success with growing normal tissue to the careful selection of tissue and the careful stripping of stroma from under

the epithelial cell layer.

In 1966 secondary non-specific epithelioid cells were described (Auersperg and Worth, 1966). These cells developed during a second wave of epithelioid cell proliferation and were noted in 9 cases of benign tissue in culture and in half of the 68 cases of invasive carcinoma in culture. They grew from the tissue fragments as cohesive monolayers with pronounced intercellular borders, and separated into single, large polygonal cells as they became removed from the explant. They would survive for months metabolising slowly without dividing. If they were subcultured during the active phase of growth they continued to grow for a few passages and then became stationary. They differed from fibroblasts because of their shape, their initial cohesion and their limited ability to proliferate. They must be benign cells because they were found in all the cultures of benign tissue, but it is unlikely that they are normal cells because they were present in cultures from tumours which did not contain similar cells in histological sections. In chromosome analysis of tumour cells in culture a chromosome number of 80 - 86 with abnormal karyotypes was seen, but the secondary non-specific epithelioid cells had no chromosome abnormalities, only a number consistent with the normal female karyotype. In addition interphase nuclei in the cells in these cultures contained structures characteristic of sex chromatin.

Growth pattern in vivo was to some extent mimicked in vitro. For example cohesive tumours grew cohesive cultures. There was a good correlation between Broder's grading of cell type and differentiation in culture. Non-cohesive cultures tended to have the highest number of chromosome abnormalities such as polyploidy. Auersperg and Worth (1966) conclude that as in histological grading of tumours, resemblance of certain carcinoma cultures to benign cultures was interpreted as evidence of squamous differentiation, but differentiation was only observed in cell sheets which were continuous with the original explant.

These results differed from previous observations by Grand (1956) and Richart (1964) which showed lack of cohesion, when the cultures were grown from differentiated carcinomas whereas excessive cohesion in vitro was associated with poorly differentiated tumours.

The secondary epithelioid cells may have been the result of objective differentiation in late appearing squamous cells because of environmental changes in prolonged primary cultures.

The surface on which the cells are grown can make a difference to the success in culture. Many growth surfaces have been tested, three of which are glass, collagen and cellulose sponge, (Auersperg and Worth, 1966).

Differences with regard to cell cohesion, differentiation, cell shape, stratification and migration of the cultures on the three different substrates have been observed, but there were no differences in the pattern of growth between cells from the same tumour. Although epithelial cell growth was faster on collagen this advantage was offset by stimulation of fibroblast growth. Cells on glass grew faster, but those cells within the sponge, when established, grew well and persisted longer than those on glass alone.

Using tissue cultures as an in vitro diagnostic test for abnormal tissue was attempted by Mellgren et al., (1967). They took samples of cervix from patients with histologically diagnosed invasive carcinoma, those with carcinoma in situ and those with dysplasia. At the same time biopsies of histologically normal tissue from the same patients were taken and grown in parallel.

Growth was either in vitro on rat collagen or the biopsies were transplanted into the cheek pouch of cortisone treated hamsters, to see if tumours could be induced.

Mellgren et al., (1967) found invasive carcinoma grew most prolifically in both models. Carcinoma in situ was not significantly different from the normal

tissue and it was difficult to measure any difference in nodule size in the hamster cheek pouch. Histologically there was some evidence of abnormal-looking cells. The normal tissue from patients with carcinoma in situ grew faster than normal tissue from invasive carcinoma, but this may have been due to the average older age group of the invasive carcinoma patients. The biopsies from dysplasia had a similar growth pattern to carcinoma in situ as though there was some suggestion that they were more like invasive carcinoma than carcinoma in situ.

Richart's observations (1964) were confirmed by Wilbanks (1969). There was more fibroblast contamination in cells from dysplasia, but intra-epithelial carcinoma grew through 16 transfers for approximately a year. It was not possible to grow a pure culture from invasive cancer because of fibroblast contamination.

In the early nineteen seventies a number of papers were published describing the ultrastructural characteristics of cervical cells in culture. (Shingleton et al., 1970, Wilbanks et al., 1970, Shingleton and Wilbanks, 1974). The cells were described as having large oval or rounded nuclei with smooth nuclear membranes and finely dispersed chromatin. The cytoplasm contained numerous mitochondria and ribosomes were present either singly or as polyribosomes. Glycogen particles were absent. Tonofilaments were present in large bundles which terminated on the inner face of desmosomes at the cell surface, which were not as regularly spaced, as in cells from tissue biopsies. Microvilli were sometimes seen projecting into the intracellular space and at times they alternated with desmosomes. Tonofibrils were either more numerous in the central part of the cells than at the periphery or they formed a lattice-like network throughout the cell.

Shingleton and Wilbanks (1970,1974) studied different areas of the monolayer radiating out from the explant. In most cultures no consistent differences were noted in the nuclei, the cytoplasmic constituents or the

cellular attachments. Glycogen, either dispersed or in pods, as well as lipid droplets, were present in the peripheral cells of cultures where differentiation had occurred.

Wilbanks and Shingleton (1970) also compared the ultrastructure of cells from the cervix in monolayer tissue culture with cells in tissue biopsies of cervix. In the tissue culture cells intercellular spaces were narrow and the desmosomes often were arranged so that their long axis was parallel to the cell surface, but in the biopsies intercellular bridges containing desmosomes joined the cells.

The same workers, publishing in 1974, compared the fine structure of the cervical intra-epithelial neoplasia in vivo and in vitro. Cells which were cultured from cervical neoplastic lesions showed prominent microvilli at their borders; tonofibrils and desmosomes were decreased. The nuclei are more irregular in contour, but the longer these cells were kept in culture the nuclear borders became smoother and apart from some dense aggregates, the chromatin had a finer distribution. The number of microvilli at the cell borders also decreased.

The cells in the middle third of a biopsy of cervical intra-epithelial neoplasia are described as having nuclei with irregular contours and chromatin coarsely distributed. The nucleo-cytoplasmic ratio was increased and there were randomly distributed ribosomal particles. There were numerous mitochondria but few tonofilaments, and desmosomes are also decreased in numbers. The number of microvilli was increased. For comparison a biopsy taken from the same level of epithelium but from a normal cervix was also described. The cells had nuclei with smooth borders and finely dispersed chromatin, a single nucleolus usually being present. The cytoplasm contained pools of glycogen and there were abundant tonofibrils, in particular at the periphery of the cell. Desmosomes were numerous and joined adjacent cells. Only a few microvilli were seen

extending into the intercellular space. The mitochondria were rare and polysomes or membrane bound ribosomes, which is what would be expected in cells taken from the intermediate layer of the epithelium.

Another method for the serial cultivation of normal cervical squamous cells was published in 1979 by Stanley and Parkinson. It depends on the presence of a non-dividing fibroblast feeder layer which provides attachment and growth factors for the epithelial cells.

They took biopsies from the cervix obtained by hysterectomy from women with negative cervical cytology. The fragments were washed and the epithelial layer stripped and minced into fine pieces. These small fragments were then either plated into explant cultures or disaggregated into single cells and plated on to irradiated fibroblast feeder layers. Subcultures were carried out when the colonies had reached approximately 1000 cells and the fibroblast feeder layer had lifted off. The cultures were grown in Dulbecco's modified Eagles medium with 10 per cent fetal bovine serum. In preliminary experiments the sera varied considerably in their ability to support epithelial cell growth. With this method they grew cultures from twenty-two of twenty-four biopsies taken.

Stanley and Parkinson (1979) found that in growth of human cervical cultures up to four transfers of epithelial cells only occurred in the presence of a fibroblast feeder layer. They also reported a growth-promoting effect with 0.5 µg/ml of hydrocortisone and 10 µg/ml of epidermal growth factor, but this only happened in the presence of the fibroblast feeder layer. Swiss 3T3 cells (Flow laboratories) also proved to be the best feeder layer as irradiated human fibroblast sheets attached too strongly and were difficult to remove when subculturing the epithelial cell colonies. They were unable to explain why the fibroblast layer promoted growth of the cervical epithelial cells, but quoted Green et al., (1977) as suggesting that the feeder layer function as an attachment substrate and that they release growth factors into the medium.

Overgrowth of the cervical epithelial colonies by fibroblasts from the stroma in the original biopsies has always been a problem with cultured cells from the cervix. In 1975 Gilbert and Migeon published a solution to the fibroblast contamination problem. It relies on the fact that some epithelial cell types have D-amino acid oxidase which enables them to convert D-valine into its L-enantiomer, but fibroblasts are unable to effect the conversion and so die out of the cultures before attaching and establishing a colony. They successfully grew fibroblast-free kidney epithelial cells with this technique.

D-valine substituted medium has been used in the culture of ectocervical epithelial cells. Vesterinen et al., (1980a).

The cells in the single layer of columnar epithelium lining the cervical canal are the target cells for certain female genital tract pathogens, such as some viruses and Chlamydia trachomatis. Short-term cultures of these cells have been grown by a culture method similar to that for ectocervical epithelial cells (Vesterinen et al., 1980b).

Cell populations derived from the endocervix formed whorled irregular colonies. There was some overlapping of adjacent cells although generally the cells grew in a single monolayer and were covered with short finger-like microvilli that could be viewed under the scanning electron microscope. Under transmission electron microscopy the nuclei were large with convoluted nuclear membranes and coarsely distributed chromatin. There were numerous mitochondria, free ribosomes and rough endoplasmic reticulum in the cytoplasm and well developed Golgi zones were seen. Small bundles of tonofilaments and tonofibrils were also detected, the latter of which were connected with desmosome-like complexes.

The study of cells in culture at first depended on the ability to maintain pieces of tissue alive in vitro, although there is some doubt the early

experiments really did show new growth of cells and not merely the addition of more cells when the crude plasma extracts were added. Then came the ability to obtain whole monolayers of cells in culture and to set up cell lines by subculture. The invention of the phase contrast microscope, (Zernicke, 1932), the production of the first commercial transmission electron microscope by Cambridge Instruments in 1965 led to a greater understanding of the growth characteristics of these cells. Biochemical, genetic and histopathological techniques were used to study the growth characteristics of these cells in culture, which has led to an ever increasing mass of data so that the cultures can be used as in vitro models for the study of in vivo mechanisms.

TISSUE CULTURE

There are three main types of tissue culture. The parent tissue can be dispersed into single cells using enzymes or mechanical and chemical agents; fragments of the parent tissue called explants may be placed in growth medium and new cells grow out from the edge of the explant; or whole organs consisting of several tissue types may be kept viable in a culture medium - for example, pieces of fallopian tube, the proportion of their beating cilia being an indicator of surviving function.

Primary cultures are those which are prepared directly from tissues of the organism. These cultures can be disaggregated using enzymes which also interfere with their attachment to the base of the culture vessel. The cells can be subdivided and replated into fresh culture conditions to form secondary cultures. If this procedure is successful and the cells can be repeatedly subcultured they are said to have formed a cell line. The cells can be kept alive for weeks or months and they may differentiate in culture, for example fibroblasts have been shown to continue to secrete collagen (Alberts et al., 1983).

Most vertebrate cells die after a finite number of divisions, for example human skin cells divide only 50 to 100 times before they die (Hayflick and Moorhead, 1961; Alberts et al., 1983). Cells from fetal tissue will go through more subcultures or passages than cells taken from an elderly adult. If cells are taken from a person suffering from Alzheimer's disease (premature senility) the cells have a shorter life span than cells from an age and sex matched donor (Alberts et al., 1983).

Cell lines can either be derived from cells which have transformed in vitro either spontaneously or after the action of a transforming virus; or they

can form tumours which are immortal in vitro and vivo. These immortal cell lines are very useful in research because they are sources of large numbers of cells of a uniform type; they can be stored at -70°C for an indefinite period.

The latest techniques in tissue culture contrast strongly with the early attempts and the uses made of cells in culture were probably inconceivable to the tissue culture pioneers.

FACTORS AFFECTING CELLS IN TISSUE CULTURE

Success in tissue culture depends on finding the optimum medium, temperature and buffering system. Tissue culture 'recipes' are usually devised by trial and error. Some tissues grow more easily than others. Fibroblasts are the 'weeds' of tissue culture and cause major problems when trying to obtain cultures of other cells from tissue with stroma attached, as in endometrial tissue from the uterus.

Equipment

All equipment should be sterile and most work on cultures is carried out in a laminar flow cabinet fitted with high efficiency particulate air filters to keep the atmosphere around the cultures free of dust, bacteria and fungi. Almost all tissues in culture require a higher than ambient room temperature for growth and incubators with attachments for controlling the carbon dioxide levels within are used.

pH, Osmolarity and Humidity

Use of a carbon dioxide atmosphere around the cells serves two functions (a) it is used for the control of pH in the culture media and (b) it

serves as a substrate for some of the essential pathways such as synthesis of purines, pyrimidines, long chain fatty acids and carbon intermediates in the tricarboxylic acid cycle (Ham, 1981). Cultures which reproduce to large numbers quickly are less likely to require extraneous carbon dioxide since they are able to produce sufficient for their requirements from their own metabolism. Cultures which only have a few cells to start with lose the carbon dioxide in the medium. The addition of a buffer, for example HEPES (N'-2-Hydroxyethylpiperazine-N'ethane-sulphuric acid) (Eagle, 1974) either replaces the need for a carbon dioxide air mixture or the buffer counteracts the effects of removing the cultures from the incubator during media changes. A 5 per cent carbon dioxide and air mixture with Eagle's minimal essential medium is the best combination for culture of human cervical epithelial cells from the human cervix.

The range of pH for optimal cell growth is usually narrow although the range may differ between different cell types, and non-transformed lines tend to require slightly more alkaline conditions than transformed permanent cell lines (Eagle, 1974). There is also a narrow range of osmolarities applicable to different cell types in culture. For example, lymphocytes survive best at very low osmolarity (228-237 mOsm/kg) (Waymouth, 1970) and mouse pancreatic epithelium at high levels (375+ 5 mOsm/kg) (Waymouth, 1978). Osmolarity depends largely on the concentrations of sodium and chloride ions in the media and therefore any additions to the media during experimentation must be carefully calculated to take into consideration an effect the addition might have to the sodium and chloride ion equilibrium. In consequence the humidity of the atmosphere is also important and most modern incubators have humidity control, because evaporation of some of the medium would lead to a rapid rise in osmolarity and eventually cell death.

Culture substrates

The culture vessel used often has an influence on the success of obtaining cell growth. Historically cell culture was done in vitro but in recent years glass has been replaced by specially treated polystyrene plastic with a similar negative surface charge. Some cell lines require a surface layer on top of the plastic such as the positively charged polymer, polylysine, which is particularly useful for cell grown in media with minimal amounts of serum (Barnes and Sato, 1980).

Growth medium

The basic medium required by most cells in culture contains salts, aminoacids in the L-form, vitamins and serum, usually horse or fetal calf, although extracts from chick embryos have also been used. In 1959 Eagle developed a minimal essential medium after extensive studies on the growth requirements of mouse L-strain, Hela and various other cell lines. The medium contains sodium chloride, magnesium (acts as a cofactor to enzymes and is a component of KATP), calcium (important in cell division), potassium and phosphate ions. Thirteen aminoacids (arginine cystine, glutamine, isoleucine, leucine, lysine, methionine, phenylalanine, theonine, tryptophane, tyrosine and valine) six vitamins (folic acid, niacinamide, pantothenic acid, pyridoxine, riboflavin and thiamine), one sugar (glucose) and choline and inositol.

This minimal essential medium requires serum to add biotin, cyanocobalamin, iron and other inorganic trace elements. Most cell lines only need a small amount of serum, less than 10 per cent. Epithelial cells from the cervix grow successfully only with relatively high amounts of serum, about 15

per cent.

It is also useful to add the non-essential amino acids (alanine, asparagine, aspartic acid, glutamic acid, glycine, proline, serine), which are used in protein synthesis, but which are usually synthesised de novo by the cells. If exogenous non-essential amino acids are not added to cells in culture, since the nitrogen source for synthesis they can use comes from the catabolism of the essential amino acids, they will use more energy and require extra essential amino acids (Ham, 1981).

Most culture media are prepared fresh from a minimal essential medium of which there are now a variety commercially available, with different buffers added to facilitate the control of pH required for different cell types in culture. Serum in various percentages is added and also non-essential amino acids, and extra L-glutamine as it tends to be unstable when stored in media for any period of time. It breaks down to the ammonium salt of pyrrolidone carboxylic acid.

Potassium and sodium: potassium ratios

Most media have sodium:potassium ratios between 24:1 and 50:1 (Ham and McKeehan, 1979), but some cell types require either very high or very low ratios.

Growth factors

Growth factors can be defined as substances that promote cellular multiplication without participating in cellular metabolism as nutrients. They are derived from several sources, for example, fibroblast growth factor is a crude luteinising hormone preparation from pituitary gland; platelet derived growth factor from blood platelets. Their precise mechanism of action is

uncertain, but it is assumed that their effects on cellular multiplication are regulatory. Some growth factors also have effects on cell differentiation.

Other factors which may effect cells in culture

Changes in the physicochemical environment of the cells particularly during preparation for culture, and during media changes may have an important effect on the viability of the cells in culture.

Temperature, illumination, oxygen tension and atmospheric pressure

Temperature: Most mammalian cell lines are cultured at between 35°C and 37°C which is mammalian body core temperature. Cells which are not normally within the body at 37°C must be cultured at a more appropriate temperature. Skin cells should be cultured at 35°C .

Fluorescent light: Although brief exposure to light seems to be beneficial to promoting cell multiplication, it has been shown that long exposure can cause chromosome damage (Sanford, 1978; Sanford et al., 1979). An effect of short wavelength visible light on culture media is to generate toxic photoproducts (Wang, 1976).

APPLICATIONS OF TISSUE CULTURE IN DIAGNOSTIC

MEDICINE AND RESEARCH

Tissue culture of plant as well as animal tissue is a rapidly growing science, possibly because in the foreseeable future the advantages of tissue culture methods will outweigh the disadvantages. In medical science in particular cultured tissues or cells are usually easier to obtain and in greater supply than whole animals, which have been the subjects of

experiments in vast numbers in the past. Tissue cultures can be experimented on and examined under the microscope while the cells are still alive. In a culture vessel cells are isolated from systemic and physiological effects that may interfere with experimental results in the whole animal. It is possible to add radioactive precursors to study basic metabolic processes and the effects of alterations in various drugs. Nutrients and environmental conditions can be altered without concern for the host. Perhaps most importantly in the case of drug testing, results are obtained from the action of the drugs on human tissue, whereas previously primary drug testing was nearly always carried out in mammals other than human. On occasions such results have been found to be disastrously misleading. Any system will have disadvantages, although tissue culture methods are becoming more and more sophisticated.

Cells in culture are in an artificial environment, which does lack the usual body controls and regulations. Sometimes the extent to which this changes the nature of the cells is unknown. Tissue culture is expensive in terms of time and money as growing primary cultures or initiating cell lines can be difficult. "No growth" for no apparent reason is a not uncommon phenomenon in tissue culture research. Reagents for media, in particular serum, are expensive, and either sterilising glass or buying disposable or presterilised apparatus also drains finances. Susceptibility of the cultures to infections by bacteria, fungi and viruses is much greater than in laboratory animals and the preventive measures to ensure against infections are both necessary and time consuming. Obtaining 'purity' of cell type can be difficult. In particular obtaining a cell culture without fibroblast contamination is difficult.

In medicine some of the applications of cells in culture have been: (a) lymphocytes used in the mixed lymphocyte reaction to test donor and recipient compatibility in tissue, blood and organ transplants (b) prenatal diagnosis of an increasing number of enzyme deficiency diseases and genetic defects is now

possible by culturing cells removed from the amniotic sac of the fetus and more recently, growing samples from chorionic villi (c) cell cultures are used in the production of monoclonal antibodies, the production of which has opened up new avenues in science (d) some cell lines are used for the isolation and diagnosis of viral infections and for the detection of Chlamydia trachomatis. In the early days of tissue culture of cervical carcinoma it was hoped to use tissue culture as a method of diagnosis.

The use of tissues, cells and whole organs in culture in research is very varied. The life cycle of cytomegalovirus, herpesvirus hominis and adenoviruses in cultures of human cervical epithelial cells has been studied (Vesterinen et al., 1975, Vesterinen, 1976, Vesterinen et al., 1978 respectively). The action of the flagellate organism Trichomonas vaginalis, which causes haemorrhagic lesions of the cervix and vagina and may be linked to premature rupture of the membranes in pregnancy, has been studied in cultures of cervical epithelial tissue (Cameron and Hawkins, 1980) and there is a large amount of data on this tissue in the literature. If in close association with a department of obstetrics and gynaecology tissue from the cervix is readily available from hysterectomy specimens. A relatively simple but successful method of short term tissue culture was developed by Thomlinson (cited by Hounsell and Hawkins, 1974). Experiments on these cultures included analysis of cell proliferation and monolayer growth using tritiated thymidine incorporation, autoradiography and area outgrowth measurements with a quantitative image analyser. This method of tissue culture was used to look at the action of Trichomonas vaginalis using the scanning electron microscope (Cameron and Hawkins, 1980). The scanning electron microscope was also used to study morphological aspects of these cells. The action of the heavy metal ions copper and zinc on cell kinetics has also been studied in an effort to quantitate the possible deleterious effects of copper

ions present in uterine contraceptive devices (Hounsell and Hawkins, 1974).

The knowledge gained from these studies and the areas of interest covered have prompted the following project: that of studying both normal and neoplastic human uterine cervical epithelial cells in short-term culture and using the normal cell cultures as in vitro models to study the action of vaginal pathogens; the fungus Candida albicans, Chlamydia trachomatis and Haemophilus vaginalis and the combined action of copper and zinc ions on cell monolayer growth. The model system with Candida albicans growing on the epithelial cells has been further developed to include a study of the actions of the imidazole antifungal drugs, clotrimazole, bifonazole and vibunazole at three stages of fungal infection, those of attachment, germ tube production and penetration of hyphal elements into cells.

CHAPTER 2

METHODS AND EQUIPMENT

The methods used for tissue culture in this study are relatively simple, but they have given consistently successful results. The procedure was first developed ten years ago by Mr J Thomlinson and detailed experiments were performed to find the optimum conditions for growth. Subsequent researchers have experimented with the technique as new procedures in tissue culture were developed, but the original method has been returned to because it gives the most successful results.

The growth medium chosen by Thomlinson is made up fresh each time from the following ingredients:

10ml of Eagle's minimal essential medium x10 concentrate (TC25

Wellcome Reagents Ltd., Beckenham, England)

15 ml fetal calf serum, batch specific (Gibco Europe Ltd., Paisley, Scotland)

1 ml non-essential amino-acids for use with Eagle's medium (Flow Laboratories, Irvine, Scotland)

1 ml L-glutamine 200mM with indicator Phenol red. (Gibco Europe Ltd., Paisley, Scotland)

30µg/ml benzylpenicillin (sodium) (Crystapen, Glaxo Laboratories Ltd., Greenford, England)

50µg/ml streptomycin sulphate (Glaxo Laboratories Ltd., Greenford, England)

69ml glass distilled, sterile water.

Modified media

For cultures of columnar epithelium, Eagle's minimal essential medium with D-valine (Gibco Europe Ltd.), substituted for L-valine was tried, because it has been found that a fibroblast-free culture of kidney epithelial cells can be obtained using this medium (Gilbert and Migeon 1975). This method is based upon the ability, lacking in fibroblasts, of many types of epithelial cells to

convert the D-amino acids into their L-enantiomers (Gilbert and Migeon 1975, 1977; White et al 1978). In our cultures it was found that the D-valine medium did not support growth even if Eagle's medium with L-valine (TC25 Wellcome Reagents Ltd) was used for the first four days of growth and then the D-valine medium used for the remaining four days.

Beef embryo extract was also added to the medium to see if the number of successful cultures could be increased. The exact chemical composition of beef embryo extract, like fetal calf serum, is not known for each animal the sample has been extracted from. For this reason it is necessary to use only one or two batches of fetal calf serum from the same source and when supplies of these batches are exhausted several new batches are tested to find the one where maximum efficiency of cell growth is obtained. During a phase of the work when the cultures were not growing, addition of beef embryo extract to the medium failed to initiate growth. When the cultures started growing again, it was without this additive and it was concluded that it was superfluous to the growth requirements of the cells.

Equipment

The equipment used included an air flow cabinet (Lamina Flow Work station, Microflow Ltd., Fleet, Hants, England) a carbon dioxide, thermostatically controlled incubator in which the humidity, expressed in terms of evaporation rate is less than 0.03 ml/cm/week. (Grant GC4, Grant Instruments, Cambridge Ltd.) and a dry oven set at 37°C (Gallenkamp size 2).

Sets of instruments are prepared each week by washing and sterilising:-

1 pair Treves' rat tooth stainless steel 13 cm forceps (Raymond A Lamb, London, England)

1 scalpel handle Swann-Morton Nickel alloy 12cm (Lamb)

1 scalpel blade Swann-Morton No 15 V>P>I> protected (Lamb)

1 scalpel blade Swann-Morton No 22 V>P>I> protected (Lamb)

2 short pasteur pipettes 150mm (Bilbate, Daventry, England)

1 embryo staining dish, glass 40mm square (Lamb)

10ml presterilised, plastic pipettes Cat. no. 133B (Sterilin Products, Teddington, England)

1ml pre-sterilised plastic pipettes Cat. No. 106B (Sterilin)

Plastic petri dishes S.60mm vented Cat.No. 123 not for tissue culture (Sterilin)

(plain plastic petri dishes are used instead of tissue culture grade, because these do not stimulate growth of cells on the plastic, and the cultures only grow on the glass coverslip)

Glass coverslips 13mm diameter No.1 Chance Propper Ltd

Glass coverslips 22 x 32mm No. 1 Chance Propper Ltd

Washing and sterilising

All laboratory glassware and instruments were soaked in detergent (Neodisher, Chemische Fabrik Dr. Weigert, Hamburg, Germany) for 24 hours after use. The glassware was then machine washed, rinsed in tap water and distilled water six times each, then oven-dried and autoclaved for 1 hour at 20 p.s.i. The instruments were handwashed, then rinsed six times each in tap water and distilled water, then dried, individually wrapped in tin foil. The embryo staining dishes were also individually wrapped in foil. Forceps, scalpel handles, embryo staining dishes and short tipped plugged glass pasteur pipettes were autoclaved in metal boxes for 1 hour at 20 p.s.i. The glass coverslips, carried in stainless steel racks before use, were soaked for 24 hours in 1 per

cent hydrochloric acid, to remove dust and grease particles, then rinsed six times in tap water, six times in distilled water, then sprayed with absolute alcohol, placed in a dry oven and autoclaved for 1 hour at 20 p.s.i.

Selection of patients

Human cervical tissue was obtained for culture from patients undergoing vaginal or abdominal hysterectomy for gynaecological reasons other than neoplasms. Pre-operatively the patient's vagina and cervix were washed with 0.02 per cent chlorhexidine gluconate. Samples of cervical intra-epithelial neoplasia were removed with a small punch biopsy forceps by the doctor (with the patient's consent) during the colposcopy clinic just before destruction of the affected area with the laser.

Cutting the specimen

The samples from the cervix after hysterectomy were cut with a scalpel with a No.22 blade. This was done by placing the blade flat on the surface of the ectocervix and slicing a thin section of the tissue about 4mm to the approximate depth of the basal layer. The correct depth was indicated by the tissue being partially translucent with areas of opacity. The samples were taken from the squamo-columnar junction. With the same technique samples of glandular tissue were removed from the cervical canal, 3 to 5 mm above the squamo-columnar junction.

Preparation of the tissue for growth in culture

Tissue samples were put immediately into the growth medium, in a sterile container. In the sterile flow cabinet, the samples were first examined for traces of blood and mucus, which were removed by washing. Any tissue which appeared too thick was cut away and discarded. The tissue samples were then washed several times with fresh medium. Using a scalpel with a No. 15 blade the tissue was minced carefully in the embryo staining dish until minute fragments which looked less than 1 mm in any dimension were obtained. These were the explants. Using a pair of forceps, a rectangular coverslip (for light microscopy), or a round coverslip (for scanning electron microscopy) was placed in each petri dish. Using a short-tipped pasteur pipette a drop of medium containing 5 to 10 explants was dropped onto each coverslip and the dishes placed in a dry incubator for approximately 20 minutes, to allow the explants to settle on the glass coverslip and for some of the medium to evaporate. The dishes were then replaced in the sterile flow cabinet and the coverslips are gently inverted using forceps and scalpel. Then 2ml of medium was pipetted gently into each dish at a point diagonally opposite to the coverslip, with minimal disturbance to the explants.

The dishes were then placed on shelves in the carbon dioxide incubator (5 per cent carbon dioxide in air at 37°C). The cultures were undisturbed for 4 days apart from checking for infection. This is indicated by a change in colour of the medium, which should be a pale orange to pink colour in normal culture and deep pink in an infected culture, or if the carbon dioxide concentration has dropped below 5 per cent. Infected cultures were examined under the light microscope and then sent to the bacteriology department for identification of the infection. The number of dishes affected was on average three out of eighty, but infection did not occur in every group of cultures.

If more than 10 per cent of the cultures were infected the whole incubator was washed and sterilised by heating to 95°C for 12 hours.

After 4 days, ten cultures at a time were removed from the incubator and placed in the sterile flow cabinet, at room temperature. There were two strip lights on inside the working area. It is not clear if the change in temperature or the light had any effect on the cultures, and for this reason only ten dishes were changed at a time so that the time out of the incubator was short. The coverslips were carefully inverted again using a sterile scalpel and blade and a pair of forceps, so that the explants lay on the upper surface. Then the medium was carefully poured off and 2 ml of fresh medium pipetted into the dish slowly, avoiding any turbulence which might disturb the explants. The medium had contained penicillin and streptomycin for the preceding 4 days; if the cultures were to be used to study pathogens, the antibiotics were omitted from the replacement medium on the fourth day. In studying metallic ions it was at 4 days that the metal ion was incorporated in the medium.

The cultures were returned to the incubator and left to grow for a further 4 days. On day 8 the cultures were used for experiments and then fixed for light or scanning electron microscopy on day eight, nine or ten, depending on the experiment.

Light microscopy; fixation and staining

The cultures, attached to coverslips were fixed for 24 hours in 5 per cent trichloroacetic acid, then washed for 5 minutes in tap water, stained in Ehrlich's haematoxylin (Lamb) made up in potassium alum for 15 minutes. The stain was then differentiated in 1 per cent hydrochloric acid in 70 per cent alcohol, blued in Scott's tap water substitute (Lamb), counterstained with 1 per cent eosin (Eosin Y aqueous solution, Lamb) for 3 minutes, washed in tap water,

dehydrated with graded alcohols, and cleared in xylene, and mounted using De Pe X synthetic resin (Lamb).

Scanning electron microscopy; fixation and preparation

For scanning electron microscopy the cultures were washed twice with phosphate buffered saline and fixed in 3 per cent glutaraldehyde (Electron Microscope grade, Agar Aids, Stansted, Essex) buffered to pH 7.4 with sodium cacodylate (Agar Aids). The cultures were then dehydrated quickly with graded alcohols. After two hours they were dried in a critical point dryer (Critical point dryer 010, Balzers Union, Leichtenstein) in liquid carbon dioxide. The coverslips were then mounted on aluminium stubs (Agar Aids) using quick drying silver conducting paint (Agar Aids).

and stored in specimen containers with silica gel crystals (Balzers Union). Just before scanning the specimens were coated with a thin layer of gold (E.M. Scope II sputter coater, E.M. Scope, Kent, England). All specimens were studied with a Cambridge Stereoscan 600 scanning electron microscope at 15 kV (Cambridge Instruments, Cambridge, England).

CHAPTER 3

GROWTH OF ECTOCERVICAL CELLS IN TISSUE CULTURE

In cervical explant cultures, growth of the cells is dependent on the explants being firmly attached to the coverslip for at least the first four days of incubation. From day one to day three only the explant can be seen. After four days small buds of cells have usually grown out of the edge of the explant (Figure 1). The cells are closely packed with light staining cytoplasm and a darker stained ovoid nucleus almost centrally placed. The nuclei may contain one or more nucleoli.

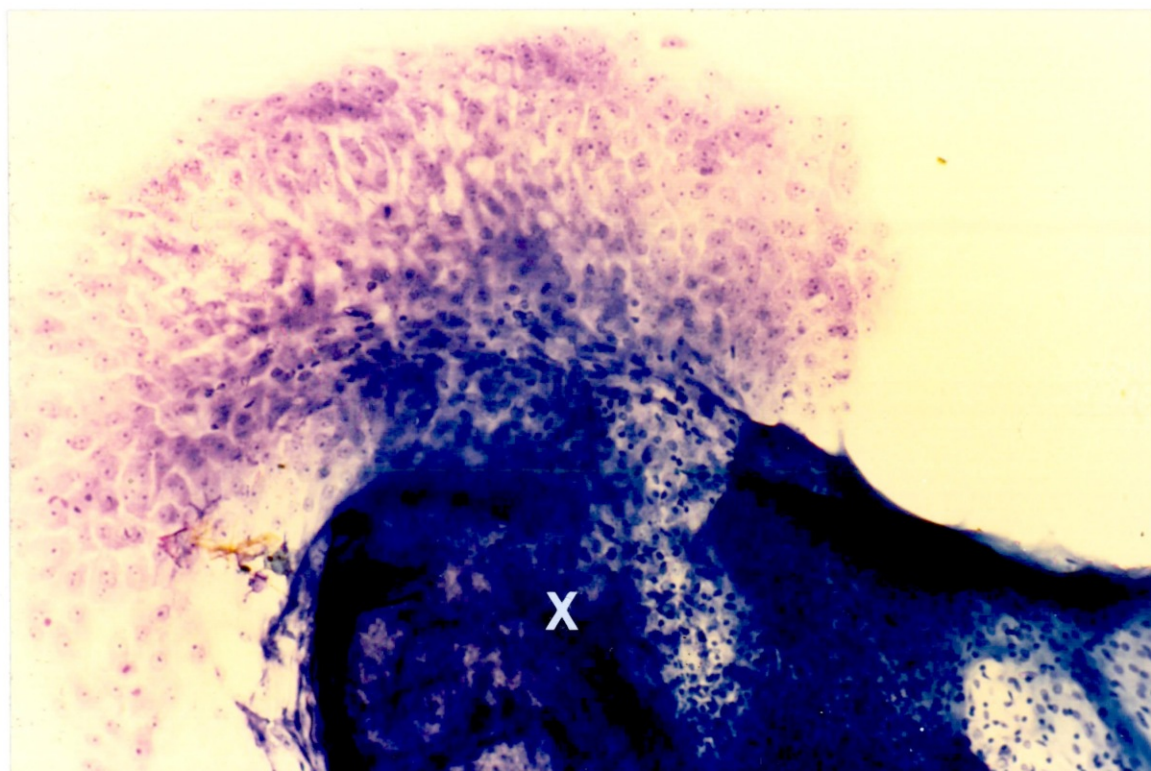
After eight days incubation an epithelial cell sheet has grown out surrounding the explant (Figure 2). The cell sheet is referred to as a monolayer, but there is some overlapping of cells. If there is no overlap the cell borders can be identified and intercellular spaces are uncommon. The cells are irregularly polygonal in shape and have an average diameter of 50 μm .

The cells stained pink with eosin appear faintly granulated. The nuclei were ovoid, approximately centrally placed and stained a deeper colour. Cells in mitosis could be seen scattered through the monolayer and all phases of mitosis were represented. The chromosomes appeared deep purple when stained. No intracellular organelles were visible under light microscopy. Occasionally, on cells at the periphery of the culture, small projections visible by light microscopy, could be seen coming from the cells; These were called 'marginal fibrils' by Cameron et al.,(1980)

Pyknotic cells

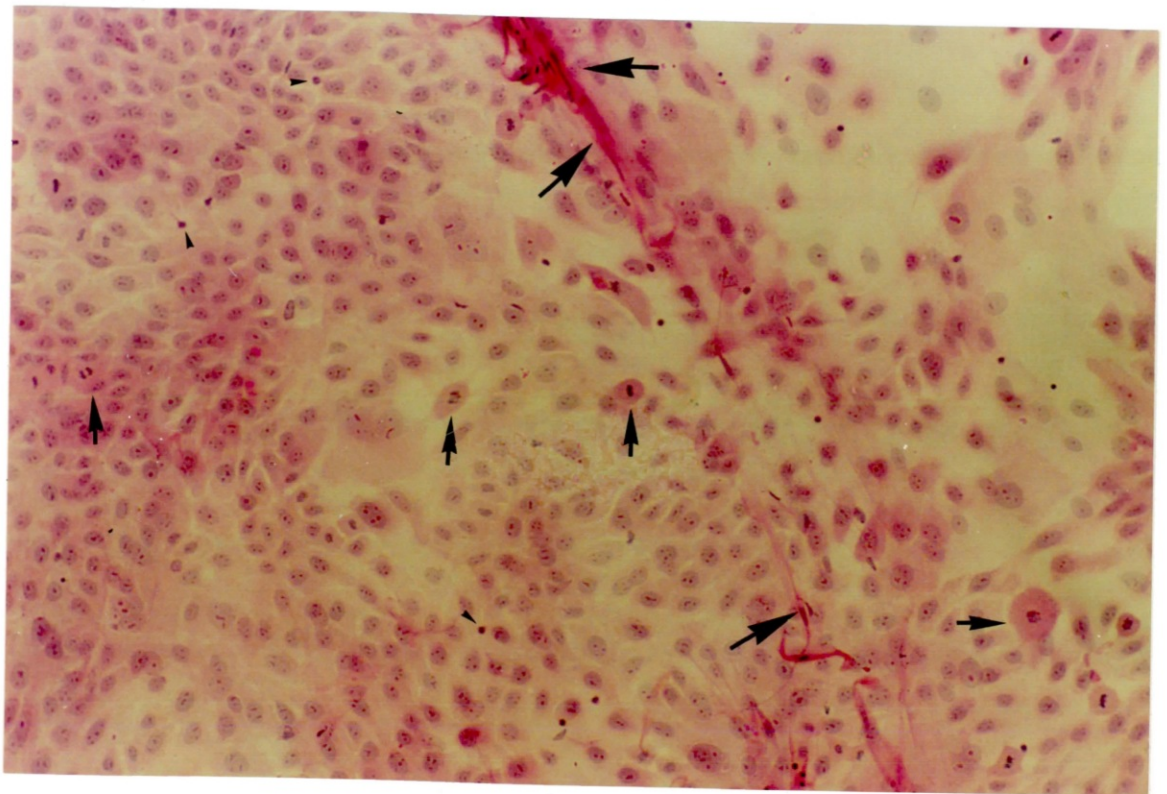
Pyknotic nuclei (diameter about 10 μm compared with 50 μm in active cells) were sometimes seen interspersed through the outgrowth (Figure 2). They

Figure 1



A bud of epithelial cells growing from the explant (X) of human uterine cervical squamous epithelium after 4 days in culture. Magnification (x55)

Figure 2



Monolayer of epithelial cells after 8 days in culture. Part of a ring of contaminating fibroblast growth (arrow) and some pyknotic nuclei (arrowheads) are characteristic features of these cultures. Cells in various stages of mitosis are indicated.(little arrows)
Magnification (x55)

were distinguished from the active epithelial cells by the small amount of darkly stained cytoplasm around the nucleus. It is likely that these pyknotic nuclei are in degenerating cells.

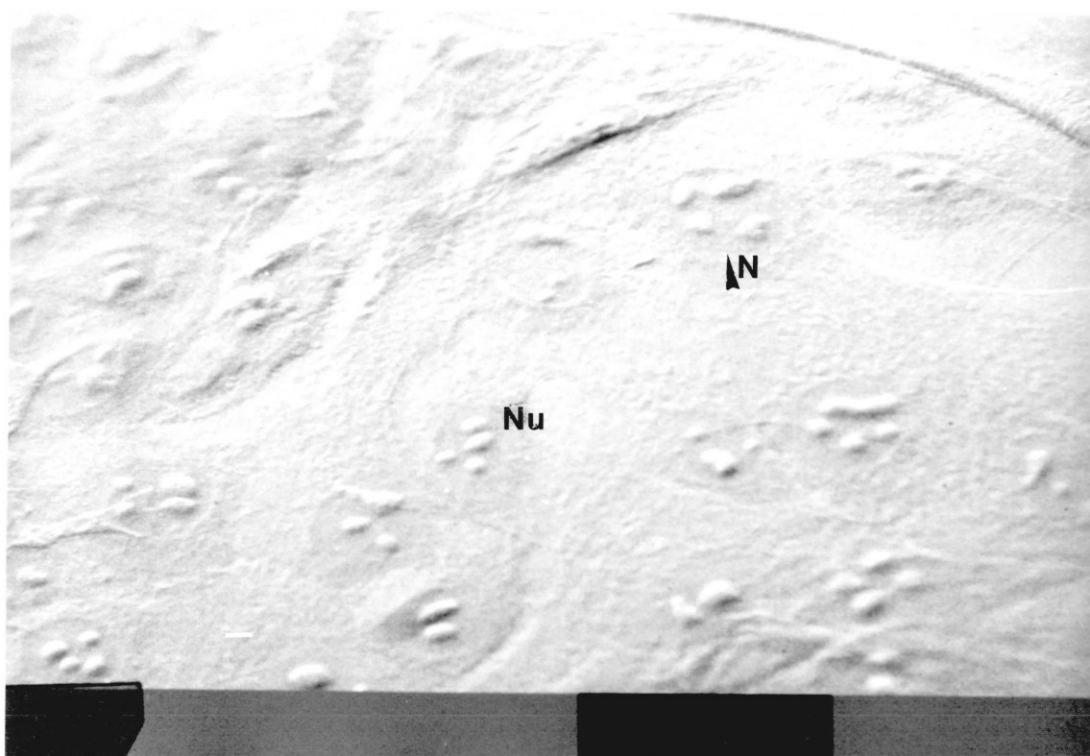
Fibroblasts

Although it was attempted to avoid including sub-epidermal stroma in the biopsies taken for explants fibroblast contamination of the cultures sometimes occurred. The fibroblasts had cytoplasm which was dendritic and scanty and had smaller nuclei than the epithelial cells. A fibroblast may lie with its long axis in any direction, but at the periphery of the outgrowth fibroblasts tend to occupy a position roughly parallel to the growing edge, lying across the epithelial cells in a thin band, several cells in from the perimeter of the monolayer. (Figure 2)

Morphology of the epithelial cells under scanning electron microscopy

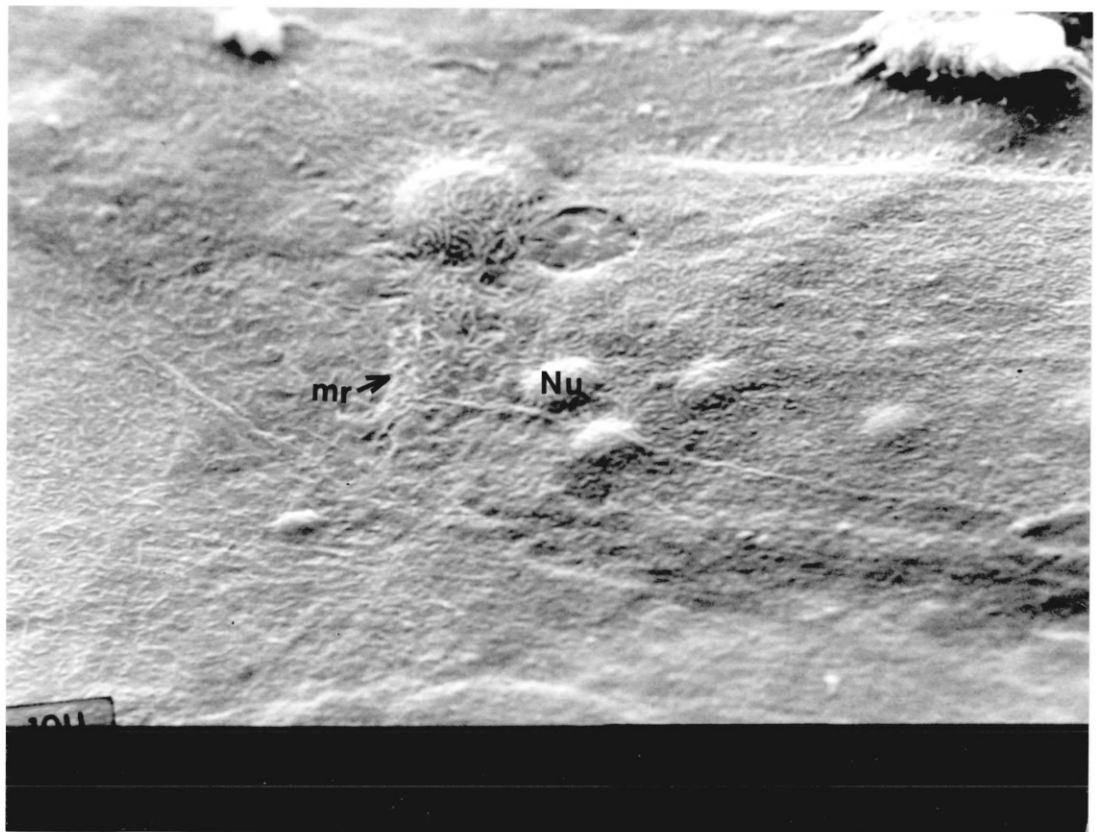
The epithelial cells of the monolayer were irregularly polygonal in shape and are flat except for a raised nuclear hump. The position of the nucleoli could sometimes be seen (Figure 3). The cell surface could be covered with short microvilli or a pattern which looks like microrugae, but the ridges do not appear so tightly packed as in cells in the original explant (Figure 4). Some cells in the monolayer appeared to have a smooth surface and these are cells in mitosis (Figure 5). Cells in later stages of division could be seen covered by microvilli (Figure 6). Cells around the perimeter of the monolayer were covered by microvilli and had long thin finger-like projections termed marginal fibrils (Figure 7), Cameron et al., (1980). These marginal fibrils have been described in a scanning electron microscopy study as either appearing in advance

Figure 3



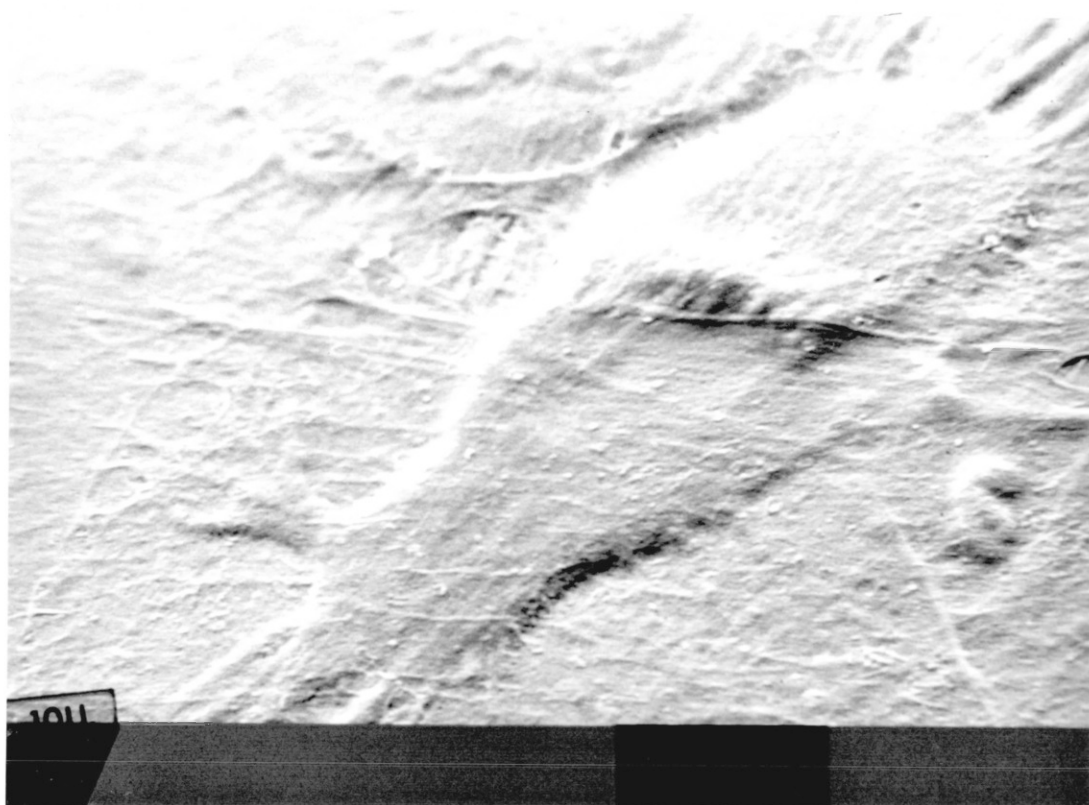
Surface of epithelial cells in monolayer culture under scanning electron microscopy. The position of the nucleus (N) can be seen with smaller nucleoli (Nu) in the middle. Bar marker 40 μ m

Figure 4



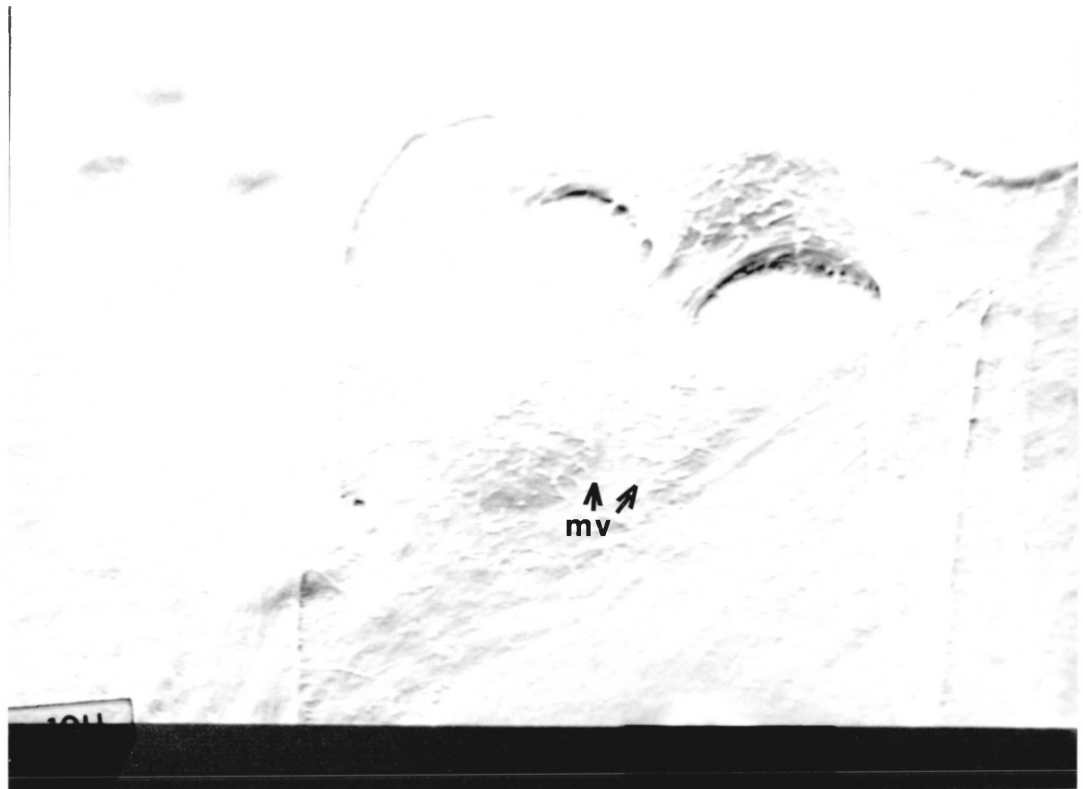
High power scanning electron micrograph of the upper surface of the epithelial cells. The surface appears to be covered by microvilli (mr), which also cover the nucleoli. (Nu)
Bar marker 10 μ m

Figure 5



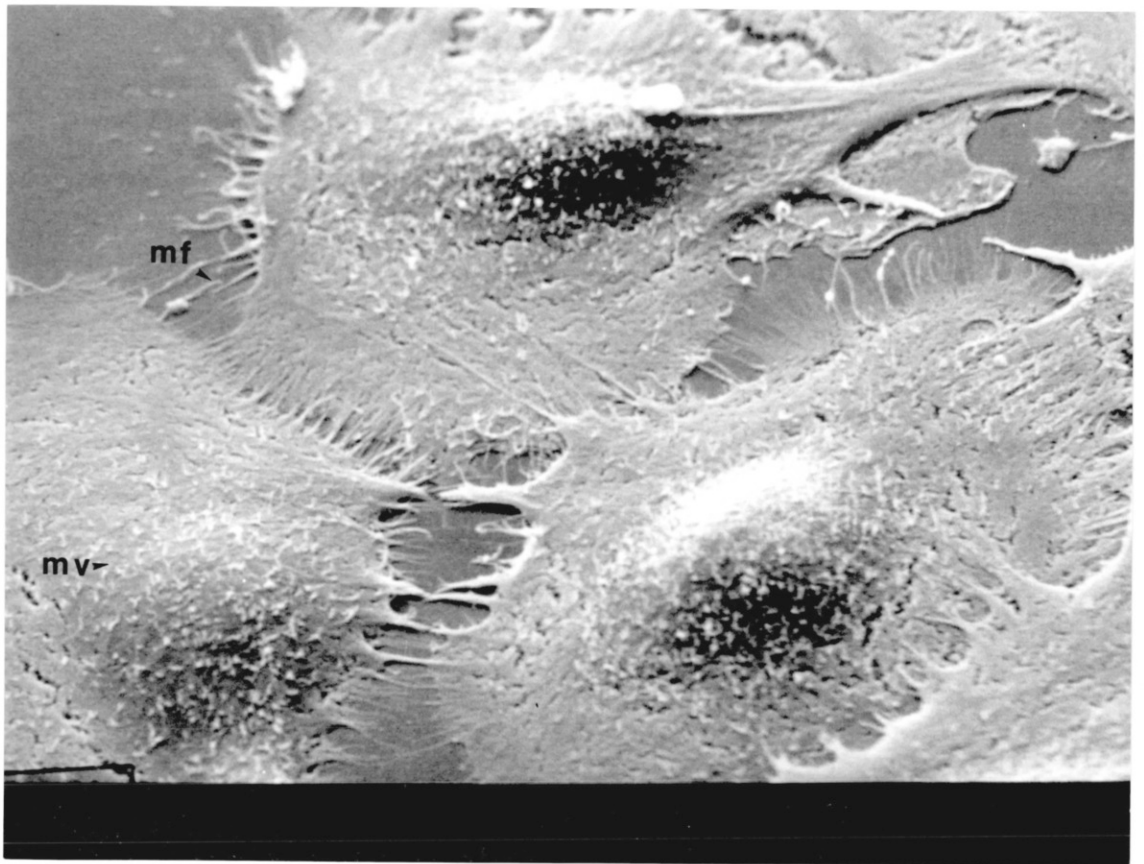
Scanning electron micrograph of an epithelial cell in metaphase. The surface of the cell is smooth, but there are marginal fibrils at the ends of the mitotic spindle axis. The cell appears to be overlying other cells in the monolayer. Bar marker 10 μm

Figure 6



Scanning electron micrograph of an epithelial cell in the final stage of division. The surface of the cell is covered by microvilli (mv). Bar marker 10 μ m

Figure 7



Scanning electron micrograph of cells on the periphery of the monolayer. The cells are domed over the nucleus and the nuclei are no longer visible. The surface of the cell is covered by short microvilli (mv). The edge of the cells protrude as marginal fibrils (mf). Bar marker 20 μ m

of the leading edge of a cell as it moves forward or as the result of the cell retracting as it rounds up. (Cameron et al., 1980) They could also be seen on cells in mitosis and once the cell was in metaphase the fibrils were mostly at the two poles of the mitotic spindle (Figures 5). Marginal fibrils were found especially frequently on the protruding edges of the flattened tongues of cytoplasm (lamellipodia) bulging out in advance of the growing cell sheet. Cells in the explant are seen in Figure 8.

Number of cells cultured

The number of epithelial cells in outgrowths was counted for each day in representative cultures from day 3 to day 8. The means are presented in Table 1.

The standard deviations are high for each mean, but regression analysis of the data for the logarithm of the number of cells on each day both show that the slope of the regression line is highly significant. ($P < 0.0001$) for the 38 pairs of values for day 4 to day 8 (Graph 1).

Regression analysis days 4 to 8 (38 pairs of values)

No. of cells on day

Log No. of cells on day

$$y = 934 + 355$$

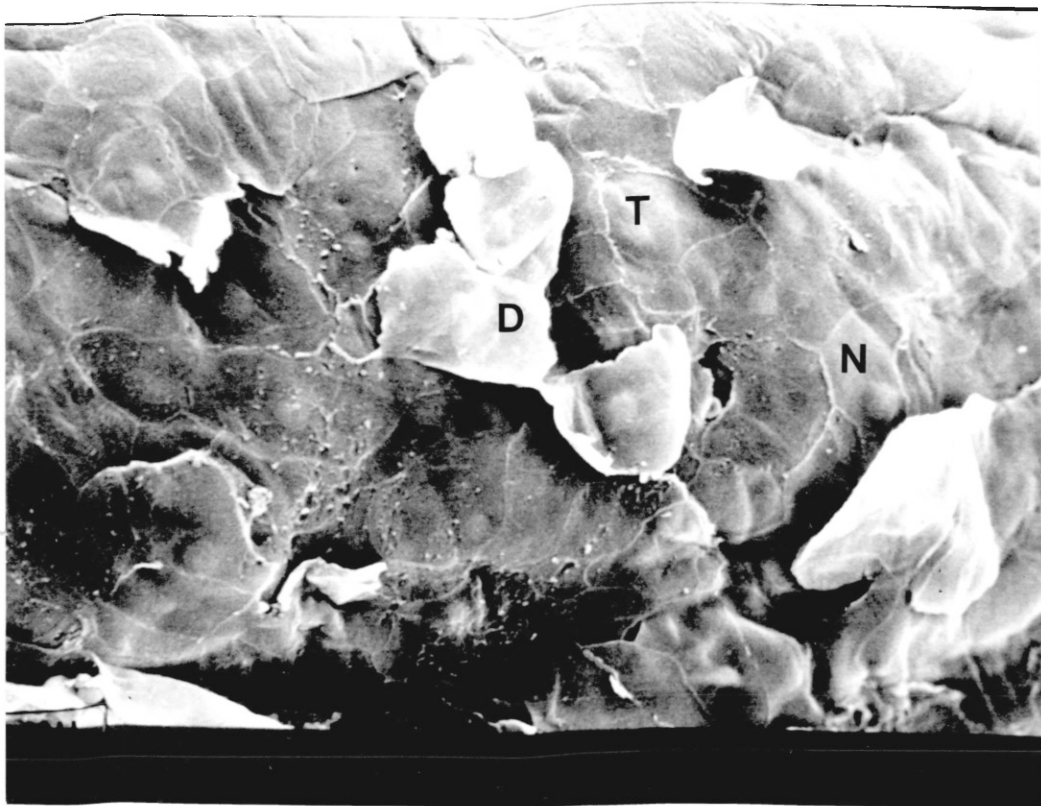
$$y = 0.21 x + 1.86$$

Regression coefficient + 0.65

Regression coefficient +0.74

t - significance of slope of line

Figure 8



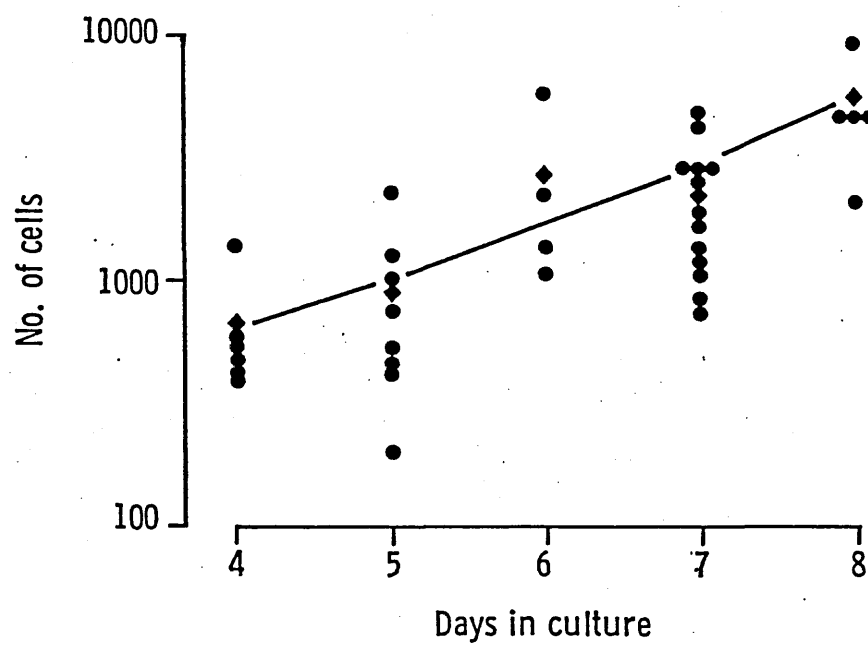
Explant of cervical epithelium under scanning electron microscopy. Pavement-like cells with raised areas demarcating the nucleus (N) and the edge of the cells marked by terminal bars (T). Some cells in the process of desquamation lie on the surface of the explant (D).
Bar marker 40 μ m

Table I

Days in culture	Number of cultures counted	Mean number of cell $\pm$ s.d.	Median
3	2	131 $\pm$ 91	131
4	6	656 $\pm$ 513	510
5	9	900 $\pm$ 651	780
6	4	2645 $\pm$ 2190	1815
7	13	2221 $\pm$ 1302	1920
8	6	5057 $\pm$ 2328	4720

Mean number of cells per day in culture.

Graph 1.



Regression line of number of cells against number of days in culture.

$t = 5.08$ 36 degrees of freedom $t = 6.67$ 6 degrees of freedom

$P < 0.001$

$P < 0.001$

y = number of cells

y = log number of cells

x = day of culture

x = day of culture

Graph 1 shows the increase in the number of cells from day 4 to day 8. A straight line was obtained on a semi-log plot. This suggests that the population of cells was in exponential growth and not in a steady state (where the number of cells being produced is proportional to the number of cells dying). In an exponential growth phase the rate of cell production remains in constant proportion to the cell population size.

Areas of the cultures

Areas of outgrowth were measured after fixing and staining the cultures from day 4 to day 8. When more than one explant in a culture produced a monolayer, the mean outgrowth was taken as representative for that culture. The points plotted for each day came from 1 donors, between 1 and 6 cultures being measured per donor. The areas were found by Thomlinson (personal communication) to be logarithmically distributed and Table II records the geometric means and standard deviations for the areas of outgrowth for day 4 to day 8 in culture. Graph 2 shows the data on a logarithmic scale.

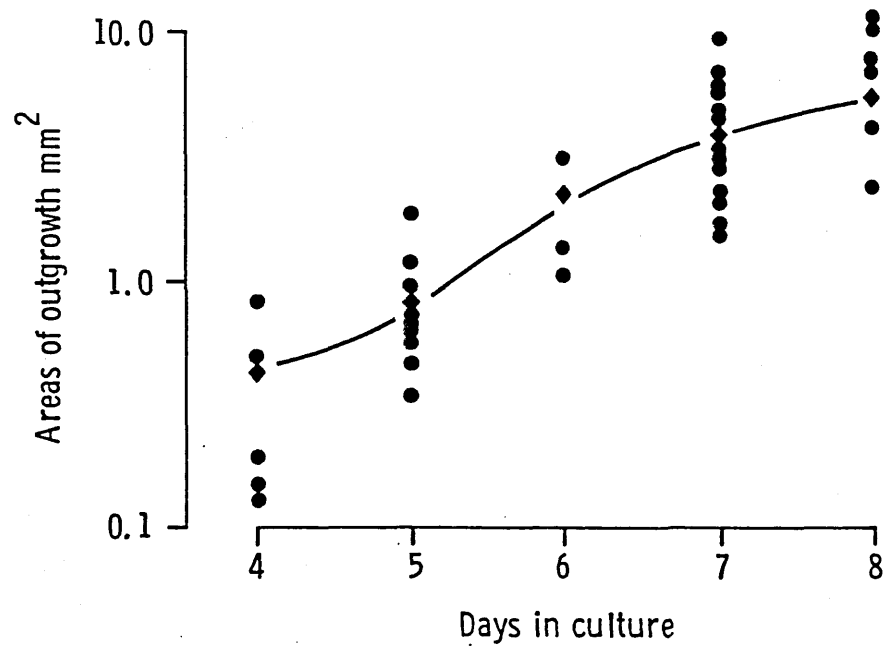
From the means it can be seen that the areas increased to day 8.

Table II

Days in culture	No. of cultures measured	Mean areas $\text{mm}^2 \pm \text{s.d.}$
4	6	0.43 ± 0.33
5	9	0.82 ± 0.46
6	4	2.17 ± 1.12
7	13	3.92 ± 2.44
8	6	5.62 ± 3.75

Mean area of monolayers per day in culture.

Graph 2



Regression curve for area of outgrowth
against number of days in culture.

From the graph the increase in area after day 7 to day 8 was not as great as between days 4, 5, 6 and 7. There was a considerable overlap of areas between day and day 8.

Regression analysis log area Day 4 to day 8

$$y = 0.32 x - 1.75$$

regression coefficient 0.86 36 degrees of freedom

t for regression = 10.5; $P < 0.001$

y = mean log area of slide

x = day of culture

Donor effects on growth

It was thought that age and parity of the donors and the point in the menstrual cycle that the specimens were taken might effect growth in the cultures.

Thomlinson (personal communication) looked for age and parity effects on growth in the cultures using the logarithms of the area measurements. The ages of a total of 32 donors ranged from 21 years to 66 years. The parity range was from 0 to 6. For day 7 cultures the partial correlation coefficients for age and parity with growth were -0.19 and +0.38 respectively for 19 donors, and for day 8, +0.24 for age and +0.26 for parity from 13 donors; none of these were statistically significant. There was no significant difference between geometric mean areas of the cultures on day 7 and day 8 between nulliparous and parous women.

Cameron (personal communication) examined the geometric means for area growth in those cultures from patients in different stages of the menstrual cycle

Table III

Menstrual status of Donors	Geometric mean of area (mm ²) on day 8 cultures $\pm$ standard error (s.e.)
Proliferative (12)	1.90 $\pm$ s.e. 0.81
Secretory (8)	0.47 $\pm$ s.e. 0.19
Pregnant (11)	2.07 $\pm$ s.e. 0.75
Post-menopausal (4)	1.5 $\pm$ s.e. 0.49

(Number of donors in parenthesis)

Geometric means of th areas of monolayers from
donors of different menstrual status.

(Table III). She compared cultures of biopsies from patients in the secretory phase of the cycle, pregnant women and postmenopausal women to biopsies from women in the proliferative phase. She was unable to detect statistically significant differences.

In the present work no donor effects on the growth of cultures of normal cervical epithelium were detected.

Number of cells per unit area

The mean number of cells per unit area were computed for the cultures and are shown in Graph 3 and summarised in Table IV. The line drawn through the means showed a decrease ($P < 0.05$) over six days in culture. There are two possible explanations for this; either the rate of cell division begins to slow down or, more likely, the decrease is due to 'spreading' of the epithelial cells across the glass coverslip, since there is no obvious decrease in mitotic indices.

Mitotic and metaphase indices

The mitotic index is estimated by fixing cultures and counting the total number of cells within the culture which are in any stage of mitosis. This number can then be expressed as a fraction of the total number of cells in the culture.

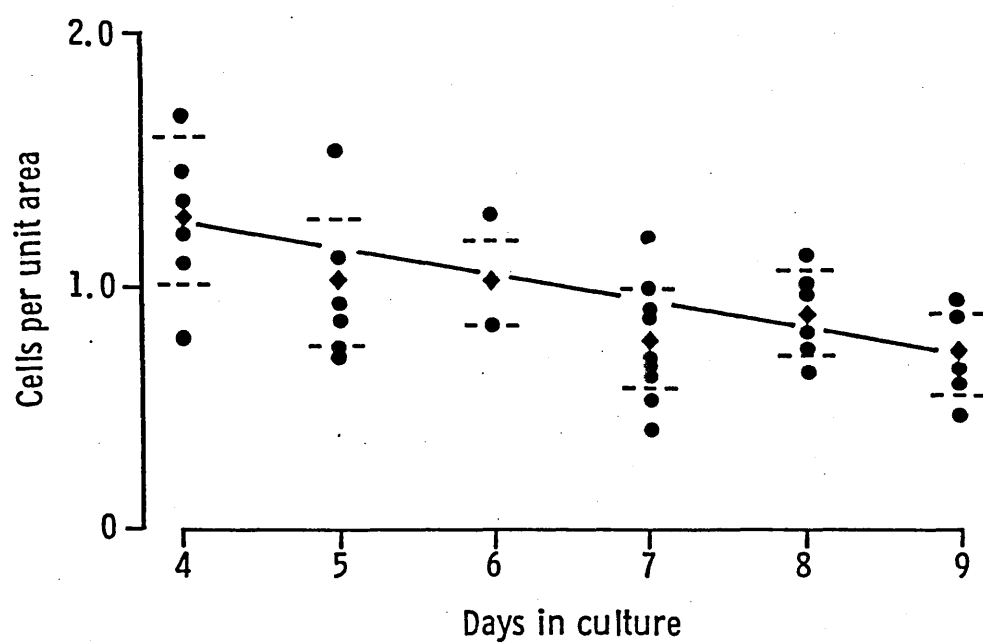
Mitotic indices were used as a guide to the rate at which the cells proliferate, but this criterion is really inadequate since the number of cells in mitosis depends not only on the rate at which cells enter mitosis and how long they remain there. If in a population the cells have a long mitotic time and the cells proliferate slowly, comparatively large numbers of cells in the various

Table IV

Days in culture	No. of cultures counted	Mean No. of cells per unit area $\pm$ s.d.
4	6	1.26 ± 0.32
5	9	1.00 ± 0.25
6	4	1.04 ± 0.17
7	13	0.76 ± 0.20
8	6	0.87 ± 0.18
9	6	0.71 ± 0.17

Mean number of cells per unit area per day in culture.

Graph 3



Mean number of cells per unit area against number of days in culture.

stages of mitosis will be seen at any one time. Stathmokinetic agents enable arrest of cell division in a particular phase of the mitotic cycle. The metaphase index can be counted as a proportion of the total number of cells, and this gives a better estimate of cell proliferation, since it is solely dependent upon the rate at which cells enter mitosis and not on how long they remain there.

Mitotic indices

In the present work mitotic indices ranged from 0.50 to 2.71 per cent of cells in day 8 cultures, while Hounsell and Hawkins (1974) found mitotic indices in day 8 cultures ranging from 0.12 to 1.92 per cent. These results and the values obtained by Cameron and Hawkins (personal communication) are presented in Table V and it is clear that the 3 studies are in broad agreement.

Rate of entry into mitosis and metaphase indices

Hounsell and Hawkins (1974) demonstrated that the appropriate concentration of vincristine to be added to the cultures to arrest mitosis in the metaphase was 30 ng/ml. With less than this there was some 'mitotic escape' and anaphases and telophases were seen. With doses larger than 50 ng/ml there was interference with cell multiplication resulting in reduced cell production rates.

In the present work, metaphase indices were estimated in 8 day cultures after addition of vincristine, 30 ng/ml and then fixation at timed intervals for up to 8 hours. Previous work (Cameron and Hawkins) suggested there may be a lag phase of about 2 hours before mitosis is arrested, but we were unable to show this in our cultures.

From the slope of the line on the graphs (4) the rate of entry into mitosis or cell production rate can be calculated (Table VI). We found the range

Table V

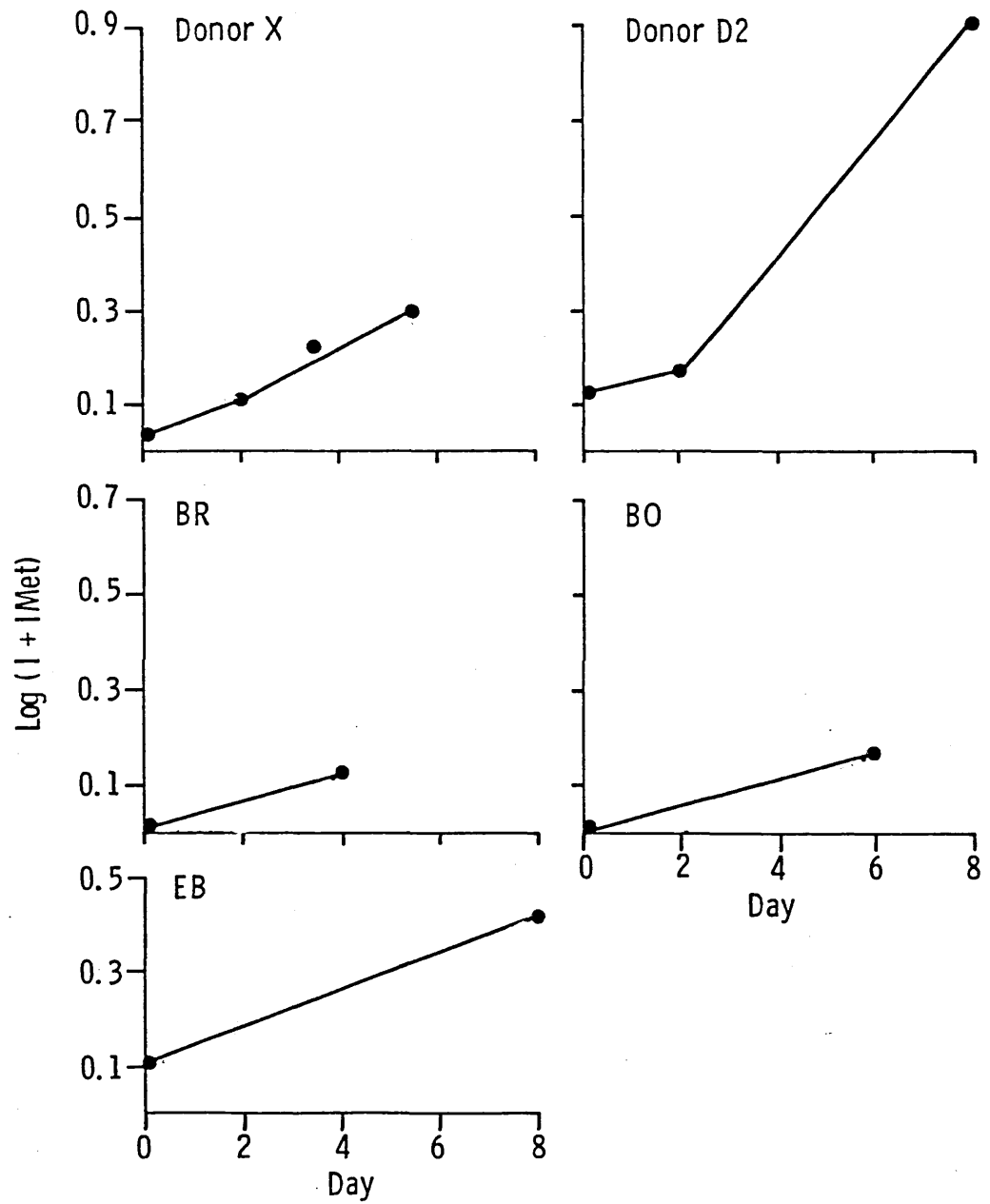
Mean Mitotic Indices for Day 8 cultures in 3 separate studies

Present Work	Hounsell and Hawkins (1974)	Cameron and Hawkins (1980)
0.50%	0.85%	0.37%
0.81%	0.12%	0.37%
0.15%	0.18%	0.54%
0.49%	0.95%	1.62%
2.29%	1.92%	0.89%
2.71%	0.84%	0.19%
0.18%	0.38%	0.27%
0.15%	1.38%	
0.35%		
0.41%		
0.39%		
0.72%		

Table VI Cell production rate and potential cell doubling time calculated on cultures from 5 donors

Donor	Hours after addition of Vincristine	Mean Mitotic index %/Hour	Mean Metaphase index %/Hour	Rate of entry into mitosis cells/100cells/hr	Potential cell doubling time (Hours)
X	0	0.12	0.10	0.07	1429
	2	0.37	0.29		
	3½	0.65	0.67		
	5½	0.81	1.00		
DZ	0	0.46	0.35	0.89	112
	2	0.72	0.47		
	8	6.96	6.96		
BO	0	0.00	0.00	0.08	1250
	6	0.49	0.49		
BR	0	0.00	0.00	1.74	56
	4	0.73	0.36		
	6	3.84	3.84		
EB	0	0.53	0.30	0.17	588
	8	1.65	1.65	0.05	

Graph 4



Log of metaphase index against number of days in culture, for cultures from 6 different donors.

of cell production rates was between 0.05 and 1.74 per cent of cells entering mitosis per hour, with the potential cell population doubling time ranging from 56 hours to 1429 hours. These results compared well with those obtained by Hounsell and Hawkins (1974)

CONCLUSIONS

The morphology of the eight day cultures under scanning electron microscopy compares favourably with that described by Cameron et al., (1982). The results of previous studies on these cells in culture which showed that the age, parity or menstrual status of the donors did not effect the growth of the cultures means that there was no problem with donor selection. Therefore any woman with a histologically normal cervix undergoing gynaecological surgery could provide specimens for culture.

Stathmokinetic studies on the cells compared well with those obtained by previous workers using the same conditions of culture. There is a somewhat wide variation between the 56 hour doubling time found for donor BR and the time of 1429 hours found with donor X, but this variation was also found by Hounsell and Hawkins, and it is probably due to the small number of time intervals measured and the small number of donors counted.

ENDOCERVICAL EPITHELIAL CELLS IN CULTURE

Little has been reported on endocervical cells in tissue culture although they may be useful for studies on viruses and pathogens such as Chlamydia, probably because of difficulties involved in obtaining specimens. Access to the cervical canal for specimens is only easy after hysterectomy, and the convoluted surface of the glandular epithelium makes it very difficult to obtain a stroma-free tissue sample.

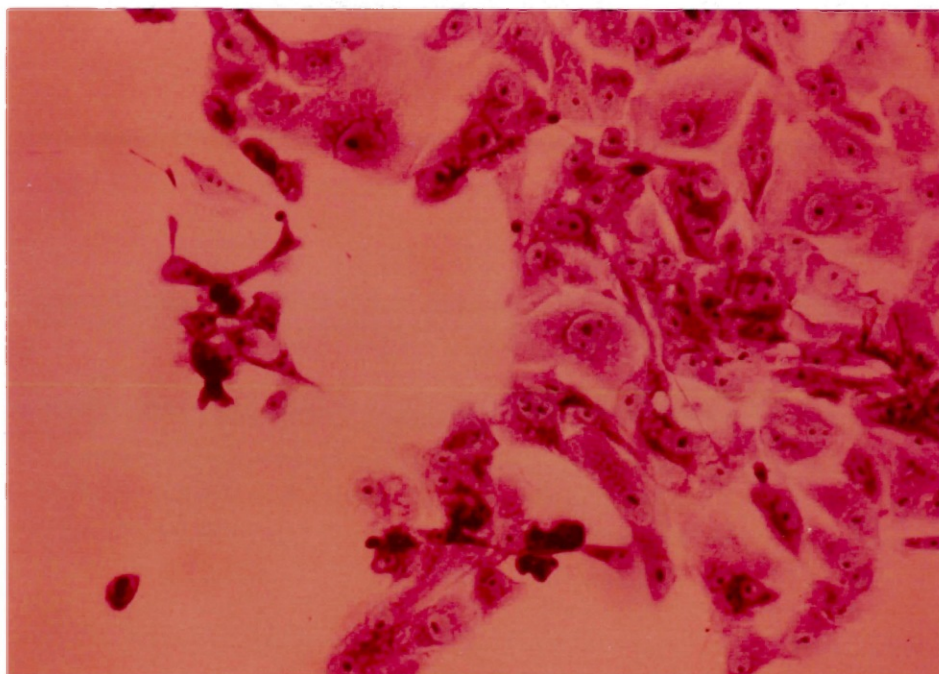
The ultrastructure of ectocervical and endocervical epithelial cells in monolayer culture was compared by Vesterinen et al (1980). They only gave a brief description of the cultured cells under scanning electron microscopy. The cellular pattern in the cultures was whorled. The cells were not covered with microrugae and only occasional microvilli could be seen.

DESCRIPTION OF THE CELLS UNDER CULTURE

Under light microscopy, 8 day cultures of endocervical cells could either be seen as a monolayer (Figure 9) of between 2000 and 4000 cells surrounding an explant, or as a patch of 20 to 40 cells lying individually or in groups, without an explant. The latter type of culture was found when the original explant had become detached during incubation. In both types of culture there was some fibroblast cell growth.

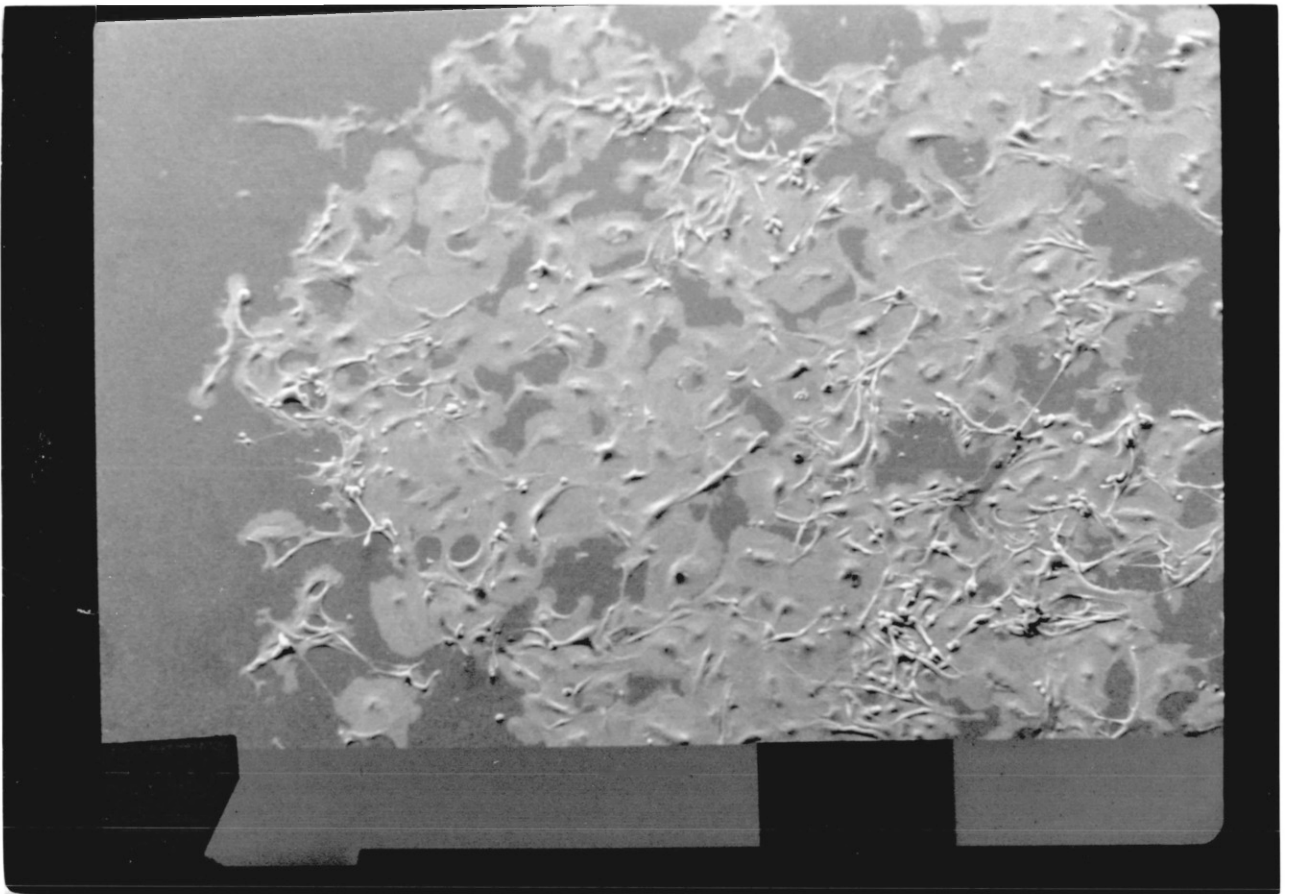
The endocervical cells stained very lightly with eosin. The cytoplasm appeared clear and not granular. The cells were larger than cultured squamous cells, with a diameter in the range of 80-100 μm , but the nuclei were of comparable size to those of squamous cells, being 10-15 μm in their longest

Figure 9



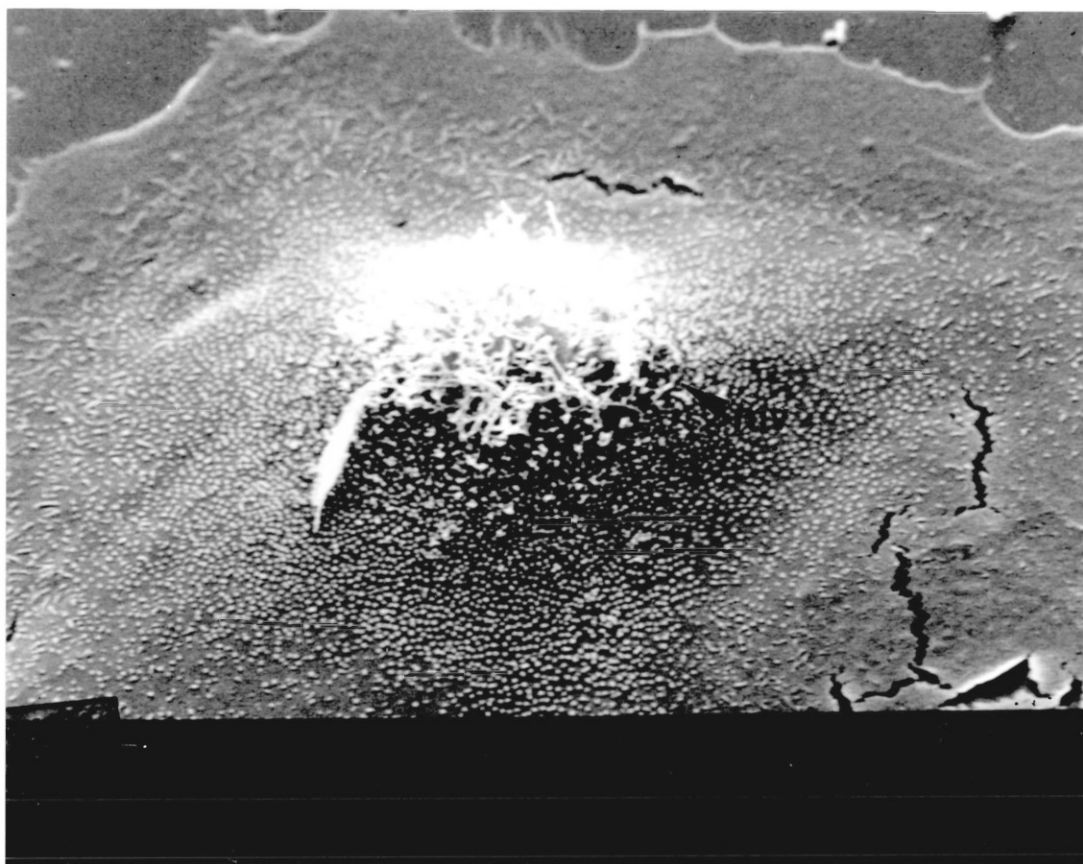
Edge of the monolayer of endocervical cells
viewed under light microscopy.
Magnification (x100)

Figure 10



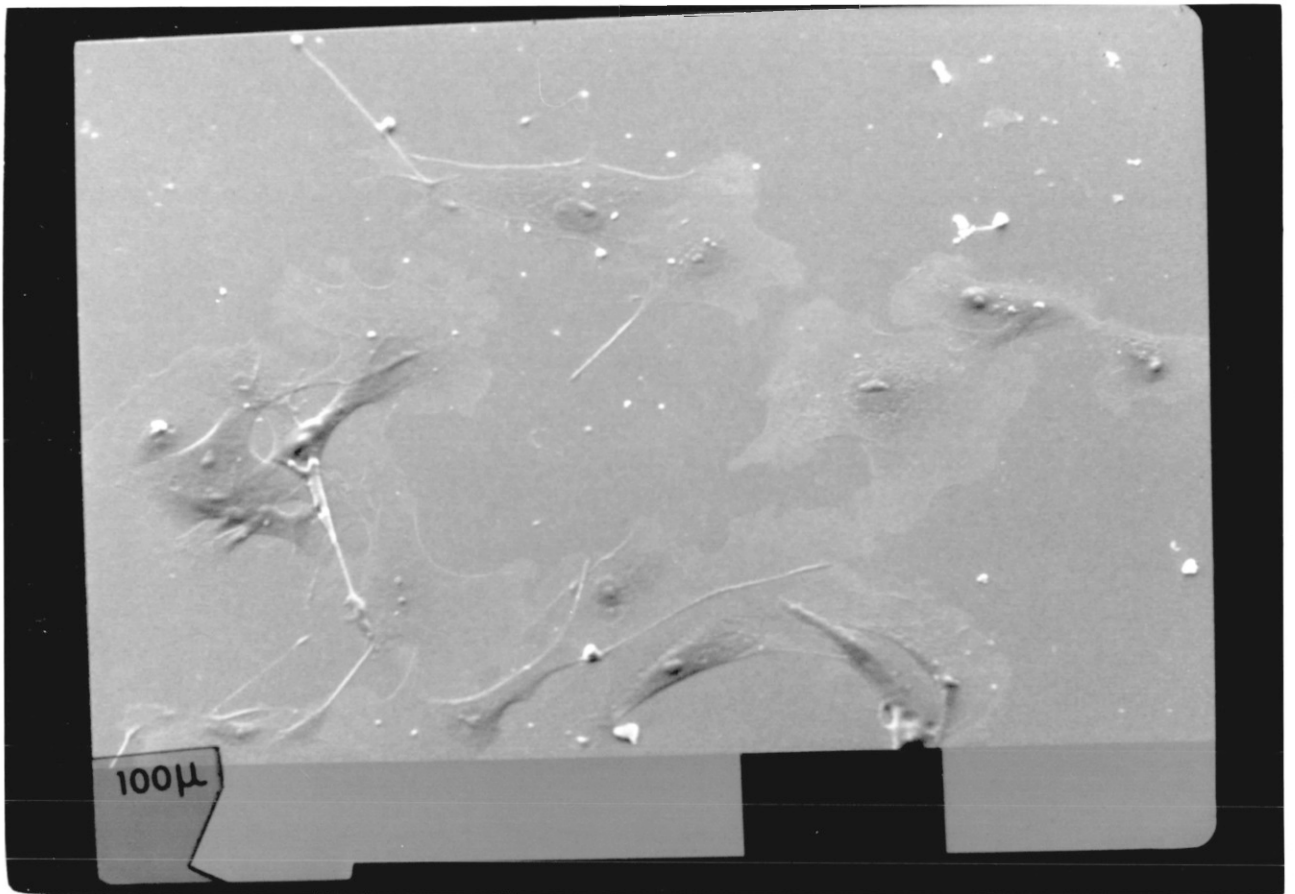
Scanning electron micrograph of endocervical cells
in monolayer culture.
Bar marker 400 μm

Figure 11



A single endocervical cell viewed under scanning electron microscopy. The surface of the cells are covered by short microvilli (mv). Over the nucleus the microvilli are longer on this cell, this is not a common feature.
Bar marker 10 μ m

Figure 12



Endocervical epithelial cells from the 'patch'
form of growth pattern.
Bar marker 100 μ m

diameter. The cells had a high cytoplasm to nucleus ratio. The nucleus was strongly eosinophilic and one or two nucleoli could be seen.

Under scanning electron microscopy in the monolayer cultures the cells were pushed together so that the nucleus could be seen as a central raised ovoid bump (Figure 10) and the nucleoli could not be distinguished. There was little overlapping of the cells except by fibroblasts which sometimes lay across the epithelial cells. The surface of the cytoplasm was smooth and no microrugae were seen. On some cells a few microvilli could be seen but these villi were shorter than those on squamous epithelium, but over the nucleus the microvilli might be long (Figure 11).

The cells which lay individually in the cultures were also studied under the electron microscope; their shape was irregularly polygonal and sometimes with long projection spread out across the monolayer (Figure 11). These lamellipodia-like projections might or might not have occasional marginal fibrils (Figure 12).

CONCLUSIONS

Obtaining pure cultures of endocervical cells was difficult because the endocervix is folded and therefore when specimens were taken it was difficult to cut thin slivers. Thick samples resulted in fibroblast growth. We did not observe the whorled pattern of growth reported by Vesterinen in all our cultures.

CHAPTER 4

CANDIDA

The isolation rate of C. albicans from the vaginas of pregnant women is from 15-30 per cent and slightly lower in non-pregnant women (Mead, 1974, Goldacre, et al., 1979). Torulopsis glabrata has an isolation rate of about 3 per cent; other yeast species also isolated in the following descending order of frequency are: Candida parapsilosis, Candida tropicalis, Candida intermedia, Candida krusei and Candida guilliermondii. (Goldacre et al., 1981) It has been said that Candidosis is the disease of the diseased. In 1925 Castellani and Taylor reported that candida is not found either in smears or in cultures of the normal vaginal secretion. (Castellani and Taylor, 1925) The proposition that Candida is only isolated from the unhealthy vagina has been supported in more recent studies (Carroll et al., 1973). However isolations of yeasts from apparently healthy vaginas has also been reported. This may be partly explained by contamination of the vaginal swab with gut flora, (Hurley, 1977). Between 13-26 per cent of women tested may not show any clinical symptoms or signs of genital mycosis, but may have microscopic and cultural results consistent with vaginitis (Morton and Rashid, 1977). This may be due to taking a swab in the interval between the onset of vaginitis and the development of overt clinical symptoms, or that the clinical symptoms have a wide variation in type and severity.

There are several conditions which appear to favour yeast infections:- pregnancy, the incidence of candidosis increasing with each trimester; diabetes mellitus; other endocrinopathies, such as hypothyroidism, Addison's disease, malnutrition, leukaemia and burns and immunosuppression. Iatrogenic related predispositions to candidosis are also found with X-ray therapy, immunosuppressive, antiparasitic, antibiotic and corticosteroid therapy. The use of exogenous hormones, in particular the contraceptive pill, has also been implicated in vaginal candidosis, but the results of three studies have not proved the association (Goldacre et al., 1979; Peddie et al., 1984; and Davidson,

1985). Some women taking oral contraceptives do have vaginal candidosis but it is unlikely that the low doses of exogenous oestrogen and progestagen alone are sufficient to influence the conditions favourably for the growth of pathogenic Candida although in some women they may have an additive effect.

The possible underlying mechanisms for the development of vulvovaginal candidosis in relation to these predisposing conditions can be studied from several points of view. Current areas of research include: the environmental condition of the vulva and vagina; pH, indigenous flora, raised temperature and humidity of the vulval area due to occlusive clothing; immunological, hormonal and nutritional factors; and study of the interaction between the yeast cell and the host target tissue.

Work on yeasts is complicated by the dimorphology of the organism. All the yeasts in the genera Candida and Torulopsis grow as unicellular round or ovoid organisms, which multiply by 'budding'. This process is asexual reproduction in which the yeast cells undergo mitosis; a section of the 'maternal' yeast cell wall balloons out in the shape of a small bud. As the bud fills with cytoplasm and cellular contents from the mother cell the bud enlarges. When the bud or daughter cell is approximately two thirds the size of the mother cell a primary septum is laid down. This is followed by secondary septa on either side. Finally the daughter cell breaks or buds off from the mother cell, leaving a bud scar on the mother cell; the bud scar on the daughter cell is virtually invisible in Candida albicans, (Shannon and Rothman, 1971).

All Candida species are characterised by their ability to produce pseudohyphae. These arise as a result of budding yeast cells remaining attached to each other and elongating to form chains of bloated tubes, pinched between each cell by constricting septa. Daughter yeast cells continue to bud either at the apex of the end pseudohyphal unit or on the side of a tube in the region just behind the constricting septa.

Candida albicans and Candida stellatoidea are unique in that in serum at 37°C they display a third morphological form. In the first stage of development a cylindrical outgrowth occurs from the yeast cell wall. This elongates and is called a germ tube. It continues to grow as a parallel-sided tube, but is divided into cell units by septa. New yeast cells bud in the region immediately behind the septa. Hyphal branches may grow out instead of new budding cells and then yeast cells bud from these branches. In peptone media at 40°C, mycelium formation without branching occurs and the round yeast cells predominate in the media.

The fact that *Candida albicans* can grow in the mycelial form which appears mechanically stronger than the pseudohyphal form, coupled with the fact that *Candida albicans* is isolated most frequently from infections has led to the theory that mycelial production is indicative of pathogenicity. However Torulopsis glabrata, which is isolated with the second highest frequency does not grow mycelia or pseudomycelia but can produce a severe inflammatory vulvovaginitis. Pathogenicity may be due to mechanical damage by yeasts and or it may be due to secreted toxins. Elucidation of the pathogenicity of yeasts must depend for a large part on the study of the interactions between yeasts and the cells they can infect.

Yeasts cells and hyphae have been observed under light, transmission and scanning electron microscopy in scrapings from the buccal and vaginal mucosa of patients with candidosis, (Montes and Wilborn, 1968; Cawson and Rajasingham, 1972; Schnell and Voight, 1976; Garcia-Tamayo et al., 1982) The yeasts are found up to 15 cell layers deep in vaginal mucosa, (Schnell, 1982) but tend to be more in the superficial layers of buccal mucosa (Garcia-Tamayo et al., 1982) The outer surfaces of the yeasts are often seen coated with a fibrillar or floccular material, which has been reported to be left on the epithelial cell surface as the yeast hyphae penetrate the cell. This finding is

disputed in a recent study in which the presence of the glycoprotein coat on intracellular yeasts was demonstrated with ruthenium red stain. (Garcia-Tamayo et al., 1982). Scanning electron micrographs of the floccular material on extracellular yeasts show it as being similar to the intercellular matrix of mucopolysaccharides described in cultured C.albicans colonies by Barns et al., (Barns et al., 1971; Garcia-Tamayo et al., 1982).

In one study tonofibrils were not seen adjacent to invading hyphae, (Montes and Wilborn, 1968), but this was disputed in another study in which hyphae were surrounded by tonofilaments. In some cases hyphae crossed intercellular junctions (Cawson and Rajasingham 1972). Pseudohyphae as well as true hyphae were observed within epithelial cells and invasion was by an apical bud with an electron dense coat (Garcia-Tamayo et al., 1982). Yeast cells in different stages of proliferation can be seen intracellularly while the vaginal epithelial cell maintains undamaged cytoplasmic membranes and desmosomes (Schnell and Voigt, 1976). Intracellular yeasts have large empty vacuoles (Montes and Wilborn, 1968, Garcia-Tamayo et al., 1982) which sometimes contained myelin figures and in S. cerevisiae and C. albicans have been shown to contain hydrolytic enzymes corresponding to those in lysosomes. It is suggested that these enzymes may play a role in the invasion of host cells by yeasts. (Montes and Wilborn, 1968).

C.albicans inoculated onto tissue cultures of mouse renal epithelial cells produced numerous short germ tubes after 1 hour, which within four hours elongate and branch, some producing pseudomycelia. In 2 hours large colonies with abundant yeast cells form (Stanley and Hurley 1967). The growth of Candida albicans on these cells was in the form of a felt which overlay and spread through and between the epithelial cells within 7-16 hours, eventually destroying the monolayer (Stanley and Hurley, 1967). Ingestion of yeast cells by macrophages was observed frequently. Once intracellular, mycelia or pseudomycelia

form which can eventually burst out of the macrophage cytoplasm and destroy the cell. Single yeast cells were seen inside epithelial cells of the monolayer but there was no evidence for active invasion by yeast cells (Stanley and Hurley, 1967).

Active invasion of endothelial cells in porcine whole blood vessel strips by yeast cells which had not produced germ tubes has been demonstrated. The invading yeast caused demonstrable damage to the endothelial cell within 15 minutes of attachment and subjacent cells became disrupted within 30 minutes. Damage to the endothelial cells was confined to the site of attachment of the yeast cell, adjacent endothelial cells without attached Candida were unaffected. Klotz et al., (1983) only say that the yeast cells pass between two epithelial cell, destroying tight junctions, they do not mention that the yeasts actually burrow into a cell. This also suggests that an exoenzyme may be released by the yeasts (Klotz et al.,1983).

Yeast cells will attach to and produce germ tubes within 75 minutes on guinea-pig vaginal epithelial cells in culture, (Persi et al., 1981) and human vaginal epithelial cells in culture (Sobel et al., 1982). Under the scanning electron microscope the adherence of yeasts was not uniform although diminished adherence in areas of active mitosis and proliferation and increased adherence to desquamating mature flat cells was reported (Sobel et al.,1982).

It is apparant that it is the relationship between the yeast cell and the host target tissues which will elucidate the mechanisms of pathogenicity.

METHODS

Fresh clinical isolates of fungi which had been subcultured once and identified were collected from the Bacteriology Department at Queen Charlottes, Maternity Hospital. The species obtained were Candida albicans,Saccharomyces

cerevisiae, Candida krusei, Candida tropicalis and Torulopsis glabrata.

The fungi were in Sabouraud's broth. This was washed off by centrifuging at 100 r.p.m. for 5 minutes. The fungal pellet was resuspended twice in epithelial cell culture medium. The fungi were examined under the microscope and the number of yeast cells per ml counted using a haemocytometer. The fungi were always in the yeast cell stage. In the preliminary experiments a smear slide of the fungi was made at 0 hours in growth medium, before and after centrifugation, for light microscopy. An inoculum of 10^4 yeasts per ml was used in all experiments.

The co-cultures were fixed at 2, 4, 6 and 24 hours after inoculation with fungal spores. Fixation was for either light or electron microscopy.

Staining with monoclonal antibody for cytokeratin.

Sections of human skin were stained as positive controls for the anti cytokeratin stain.

Cultures on coverslips were fixed in methanol washed with modified phosphate buffered saline. 40µl of monoclonal antibody (Becton Dickinson, California, U.S.A.) was pipetted on to each culture and the cultures were incubated for 30 minutes. Then the cultures were again washed with phosphate buffered saline. The second layer, 11 µg/ml of horse anti-mouse IgG Biotin (Vector, California, U.S.A.) was added to the cultures and they were incubated for another 30 minutes. ABC solution (Vector) was added and incubated for another 30 minutes. Diaminobenzidine hydrochloric acid (DAB) was used to develop the peroxidase stain. The cultures were then counter-stained with Hematoxylin rinsed in distilled water and dipped in ammonia water, before a final rinse in distilled water and mounted.

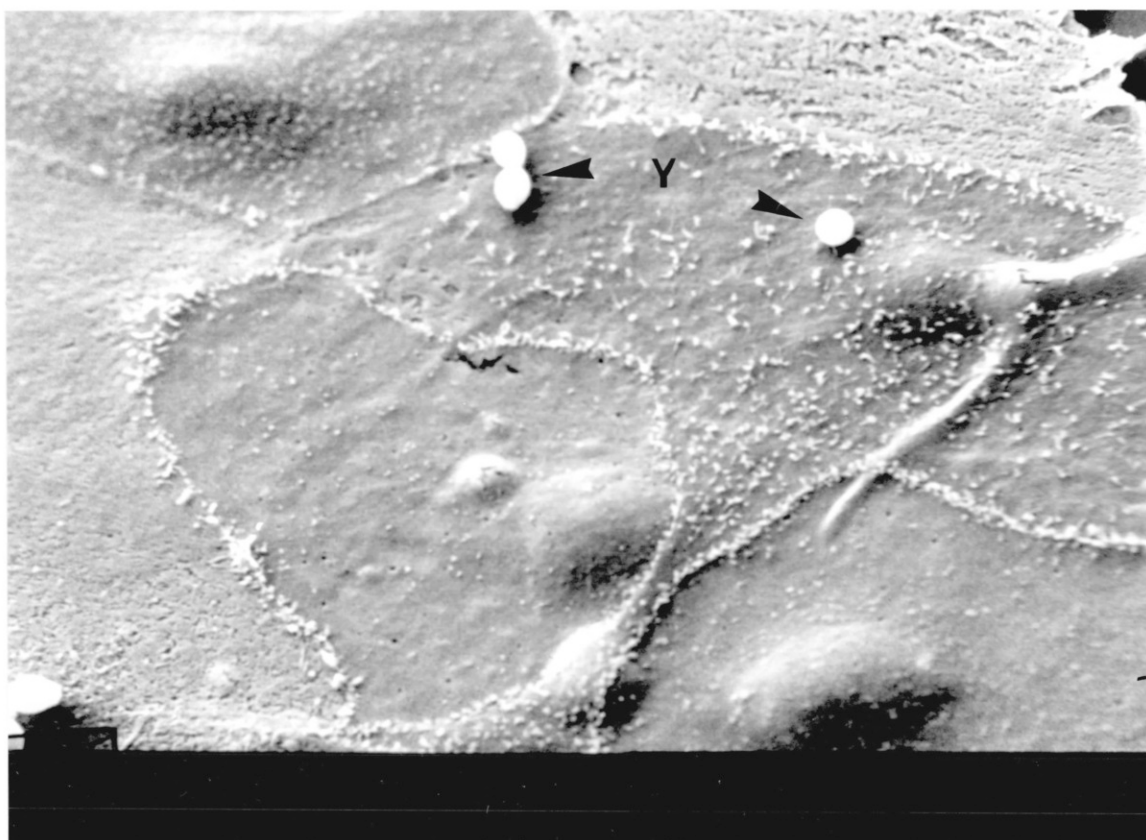
RESULTS

The blastospores settled at random on the epithelial monolayer, either individually (Figure 1) or in groups of about 4 or 5. There did not appear to be any preference for attachment to cells in mitosis. Under scanning electron microscopy the yeast cells were smooth, white and were often in the process of budding daughter cells (Figure 2). We found no morphological evidence of the attachment mechanism of the yeast cells to the epithelial cells other than the fact they were not washed away during fixation. Light microscopy generally showed more yeast cells (Figure 3), probably because the fixation process is short and the cells were not disturbed as much as they are during the many stages of fixation for scanning electron microscopy.

Between one and two hours after inoculation germ tubes were produced (Figure 4) and another hour later the mycelial form of the yeast predominated, (Figure 5). The hyphae either grew longer before penetrating the upper surface of the epithelial cell wall (Figure 6) or they started to invade as they were produced, in which case the hyphal thread might pass into the epithelial cell, re-emerge across the surface of the cell and then disappear again into the epithelial cytoplasm (Figure 7).

Near the point of invasion or emergence deposits of floccular material were sometimes seen on the surface of the epithelial cell surrounding the hyphae (Figure 8). No floccular material was seen in relation to hyphae which had grown away from the monolayer and were lying directly on the glass coverslip supporting the culture. A true hyphae may be seen separated from the 'mother' cells by what appears to be a track (Figure 7). Sometimes the track looks like a discarded second skin (Figure 9) and sometimes it looks as though it is just a collapsed hyphae inside an epithelial cell (Figure 8).

Figure 1



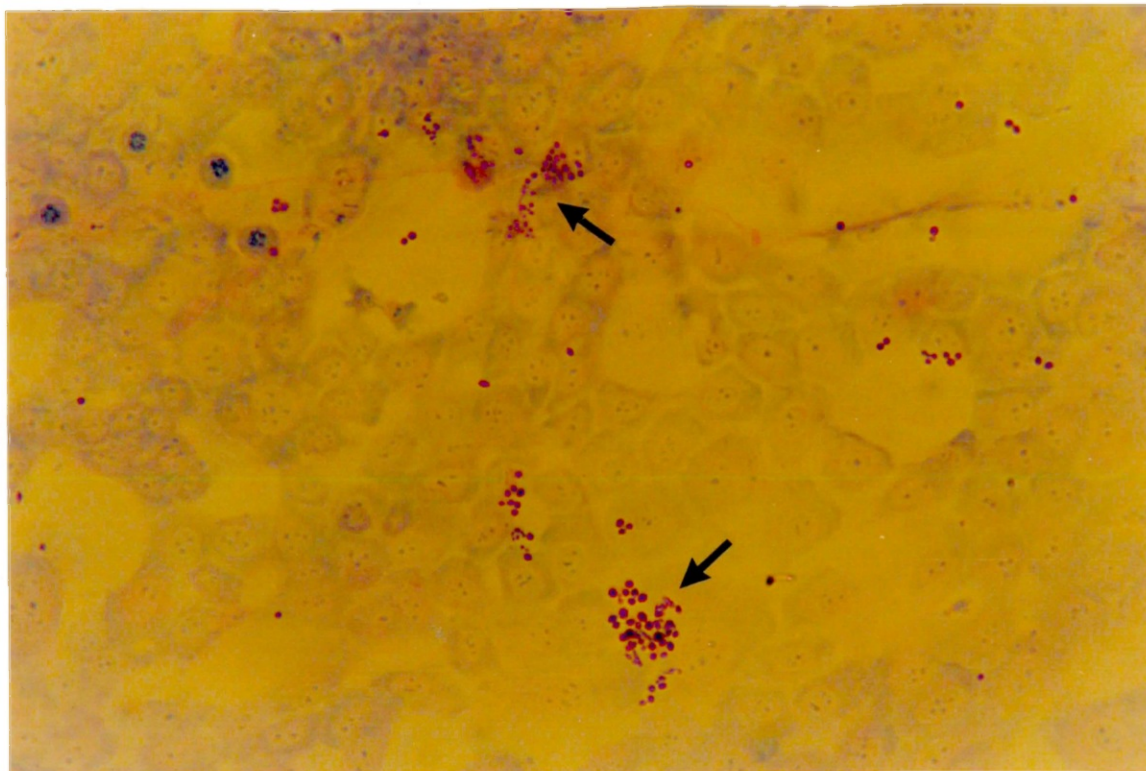
A single yeast cell (Y) of Candida albicans and budding cells (Y) on the surface of cervical epithelial cells in culture.
Bar marker 20 μm

Figure 2



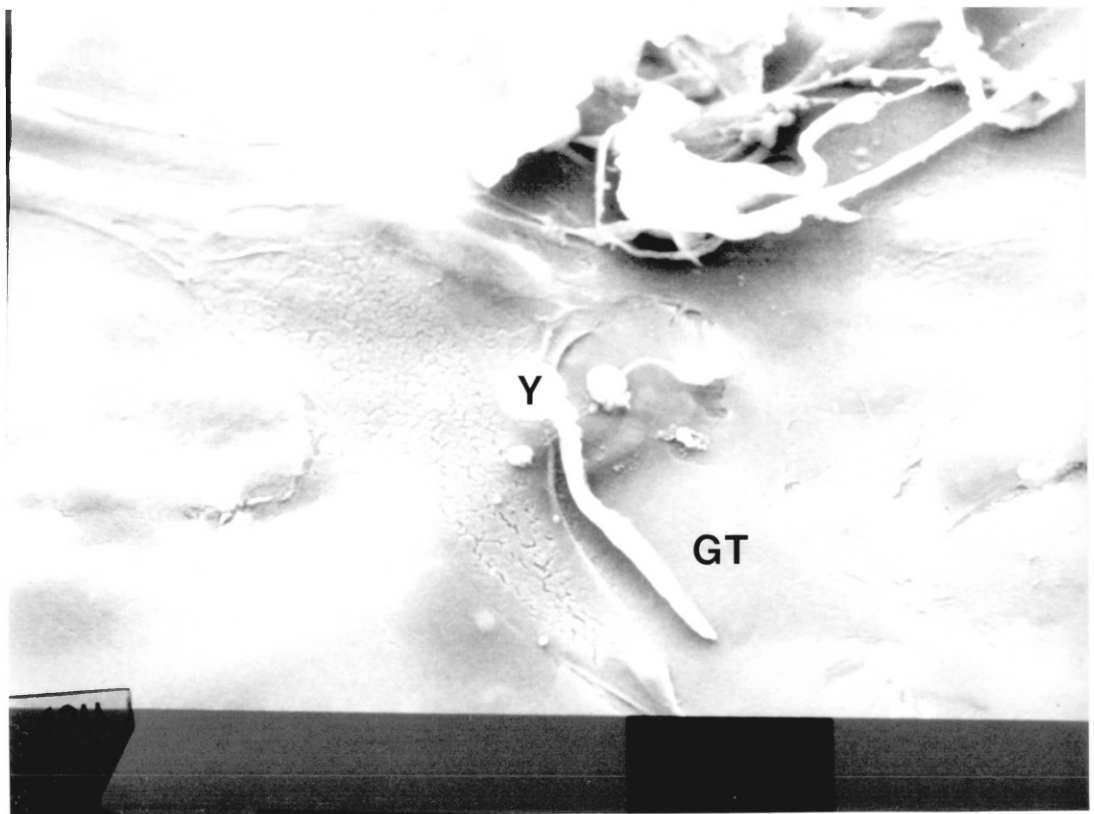
A group of budding yeast cells (Y) on the surface of epithelial cells in monolayer culture. Bud scars remain after the daughter cells have detached. Bar marker 10 μ m

Figure 3



Yeast cells of Candida albicans on epithelial cells
in monolayer culture viewed under light microscopy.
The yeast cells (arrows) are distributed in groups
or individual cells.
Magnification (x100)

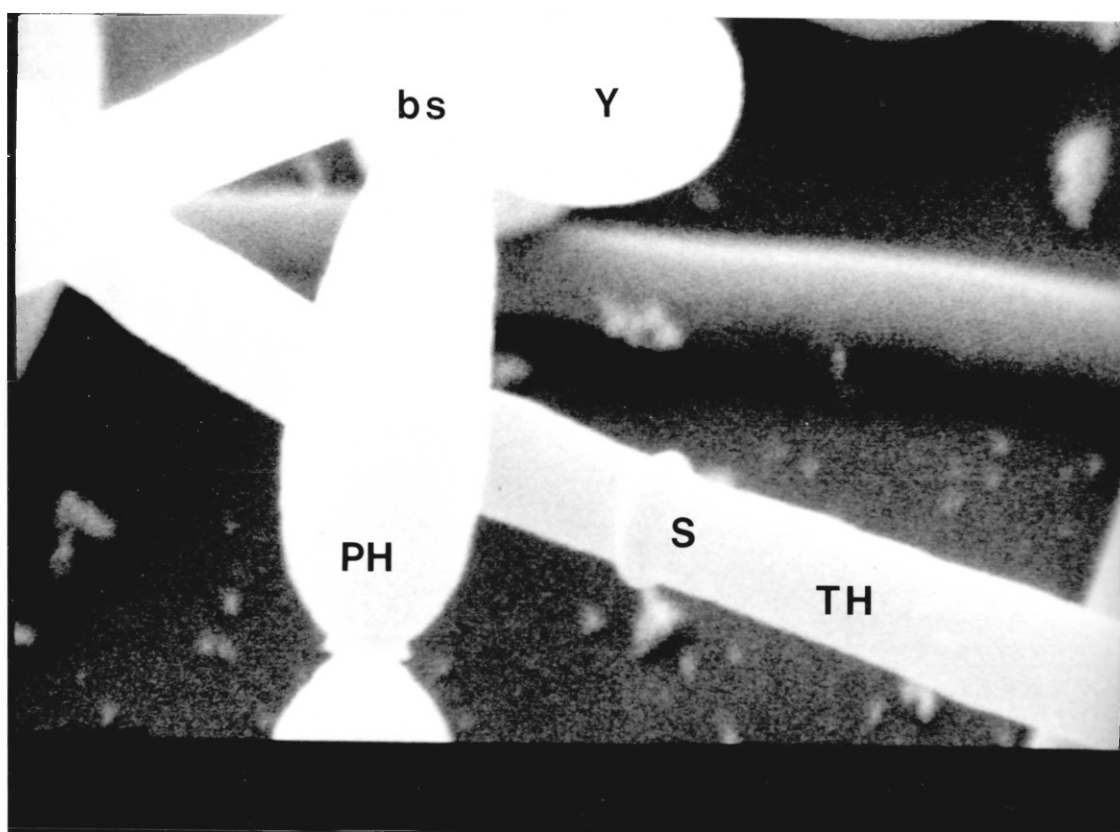
Figure 4



Germ tubes (GT) are produced by yeast (Y) cells
two hours after inoculation onto the epithelial
cells.

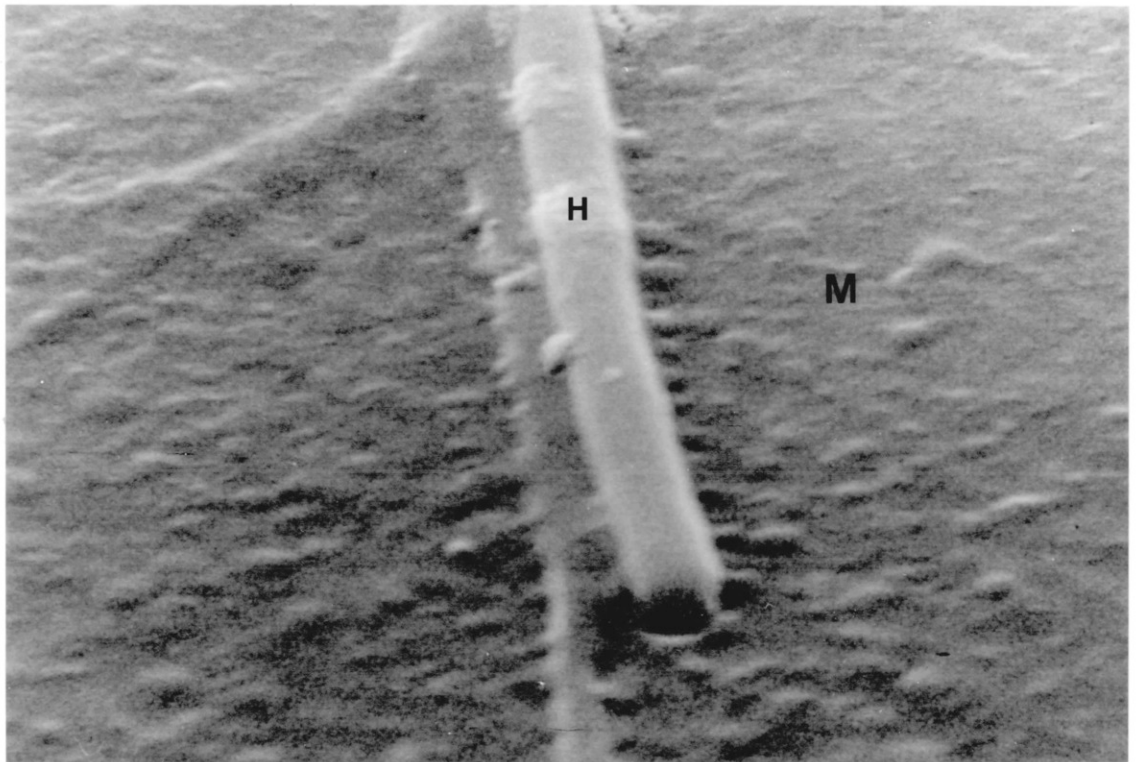
Bar marker 40 μm

Figure 5



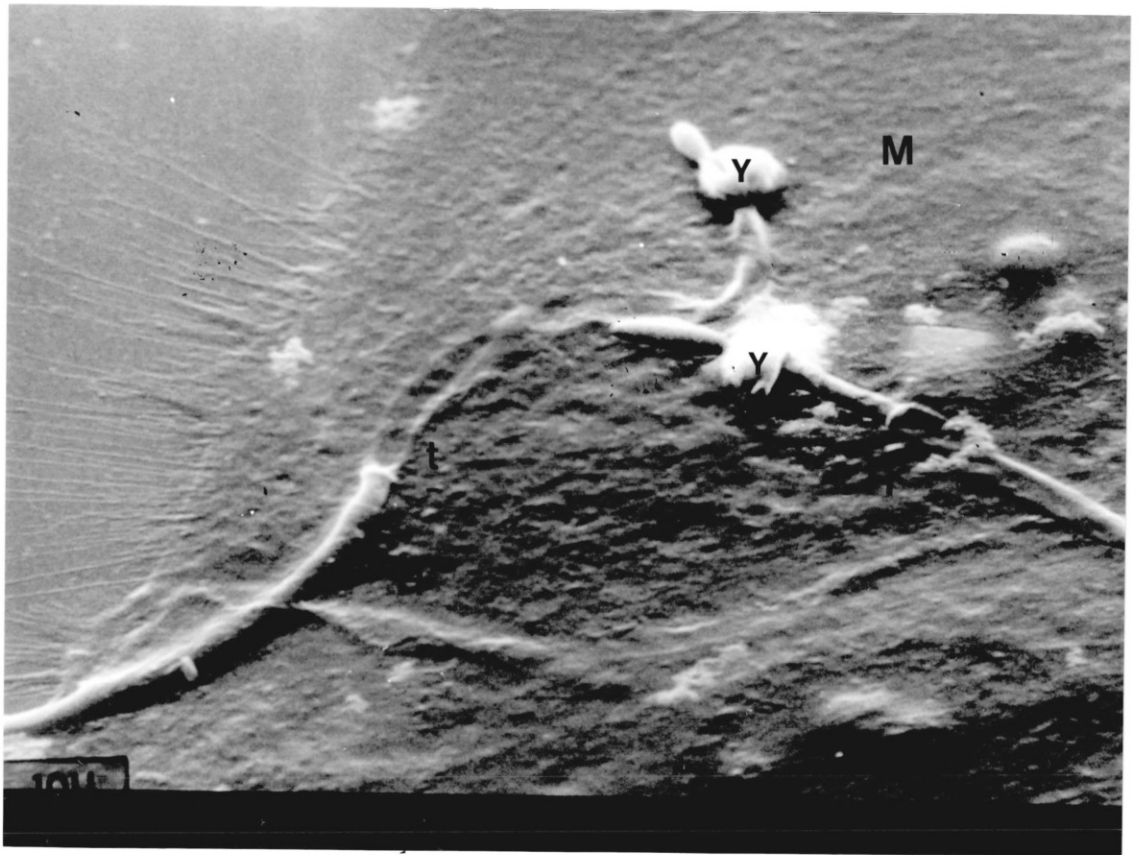
High magnification of true hyphae (TH) of *C. albicans* showing the septa (S) dividing individual hyphal units and bud scar (bs) of a budding yeast cell. Pseudohypha (PH) is also present.
Bar marker 2 μ m

Figure 6



The hyphal tip of *C. albicans* (H) can be seen penetrating the upper surface of the cervical epithelial cellin monolayer culture (M).
Bar marker 4 μ m

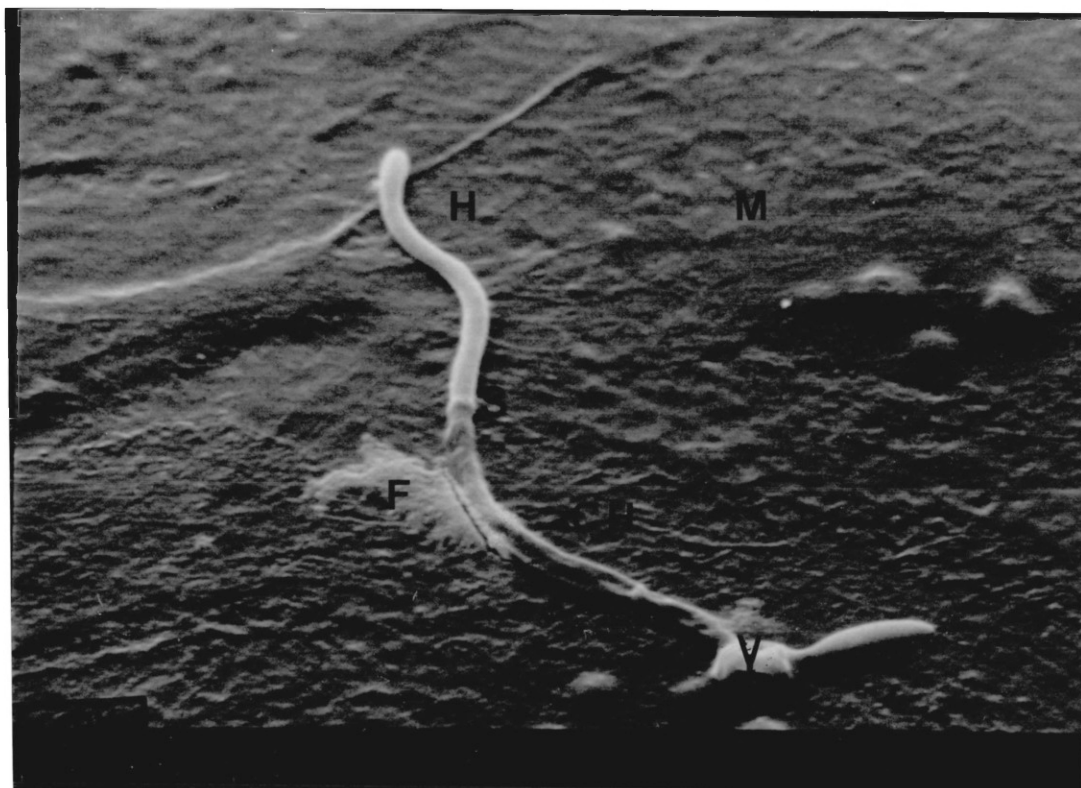
Figure 7



Six hours after inoculation of yeasts (Y) of Candida albicans onto the epithelial cell monolayer (M) the hyphae (H) from the yeast weave into and out of the monolayer. Some of the hyphae are collapsed (CH), tracks of the previous position of hyphae (t) can sometimes be seen.

Bar marker 10 μ m

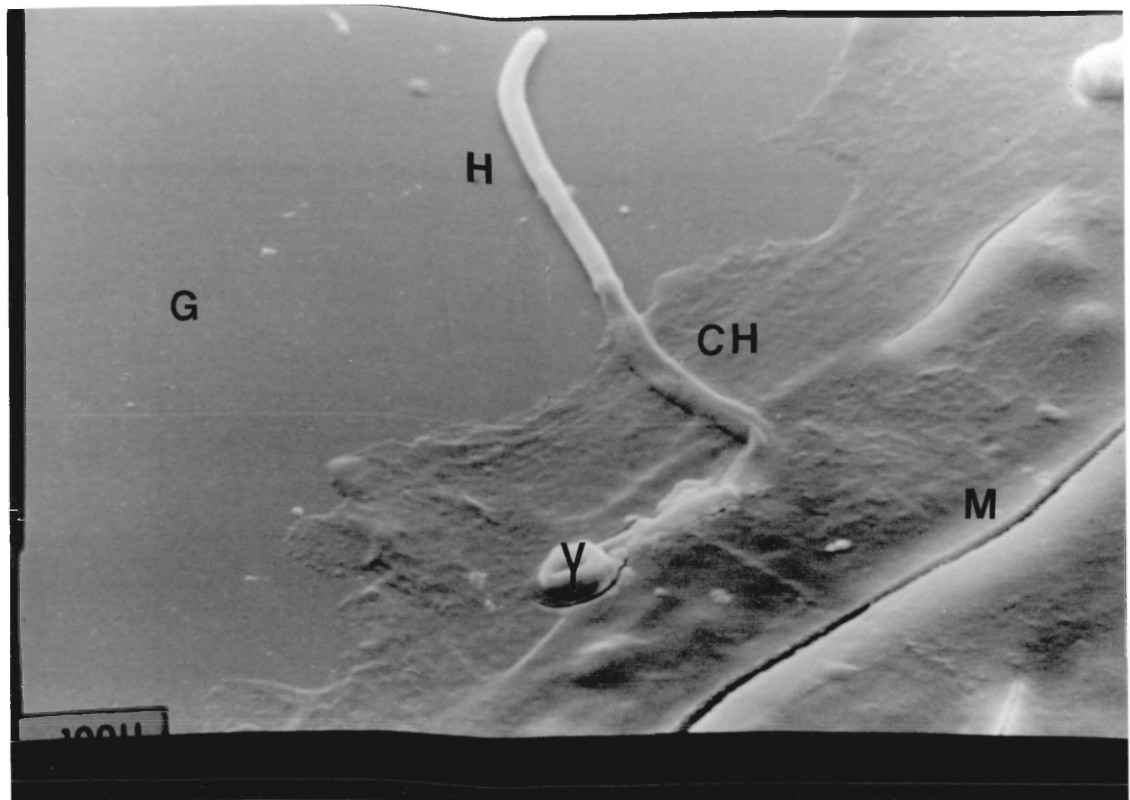
Figure 8



A portion of hypha (CH) appears to have grown from the yeast cell (Y) through the monolayer (M) re-emerged at the position of the second septa (S) from the yeast cell. At which point deposits of floccular material (F) can be seen on the monolayer. The portion of the yeast hypha (CH) which is in the cell appears collapsed, but not that portion of the hypha (H) which is lying on the surface of the cell. The position of the epithelial cell nucleoli are marked (Nu).

Bar marker 10 μ m

Figure 9



A hypha(CH) which has grown through the epithelial cell monolayer (M) and out of the edge of the cell (Y) and that part of the hypha (H) which is resting on the glass coverslip (G)
Bar marker 10 μ m

Within six hours of inoculation, 90 per cent of the yeast spores had produced long hyphal threads, which, if they were in contact with the epithelial cells, wove into and out of the epithelial cell cytoplasm. The mechanical action of these hyphae was probably the cause of cracks, ruffling, tears and overfolding in the monolayer. The resulting breaks in the epithelial monolayer may look like wounds. Where only the upper surface of the cell cytoplasm is broken, hyphae can be seen lying across the two sides of the crack and the cell cytoplasm can be seen underneath (Figure 10). In other areas the cell monolayer is unaffected by hyphae growing over the cell surface.

At the border of the cultures, hyphae can be seen passing underneath the cell so that the cell lies like a blanket on top. Sometimes marginal fibrils on the epithelial cells are seen wound around the hyphal threads (Figure 11).

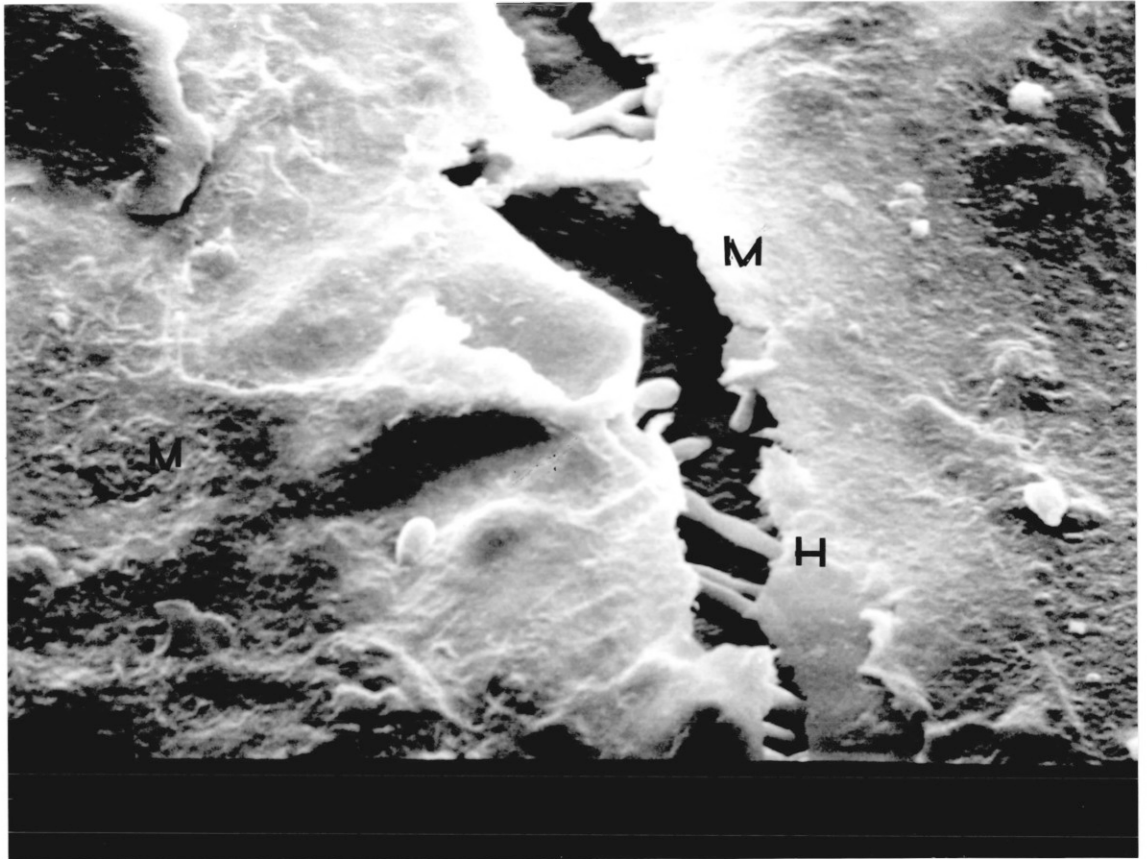
Yeast cells can be seen budding from an intracellular hypha (Figure 12). The budding yeast cells are extracellular.

Very thin threads were observed interconnecting between hyphal branches (Figure 13). The origin of one of these threads from a site near bud scar on a yeast cell (Figure 14) shows that the threads are from the Candida and not the epithelial cells.

Other species of vaginal fungi

Preliminary observations were made on five other species of vaginal fungi: C. krusei, C. tropicalis, T. glabrata and Saccharomyces cerevisiae. At least two samples of each specimen were inoculated onto three coverslips of epithelial cell growths. In the cultures infected with the first four species only yeast cells were seen scattered randomly over the surface of the culture or in small groups of 5 to 10 cells (Figure 15). No phagocytosis of blastospores by the epithelial cells was seen under light or

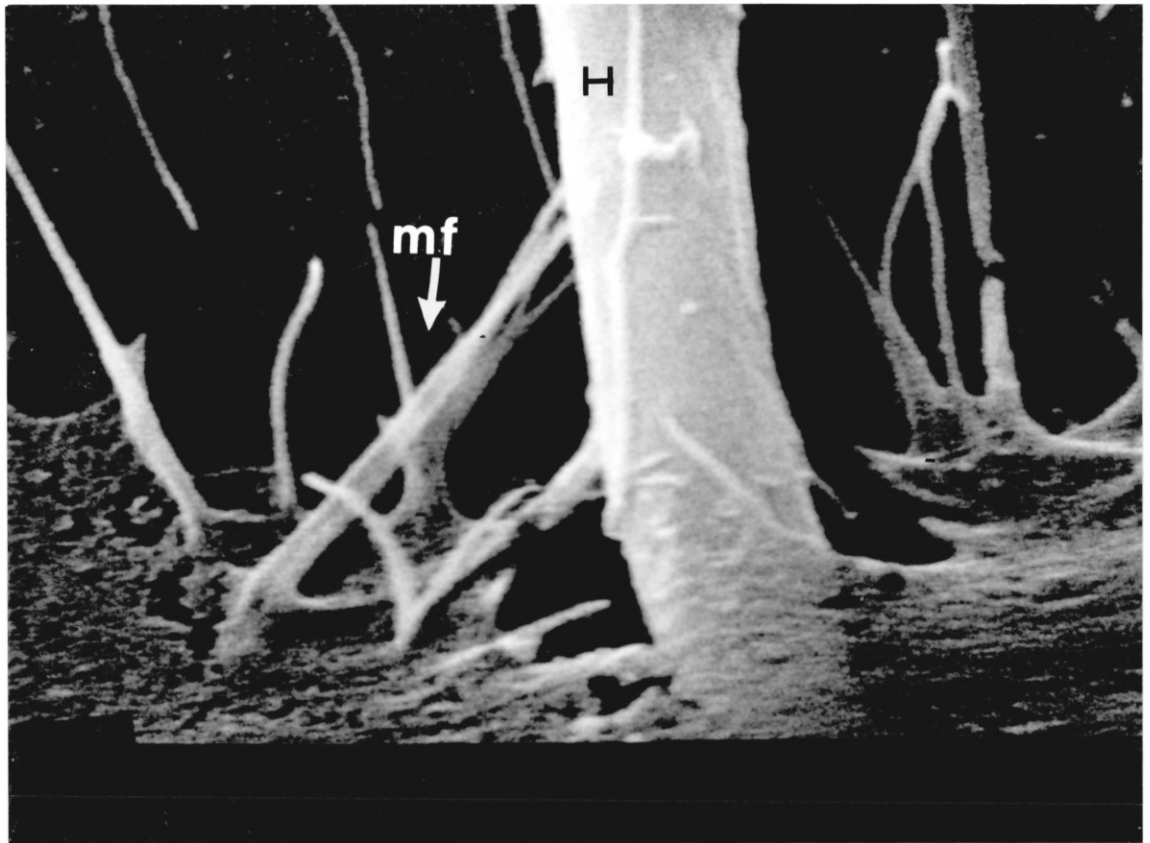
Figure 10



Twenty four hours after the inoculation of yeasts on to the monolayer (M) of epithelial cells areas of the monolayer are invaded by yeast hyphae (H). Occasionally cracks in the upper surface of the cells reveal hyphae lacing across the two sides of the wound.

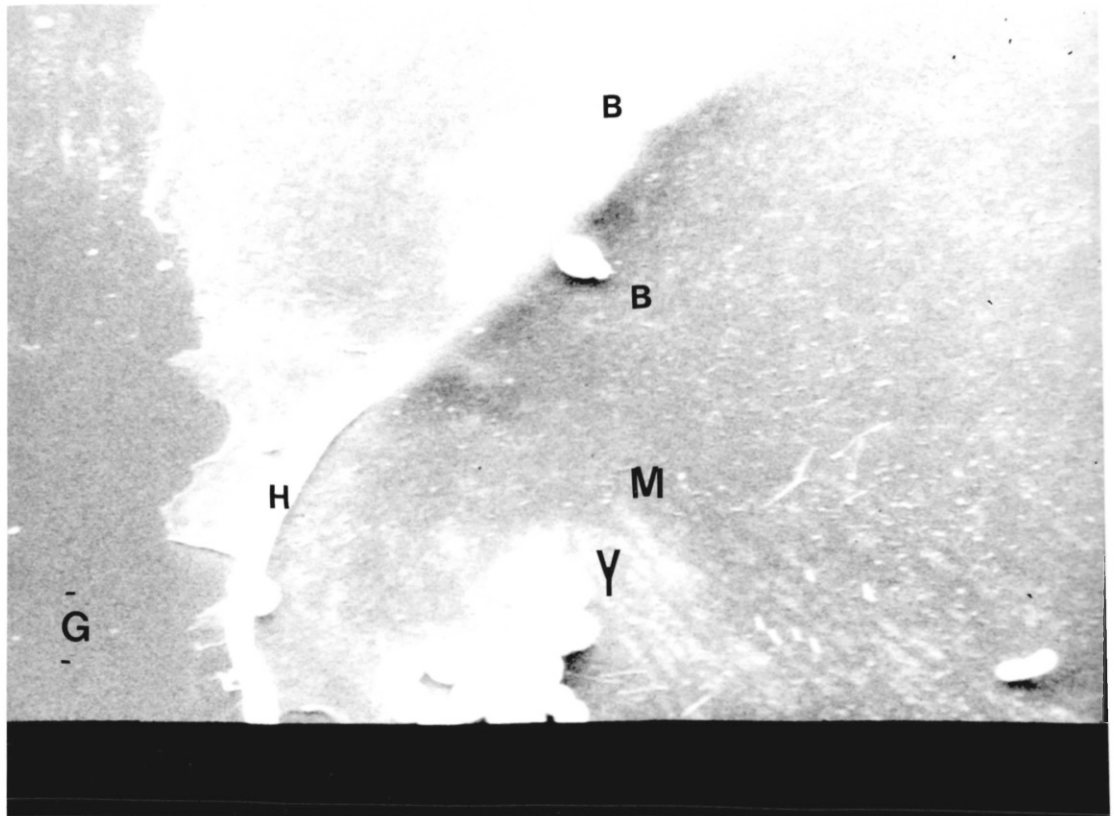
Bar marker 10 μ m

Figure 11



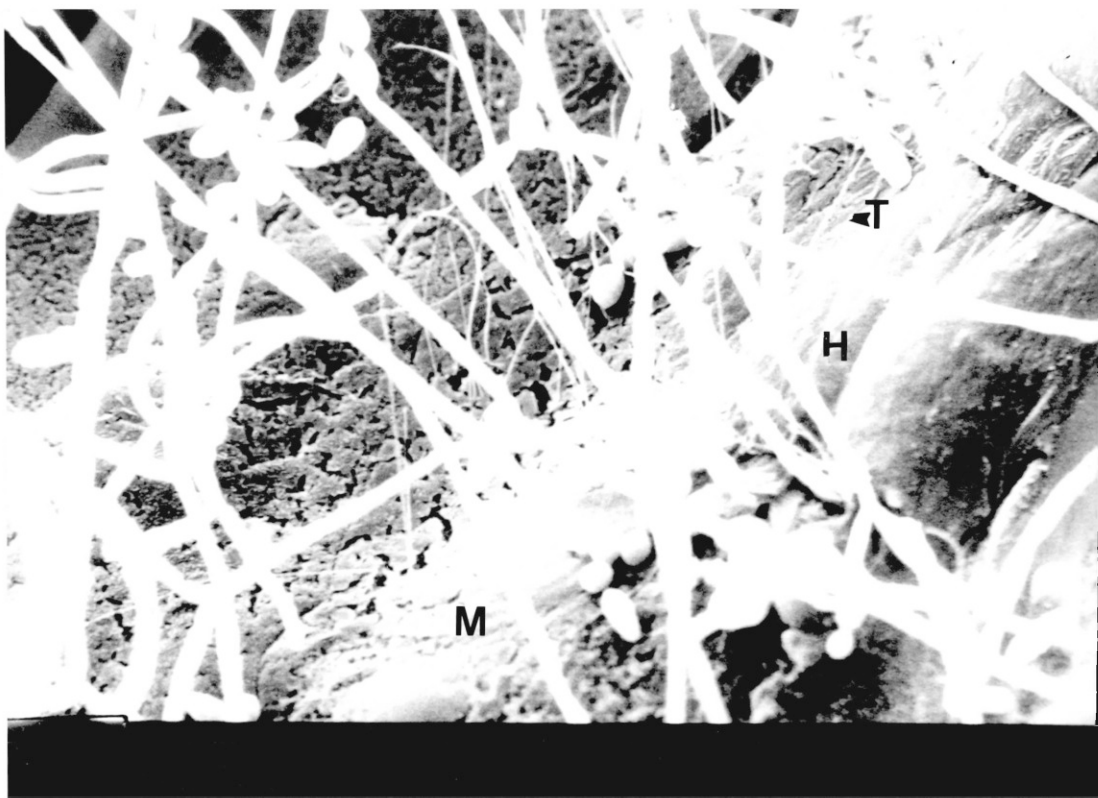
At the periphery of the epithelial cell monolayer marginal fibrils (mf) wind around a yeast hyphae (H) as it emerges from under the monolayer.
Bar marker 2 um

Figure 12



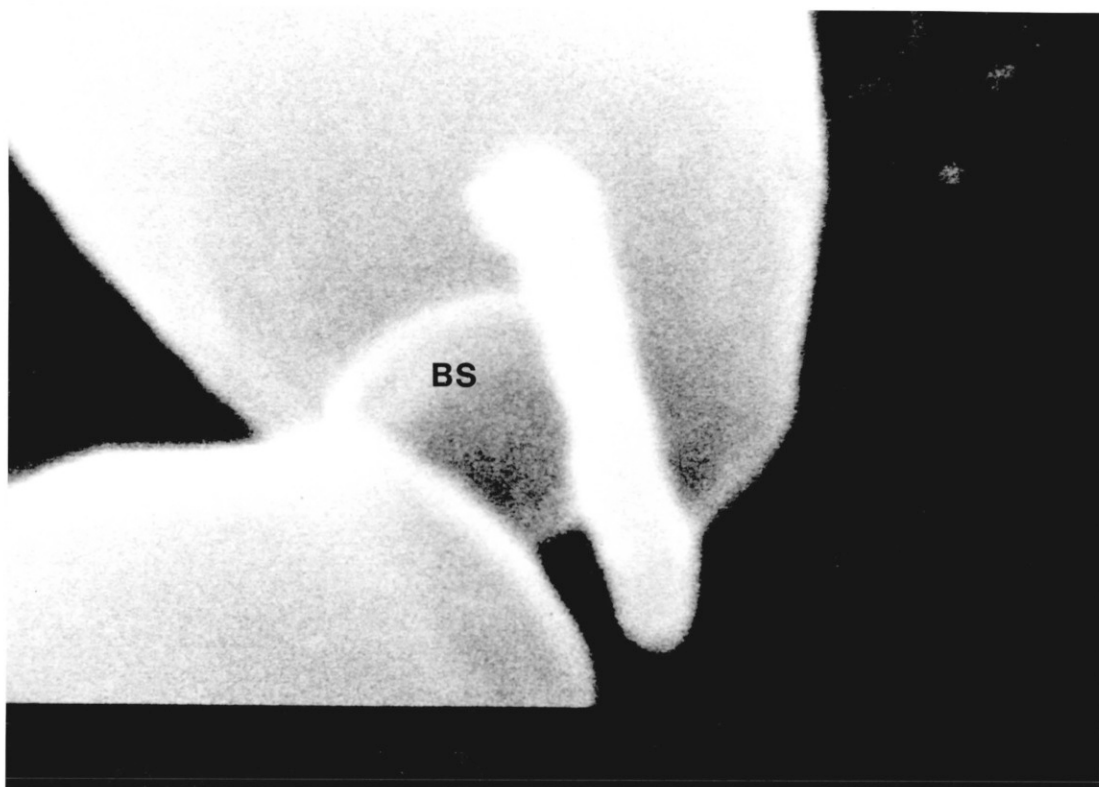
The edge of a monolayer (M) of epithelial cells on a glass coverslip (G) invaded by a hypha (H) of C.albicans. Extracellular budding yeast cells (B) can be seen growing from the portion of the hypha which is within the epithelial cell. A group of budding yeast cells (Y) lie on the monolayer. Bar marker 10 μ m

Figure 13



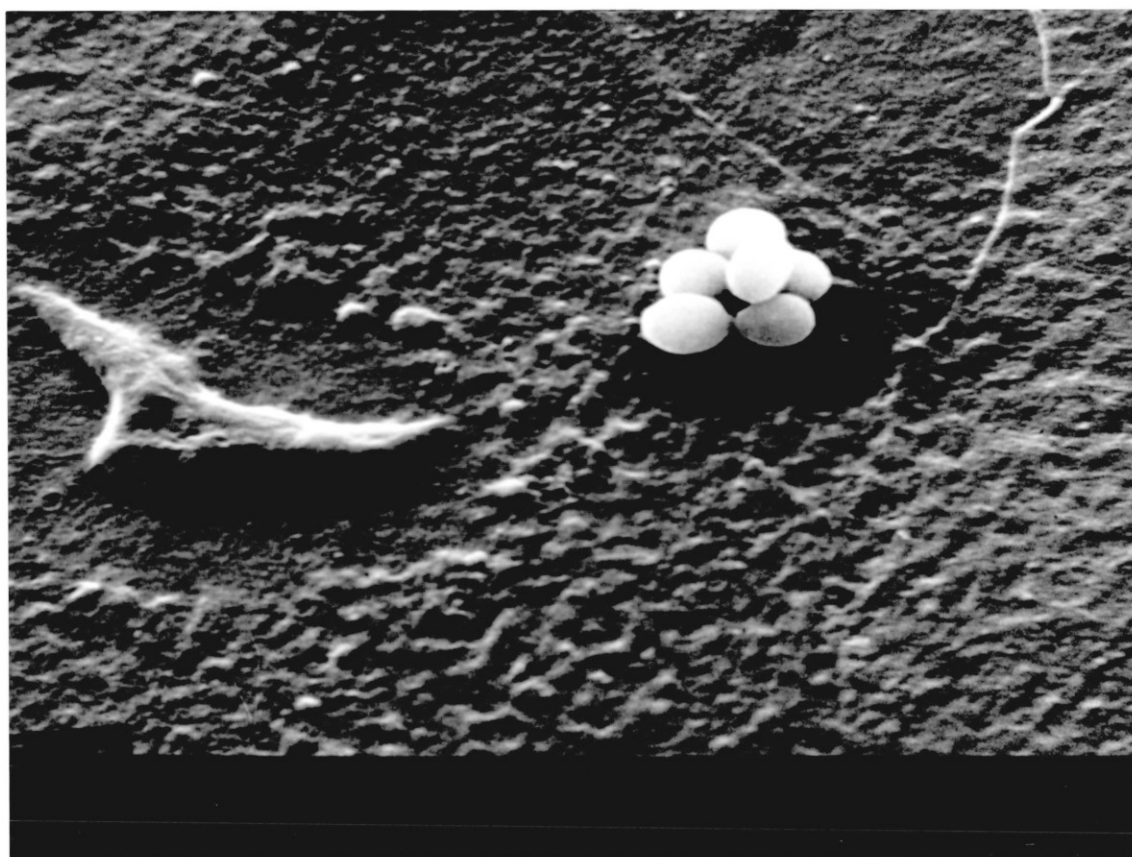
Hyphae (H) with a mass of thin threads (T) lie across the monolayer.
Bar marker 20 μm

Figure 14



It appears in this photograph that the thin threads originate on the yeast cell. The bud scar (BS) in this case is in close proximity to the protrusion.
Bar marker 0.4 μm

Figure 15



A group of Candida tropicalis yeast cells on the surface of the monolayer. The budding cells are smaller than those of Candida albicans. Length of about 4 μm or less.
Bar marker 10 μm

scanning electron microscopy. There was no evidence of invasion of epithelial cells with C. krusei, C. tropicalis, or T. glabrata even after 24 hours in the epithelial cultures. The monolayer remained mostly unchanged.

Saccharomyces cerevisiae was the only species which produced pseudohyphae in the cell cultures. The yeasts were inoculated and the cultures incubated for 24 hours before fixation. In that time almost all the blastospores produced pseudohyphae. Dense clumps of pseudohyphae could be seen on the epithelial cell monolayer (Figure 16) and on the glass coverslip surrounding the monolayer.

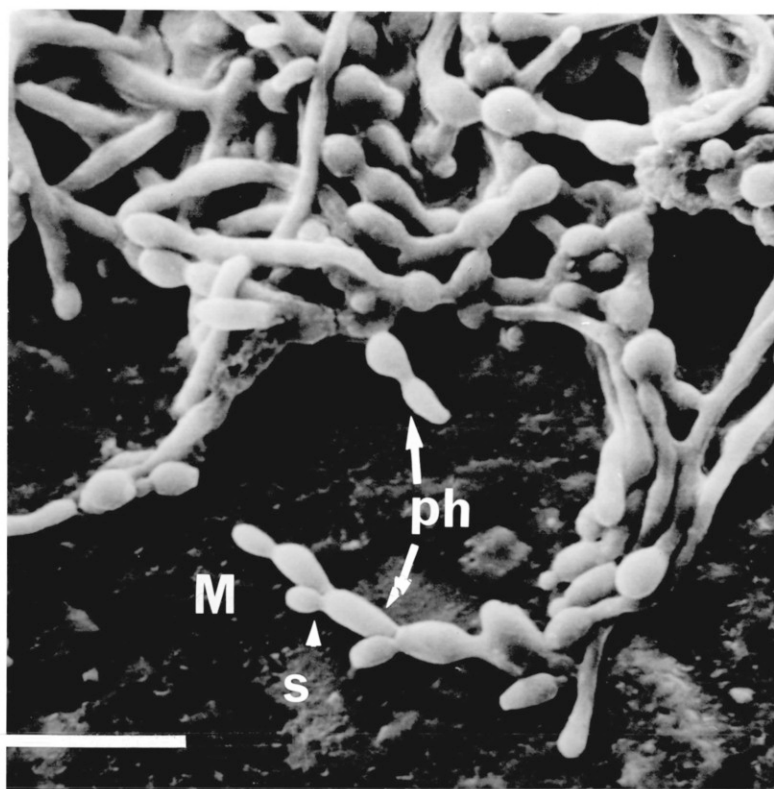
Under higher magnification the pseudohyphae were seen to be mostly lying on the surface of the cells and there was only occasional, very limited, invasion of the epithelial cells, with just the tips of the hyphae penetrating the upper surface of the cell (Figure 17).

In the areas where the Saccharomyces was in contact with the epithelial cells, which included the entire edge of the culture, the epithelial cells appeared darker in colour (Figure 18) than adjacent areas with no fungi, and with no microvilli. This might represent a fixation artifact or might be due to an effect of the fungus. The latter could have been releasing some substance which brought about the colour change. Around some of the pseudohyphae lying on the glass coverslip was a fluid deposit (Figure 19) which could be an exudate from the Saccharomyces.

Anticytokeratin staining

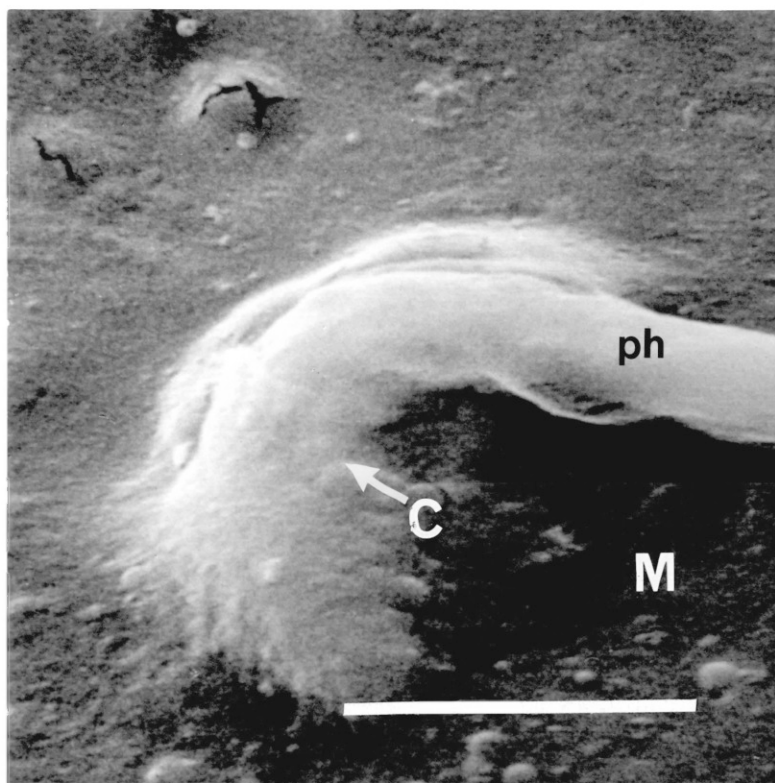
Approximately 90 per cent of the epithelial cell monolayer stains

Figure 16



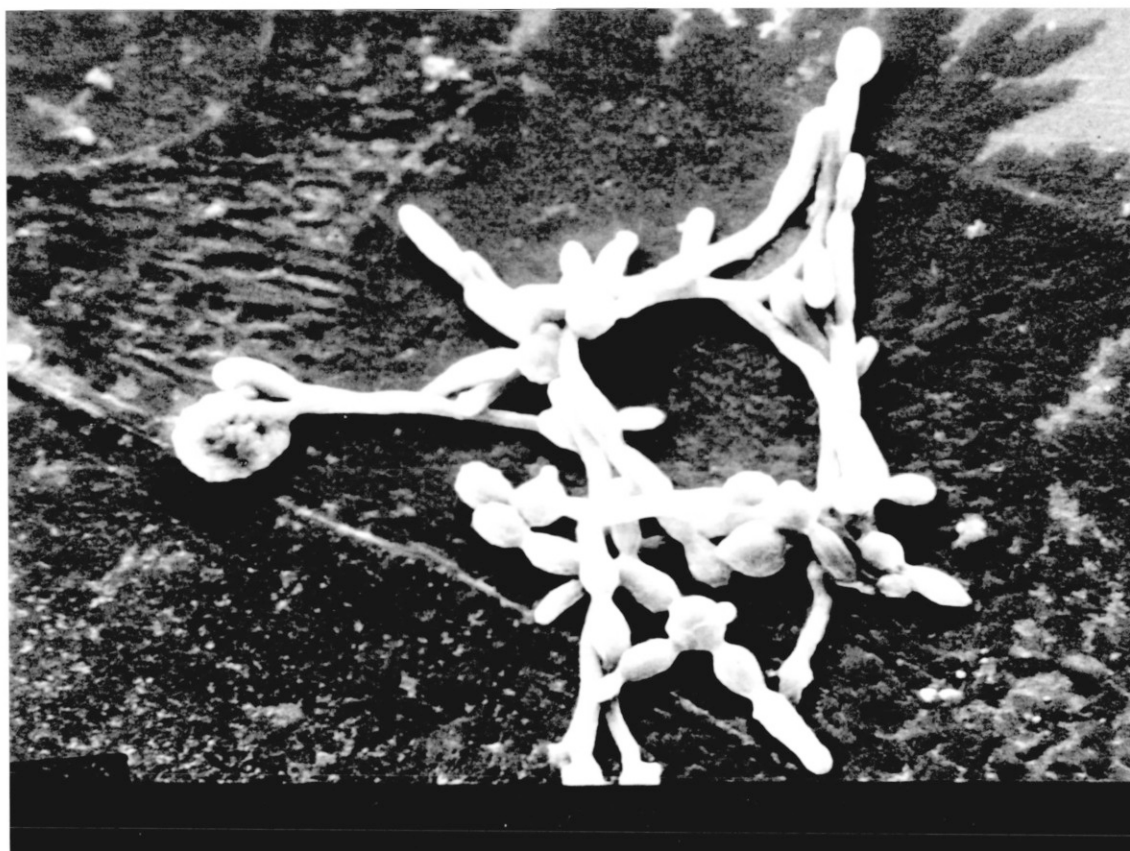
Budding branches of pseudophae (ph) of *Saccharomyces cerevisiae* with constricting septa (s) between discrete cells. The monolayer (M) is stained dark in places.
Bar marker 20 μm

Figure 17



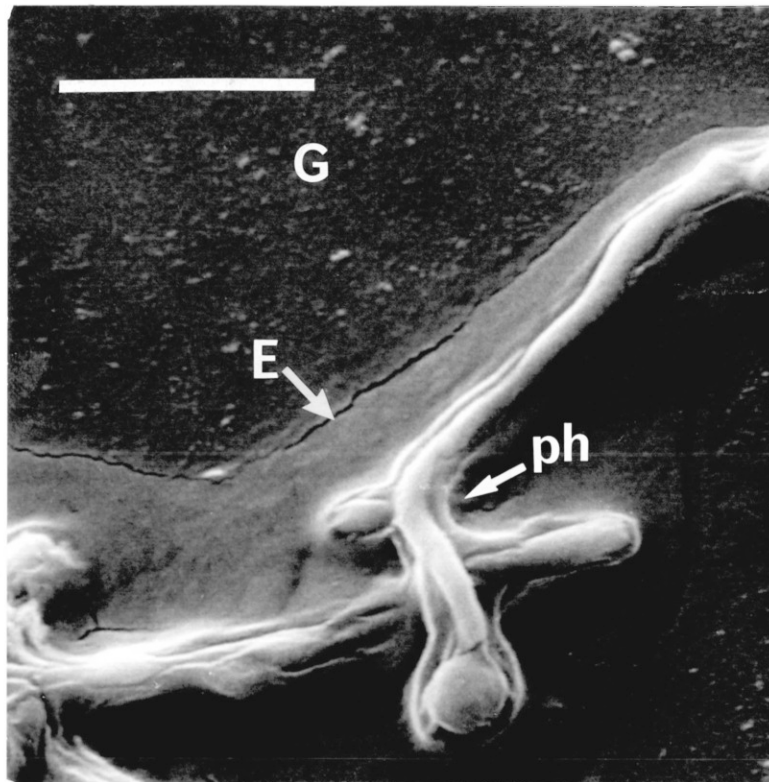
Tip of pseudohyphae (ph) of Saccharomyces cerevisiae just passing under the upper layer of cytoplasm (C) of a cell in the epithelial monolayer (M).
Bar marker 4 μ m

Figure 18



Dark stain on the epithelial cell monolayer in the area of a group of pseudophae of Saccharomyces cerevisiae.
Bar marker 20 μ m

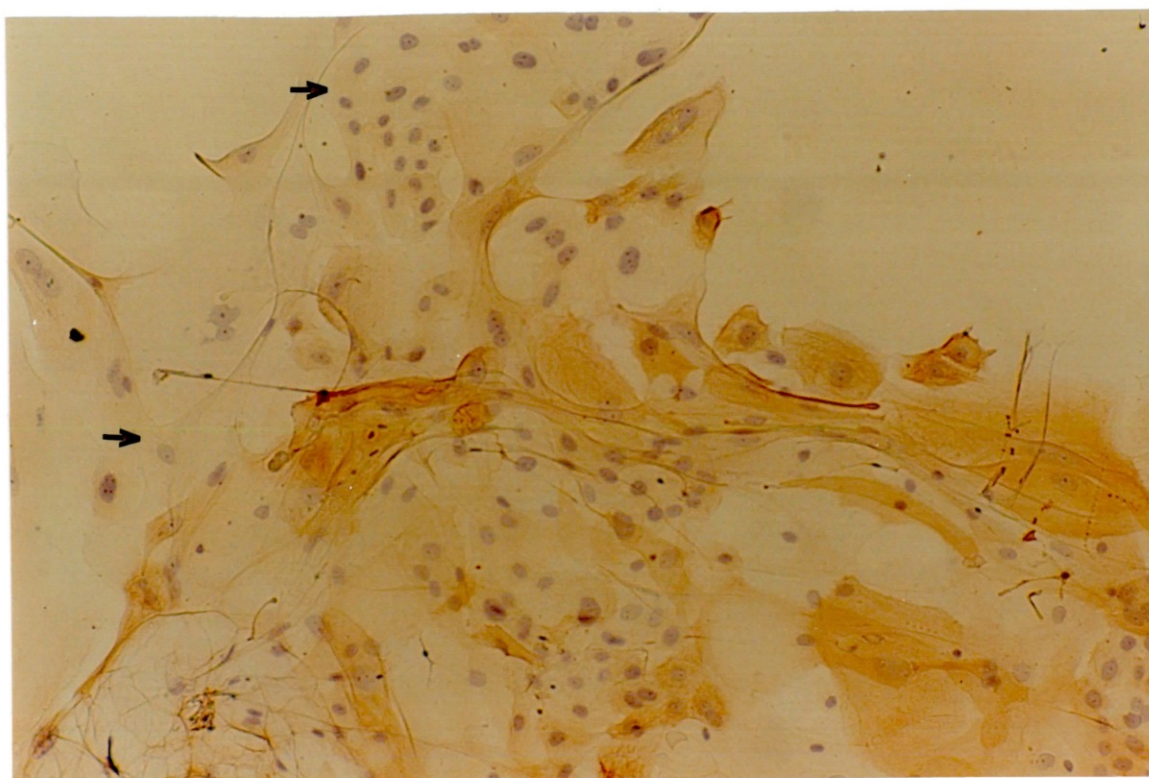
Figure 19



Strands of Sacharomyces cerevisiae pseudohyphae (ph) on the glass coverslip (G) are surrounded by exudate (E).

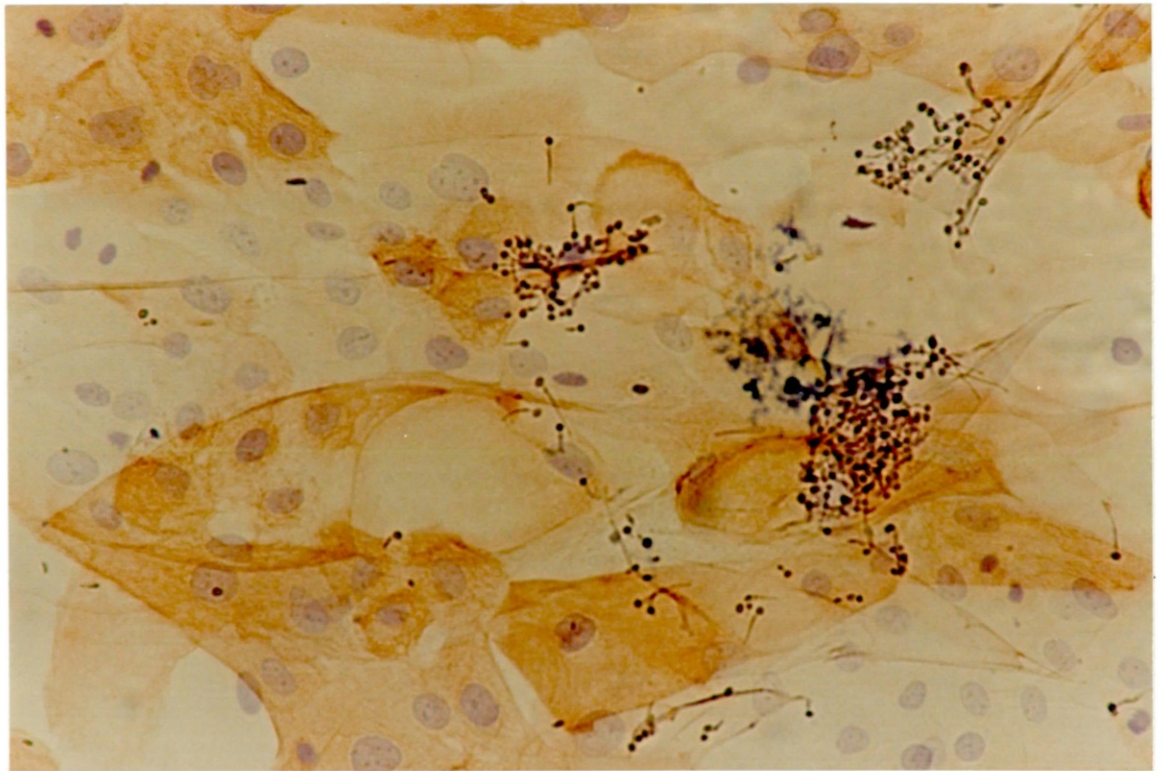
Bar marker 10 μ m

Figure 20



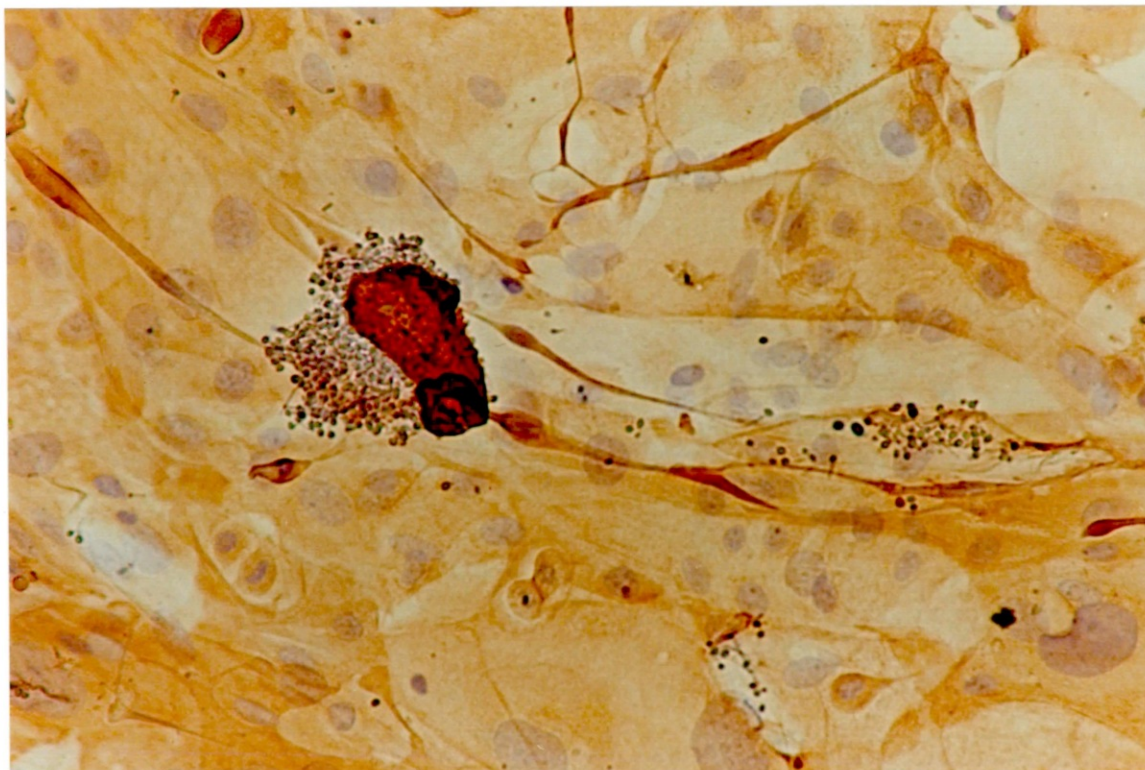
Monolayer of cervical epithelial cells under light microscopy stained with a monoclonal antibody against cytokeratin. Most cells in the monolayer stain positive with varying degrees of intensity, but there are areas in which there appears to be no stain (arrows)
Magnification (x50)

Figure 21



Yeast and germ tubes of Candida albicans attach
in greater numbers to epithelial cells expressing
a high amount of cytokeratin.
Magnification (x100)

Figure 22



Yeast cells and germ tubes of Candida albicans
clustering in high numbers to an epithelial
cell which seems to have differentiated and
keratinised in culture.
Magnification (x100)

positively for cytokeratin, (Figure 20). The distribution of yeast cells appears to correspond to the intensity of anticytokeratin (Figure 21). Occasionally individual, wrinkled epithelial-like cells, which stained very deeply for cytokeratin were seen and they were always covered with yeast cells and hyphae (Figure 22).

DISCUSSION

Distribution of yeast cells on the monolayer

Under light microscopy with haematoxylin and eosin staining the distribution of the yeasts across the monolayer appeared to be random. More yeasts were seen on the glass coverslip than on the epithelial cells. The significance of the apparent attraction of yeasts for glass is not known, but adherence of yeasts to glass beads has been shown in adherence experiments (Reinhart *et al.*, 1985). In our study there was no significant decrease in attachment in areas surrounding cells in mitosis, differing with that which has been reported in previous studies (Sobel *et al.*, 1982). In the same study of attachment of yeasts to vaginal epithelial cells in tissue culture Sobel *et al.* reported an increase in attachment to mature, flat cells in the process of desquamation. This appeared to be the case in our study. Staining our cultures with a monoclonal antibody against cytokeratin showed that yeasts were found in greater numbers in areas which stained positive for cytokeratin. It is possible that the wrinkled cells covered in yeasts are epithelial cells which have differentiated, keratinised and are in the process of separating out of the

monolayer.

Appearance of the yeasts under scanning electron microscopy

Yeast cells under scanning electron microscopy had the typical appearance reported in previous studies (Barnes et al., 1971). An intracellular matrix was observed, this has been described in colonies of *Candida albicans* grown on agar (Joshi et al., 1973; Joshi et al., 1975). It has been suggested that the matrix consists of strands of cell wall material, perhaps left behind after budding or from degenerating yeast cells (Joshi et al., 1975). A similar matrix has been described in *Candida albicans* on exfoliated human vaginal epithelial cells (Garcia-Tamayo et al., 1982). Ruthenium red stain showed the presence of mucopolysaccharides in the matrix, and in an adherent coat on yeast cells and hyphae. This layer could be seen on intra- and extra-cellular yeasts (Garcia-Tamayo et al., 1982).

This is in contrast to earlier studies in which it was reported that adherent floccular material on yeasts was deposited on the surface of epithelial cells as the yeasts invaded (Montes and Wilborn, 1968; Cawson and Rajasingham, 1972). A friable floccular material was seen in our infected cultures. Whether or not it is the same material as that which forms the intercellular matrix or it is the digested remains of tonofibrils in epithelial cells, as has been suggested by Montes and Wilborn (1968), is not known. There is evidence that the friable material which covers the surface of yeasts cells is the same as the intercellular matrix. The evidence for this is firstly that the intercellular matrix and floccular material both contain mucopolysaccharides, as shown with the use of ruthenium red (Garcia-Tamayo et al., 1982). Secondly Cawson and Rajasingham did see tonofibrils next to invading hyphae, (Cawson and Rajasingham, 1972). The fact that Montes and Wilborn did not see tonofibrils near invading

hyphae could be explained that tonofibrils are not always present in epithelial cells, which are several layers deep in cervical tissue. Disruption of tonofilaments and other cytoplasmic elements is more obvious in biopsies of cervical tissue with candida infestation.

Invasion of epithelial cells by yeasts

There is no evidence for invasion of epithelial cells by yeast cells in our cultures. It is possible that any yeast cells which are intracellular have been left behind after they have budded from hyphae. It is also possible that epithelial cells can move across the monolayer and cover yeast buds. Invasion of porcine vascular endothelial cells by yeast cells has been demonstrated with transmission electron micrographs. (Klotz *et al.*, 1983). Intra-cellular yeasts in exfoliated vaginal epithelial cells (Schnell and Voigt, 1976) could also be the result of budding from invading hyphae, which have grown out of the cell.

Hyphae invading macrophages can burst out of the cells destroying them in the process (Stanley and Hurley, 1967). Complete destruction of the epithelial cells in our monolayers after invasion by a single hyphae does not always occur, although incubation of epithelial cells with a high inoculum of yeasts does lead to mechanical destruction of the monolayer.

The tracks left in epithelial cells behind a hyphae which is either still in the cell or has re-emerged on to the surface of the cell are difficult to explain. Hyphae possibly 'move' through the cells by growing longer, but the fate of intracellular hyphae is unknown when the only visible evidence of the hypha is an empty sac or track. Intracellular yeasts do have large vacuoles which have been shown to contain hydrolytic enzymes which may be released by the yeast as it invades host cells. There is a high correlation between destroyed

host cells and intracellular yeasts with vacuoles, (Garcoia-Tamayo et al., 1982). It is possible that the enzymes carried in these vacuoles also kill the yeast cell or hyphae either as a result of some internal clock regulating the life span of the yeast or by some defensive action on the part of the host cell.

CHAPTER 5

AZOLE ANTIFUNGAL DRUGS

Imidazole derived antifungal drugs were first introduced in the nineteen sixties. They have been used successfully to treat a wide variety of fungal infections including vaginal candidosis. Most of the azole compounds are only lipid soluble and therefore they are used topically or administered intravenously. Some recently developed water soluble azole compounds which can be taken orally have been shown to alter liver enzymes levels to the extent that they can be found to have liver toxicity (Horsburgh and Kirkpatrick, 1982). New formulae are under development for oral azole antifungal agents.

The formulae of three azoles developed by Bayer Pharmaceuticals are shown in Figure 1. Clotrimazole and bifonazole are topical agents, vibunazole is a new oral agent. The unsubstituted imidazole (or triazole) ring and the covalent nitrogen - carbon bond between the heterocyclic ring and the rest of the molecule give the antifungal properties. The rest of the molecule linked via the central carbon atom influences the pharmacological properties (Plempel, 1979).

Their primary mode of action is to inhibit ergosterol biosynthesis. This leads to a build up of non-planar ergosterol precursors, which results in alterations in lipid-sterol interactions in the membranes so that temperature-dependent phase transitions of the lipids are induced. Their secondary mode of action results from changes in the membrane structure which induces differences in specific activities of membrane bound enzymes (Berg *et al.*, 1984).

The biosynthetic pathway for ergosterol is presented in Figure 2. It is common to the pathway for the production of cholesterol in human cells up to lanosterol. In fungi the side chain alkylation of lanosterol in the presence of ^{35}S -adenosylmethionine changes the direction of the biosynthesis to

Figure 1

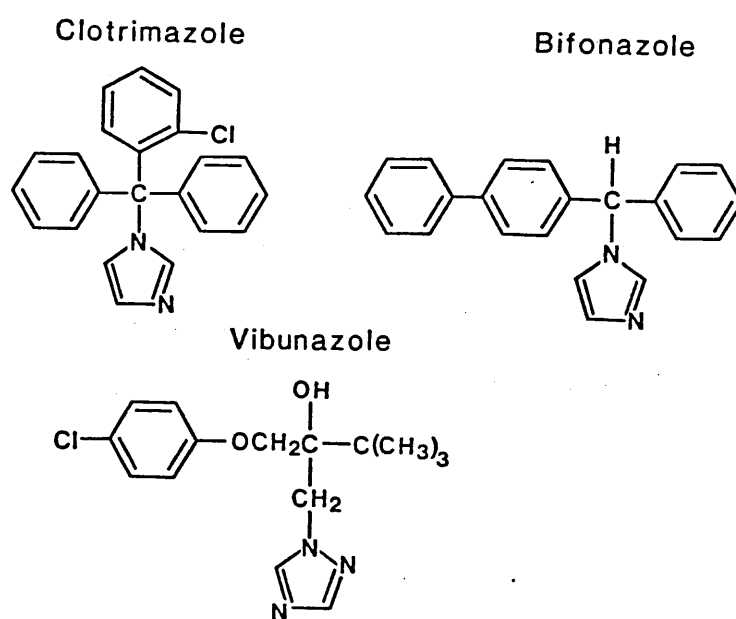
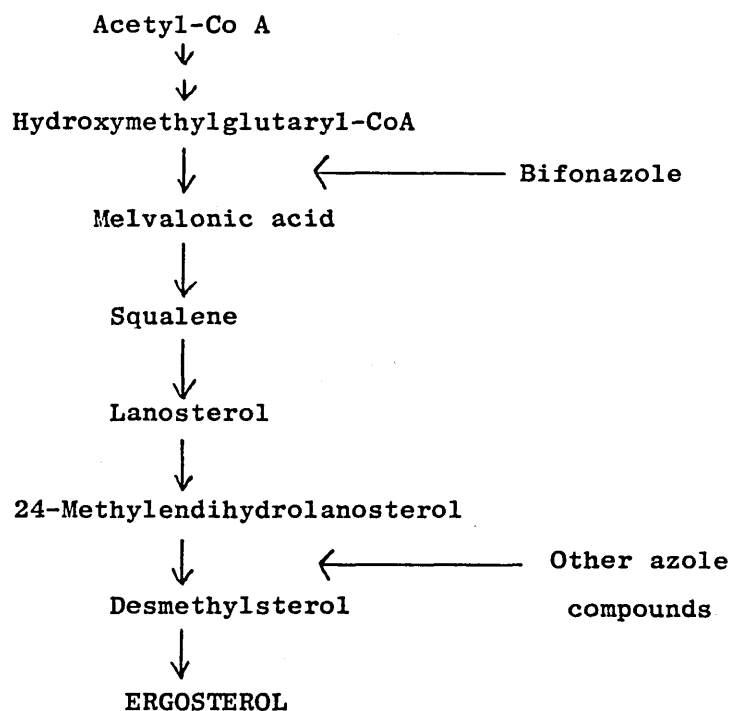


Figure 2



Biosynthetic pathway of ergosterol, points at which azoles can inhibit the next stage in the pathway are marked.

ergosterol. The azole attaches to the cytochrome P⁴⁵⁰ system altering its active site and thereby interfering with catalysis of the demethylation of trimethylsterol (Berg and Plempel, 1984).

This interference in ergosterol synthesis explains why filamentous fungal elements such as mycelia of candida are more sensitive to azoles than budding cells (Niimi et al., 1985; Sobel et al., 1981; Sobel and Obedeau, 1983). Growing yeast require ergosterol for new cell walls consequently they are more sensitive to azole treatment than stationary phase yeasts (Kerridge et al., 1976; Cope 1980).

Maximum uptake of the drug by yeast cells occurs 30 minutes after administration to growth medium and levels are still detectable in the yeast cell walls 70-100 hours later (Berg et al., 1986; Rumler 1984). The lag phase of 30 minutes is probably due to depletion of existing pools of ergosterol.

Although much research has centred on the biochemical mechanisms by which the azoles affect the yeasts, the stage at which the drugs act during invasion of host cells by Candida is not clear.

Infection of epithelial cells with candida can be described in three steps; firstly attachment of yeast cells to host cells, then production of germ tubes and invasion of cells by mycelia. Our experiments were designed to look at the action of the three azoles: clotrimazole, bifonazole and the oral agent vibunazole on two of these pathogenic stages of pathogenicity, using our human cervical epithelial cell tissue cultures inoculated with candida, the stages of attachment and germ tube growth.

Organic solvents such as dimethyl sulphoxide and hydrochloric acid have been used to obtain high concentrations of these highly insoluble drugs in previous studies of the action of azole antifungals on yeasts (Ansehn et al., 1974; Niimi et al., 1985; Odds et al., 1985; Sobel and Obedeau, 1983;

Yamaguchi et al.,1983 a and b). In our work, the possibilities that organic solvents may have detrimental effects on the epithelial cells, and that the solvents might affect the action of the drugs, led to these experiments being carried out with the drugs dissolved in neutral aqueous solution. The concentration of drug used in each experiment was determined by high performance liquid chromatography. The maximum solubility attained for clotrimazole and bifonazole was 1 µg/ml and for vibunazole up to 400µg/ml.

METHODS

Tissue cultures of cervical epithelial cells were set up as described in Chapter 2.

Candida

Fresh clinical isolates of Candida albicans were obtained from the Department of Microbiology at Queen Charlottes Maternity Hospital. The proportion of yeasts attaching to cells were found to vary considerably with different strains of Candida albicans, so to standardise the experiments one strain (coded SP) was maintained in culture for use throughout the experiments.

The yeast was obtained on Sabouraud's agar and subcultured into Sabouraud's medium 24 hours before addition to epithelial cell cultures. The medium was washed off by centrifugation at 1000 g for 5 minutes. The candida were then resuspended in Williams E medium (Gibco, Europe) without serum. The yeast cells were counted in a haemocytometer and the inoculum size adjusted to give 10^4 yeast cells per ml in each culture dish.

Azole antifungal drugs

Three azole fungicides were studied. Clotrimazole and bifonazole, both relatively insoluble in water at neutral pH, and the orally active compound vibunazole were all supplied by Bayer Pharmaceuticals, West Germany. Suspensions of each drug were made up in Williams E medium without serum at 20°C and left to stand for 1 hour after which they were filtered before use through 0.45 micron sterile filters.

Drug concentrations achieved were estimated by high performance liquid chromatography using a Microbondapak C18 column (Millipore Waters Associates). Standard concentrations of the three drugs were made up in methanol in which they are highly soluble. The two least soluble drugs, clotrimazole and bifonazole, were extracted from the Williams E medium using a mixture of 1:1 hexane and diethyl ether to extract clotrimazole, and hexane alone to extract bifonazole. The solvents and drug in Williams E medium were centrifuged at 2000g for 5 minutes and the solvent then evaporated under a stream of nitrogen. The residues were dissolved in methanol and injected on to the chromatography column after their respective standards. The flow rate was 1.5 ml per minute and detection for clotrimazole was at 210 nm wavelength and bifonazole at 258nm wavelength (W. Ritter personal communication).

The vibunazole, being soluble, was not extracted but injected directly on to the column in Williams E medium solution after the relevant standards and detected at a wavelength of 20 nm (W. Ritter personal communication).

The maximum concentration obtained for clotrimazole and bifonazole in aqueous solution at neutral pH was 1µg/ml and for vibunazole, up to 400µg/ml. Concentrations used in attachment experiments ranged from a saturated solution through tenfold serial dilutions in Williams E medium solution to a minimum concentration of 1 ng/ml.

Attachment experiments

Yeasts were added to the range of drug dilutions so that a final inoculum of 10 yeast cells per ml was used in experiments. The epithelial cell growth medium over the cell cultures was removed, and the epithelial cells were washed with Hanks' balanced salt solution removing the medium containing fetal calf serum, the albumin of which binds to yeast cells. Then 2ml of Williams E medium without serum containing yeast cells and drugs was added to each culture dish.

To ensure that attachment of the yeast cells to the epithelial cell cultures was not simply due to the yeasts settling out of suspension because the medium they were in was static, the petri dishes containing the epithelial cells and yeasts were constantly agitated throughout the incubation period of 2 hours on a rotating table set at 15 revolutions per minute inside a dry incubator at 37°C. This was enough to stir the medium over the epithelial cells but not enough to put a significant centrifugal force on the yeast cells.

The experiments were terminated after 2 hours by decanting off the salt solution, washing twice with phosphate buffered saline and fixing the cultures in absolute alcohol. The cultures were stained with Giemsa. The pH throughout the experiments was 7.2 - 7.5.

Quantitative analysis of attachment

Under light microscopy the number of yeast cells attached was counted using a graticule. The area of monolayer counted was then measured using colour television quantitative image analysis. The results of the adherence experiments were calculated as the total number of yeast cells attached per mm² area of monolayer. The effect of azoles was assessed by comparisons with

attachment in control cultures from the same donor not treated with azoles.

Germ tube experiments

An inoculum of 10^4 yeast cells per ml was added to the epithelial cells and incubated for 2 hours. Saturated solutions of the drugs were made up in epithelial cell culture medium without antibiotics and left to stand for 1 hour. Filtered media containing the drugs were added to the dishes containing yeast cells which had been incubated on epithelial cells for 2 hours, resulting in the following concentrations of drugs in the media: 0.5, 0.05, 0.005, 0.0005 μg per ml of clotrimazole and bifonazole and 200, 100, 50 and 5 μg per ml for vibunazole. The cultures were incubated for a further 2 hours in a CO_2 incubator at 37°C . The cultures were then washed, fixed and stained as described previously.

The following parameters were assessed:

- (a) The total number of yeasts attached to the monolayer.
- (b) The number of germ tubes produced from the yeast cells which had attached.
- (c) The germ tube length produced.

Mean germ tube lengths were obtained only from cultures treated with 0.0005 μg per ml of clotrimazole and bifonazole and 5 μg per ml of vibunazole and with 0.5 μg per ml of clotrimazole and bifonazole and 200 μg per ml of vibunazole of azole. Ten germ tubes from five fields for each concentration were measured.

RESULTS

Attachment of Candida cells

Candida cells attached to the surface of the epithelial cells within two hours. In control experiments the number of attachments ranged from 2 yeast cells/mm² of epithelial cells to 200 yeast cells/mm² with a median of 39 yeast cells per mm. The yeasts were not uniformly distributed over the epithelial cells. Sometimes they were seen lying in groups of 3 to 4 or much larger groups of 30 to 40 yeasts.

Effect of azole antifungal drugs

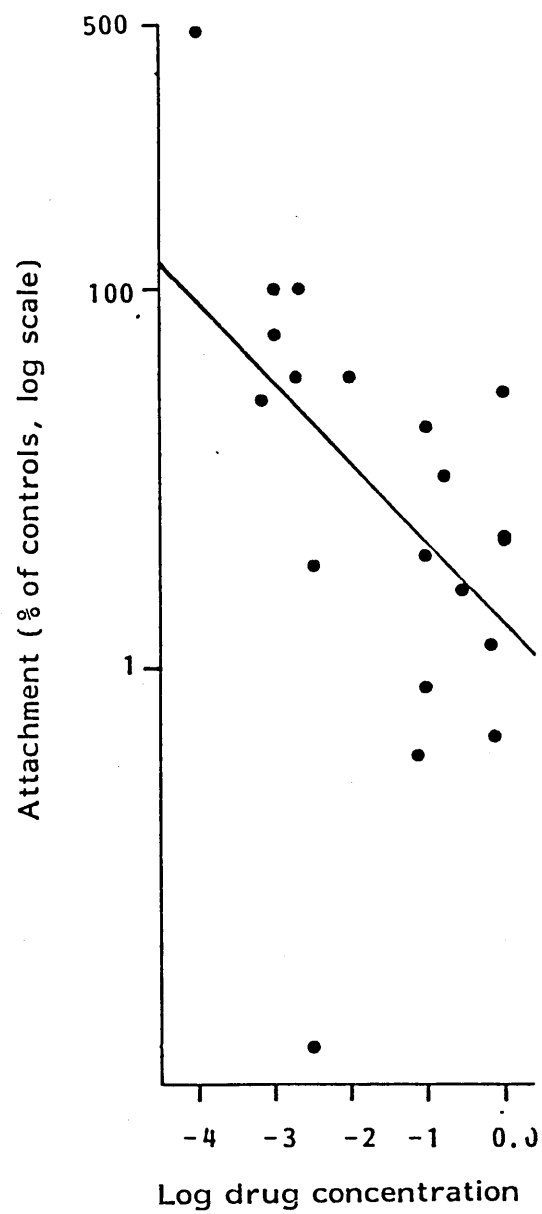
Clotrimazole in solution in Williams E medium in concentrations between 0.0001 and 0.95 µg/ml (0.0001 - 3 µmol/ml) reduced yeast cell attachment to the epithelial cells as drug concentration increased (Figure 3). The median reduction in adherence by clotrimazole at 0.95 µg/ml (3 µmol/ml; the maximum concentration obtainable) was 78 per cent.

The activity of bifonazole was similar although it appeared to be slightly more potent. Concentrations between 0.0001 and 0.3 µg/ml (0.0003 - 1.1 µmol/ml) reduced yeast attachment (Figure 4). At the maximum concentration of bifonazole used of 0.28 µg/ml (0.9 µmol/ml) the median reduction in yeast spore attachment was 78 per cent.

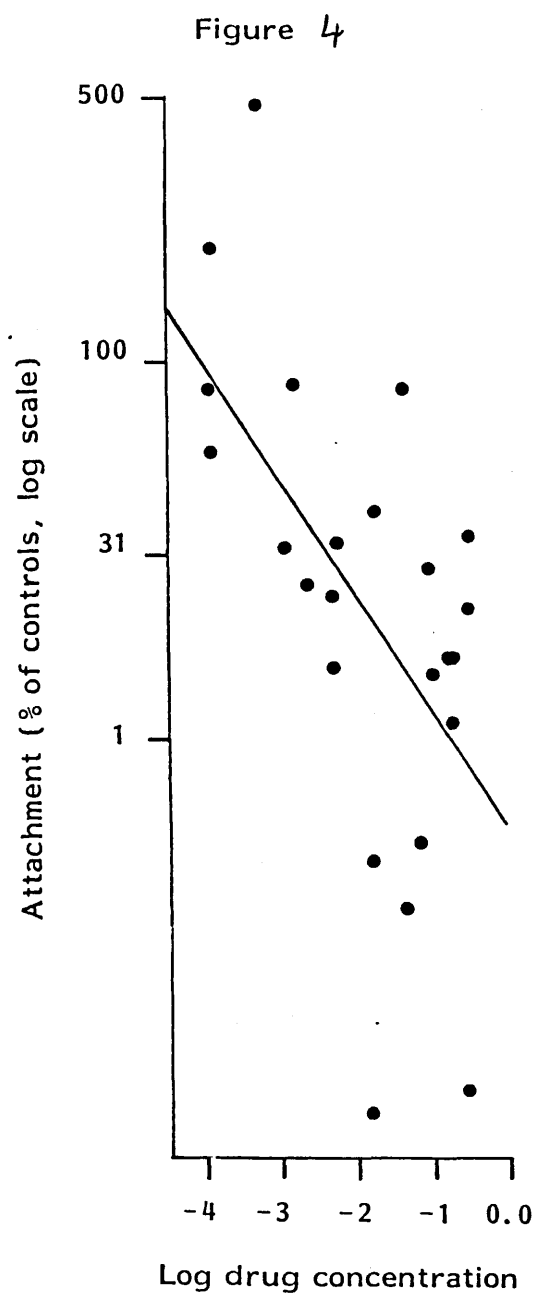
The oral agent vibunazole had similar effects, but was less active. Concentrations between 1 and 400 µg/ml (3.3 µmol/ml - 0.6 mmol/ml) produced reductions in attachment of between 64 per cent (median for 1 - 10 µg/ml) and 80 per cent (median for 20 - 200 µg/ml)

Although there is much inherent variation in the method as shown by

Figure 3



Effect of clotrimazole (log $\mu\text{g/ml}$) in reducing number of yeasts attached per mm^2 of epithelial cells.



Effect of bifonazole (log $\mu\text{g/ml}$) in reducing number of yeasts attached per mm^2 of epithelial cells.

the wide scatter of points (Figures 3 and 4), the slopes of the logarithmic regression lines of percentage reduction in attachment on concentration of azole were significant for clotrimazole ($P < 0.05$) and bifonazole ($P < 0.05$). The slopes of the regressions for the three drugs did not differ significantly (Figure 5) and this permitted an approximate comparison of potency, though the error limits of the potency ratios would be wide . The potency of bifonazole was of the same order as that of clotrimazole . Vibunazole was 100 fold less active (estimates of potency ratios with respect to clotrimazole 1.0, bifonazole 1.8, vibunazole 0.014).

Effect of the azoles on the epithelial cells

All three azoles tested were administered to the epithelial cells without Candida albicans at the highest concentrations used in the candida experiments and incubated for comparable periods of time. No difference in morphology from control cultures was seen.

Comparability of cultures used to assess germ tube growth

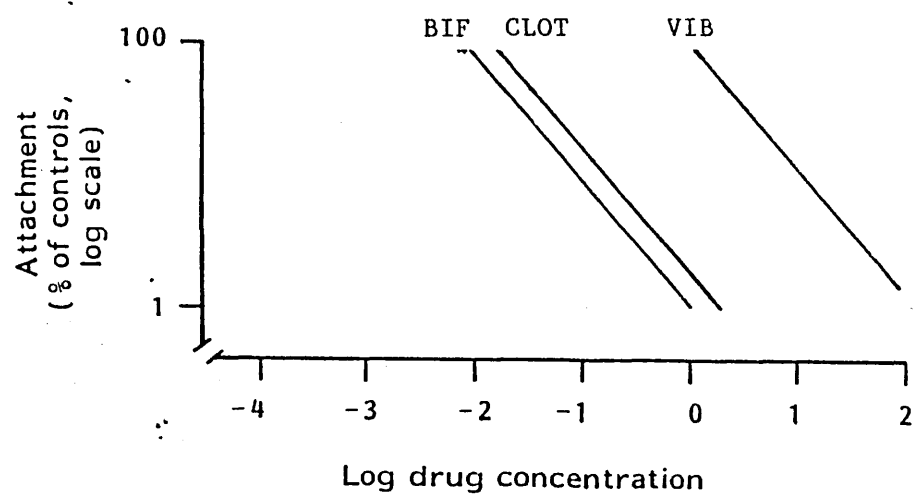
The number of germ tubes per mm^2 area of epithelial cell monolayer in these cultures was not significantly different from the controls.

Germ tube length

The difference in length of germ tube produced in the control cultures and those in the cultures treated with the azoles was noticeable by observation of the cultures under the light microscope

The mean germ

Figure 5.



Concentration-action lines for the three drugs; estimated potency relative to clotrimazole (1.0):-
bifonazole, 1.8; vibunazole, 0.014.

MEAN NO. OF FIELDS	CONTROL	CONTROL	CLOTRIMAZOLE	CLOTRIMAZOLE	BIFONAZOLE	BIFONAZOLE	VIBUNAZOLE	VIBUNAZOLE
DRUG CONC.	0	0	LOW	HIGH	LOW	HIGH	LOW	HIGH
1	0.029	0.05	0.017	0.01	0.019	0.019	0.013	0.019
2	0.037	0.04	0.015	0.011	0.015	0.02	0.014	0.01
3	0.04	0.046	0.013	0.012	0.015	0.02	0.01	0.02
4	0.04	0.049	0.014	0.011	0.019	0.02	0.01	0.014
5	0.05	0.047	0.015	0.013	0.016	0.02	0.015	0.014
MEAN	0.039	0.046	0.015	0.011	0.017	0.019	0.012	0.015

Table I Means of germ tube length in five fields for treated and untreated yeasts on epithelial cell monolayers.

tube length for the controls was significantly longer than the treated germ tubes (Table I). The mean length of germ tube in the controls was 0.045 μm with a range of 0.015 to 0.07. The cultures treated with any of the three drugs for showed a mean germ tube length of 0.015 with a range of 0.005 to 0.03 μm . We were unable to detect differences in the length of germ tube related to the between concentrations of drug used. Analysis of variance performed on the data for germ tube length to evaluate possible "between fields" variation and "between culture" variation showed no significant difference.

DISCUSSION

Preliminary experiments were carried out to determine the best protocol for the most repeatable results. An inoculum size of 10^4 yeast cells per ml was used because at 10 yeast cells per ml the number of yeast cells attached was too small to detect differences between controls and low concentrations of the drugs used. Two strains of Candida albicans were assayed in parallel for the first three experiments, but higher attachment across controls and treated cultures was obtained with SP strain, making it easier to interpret the assays. Adherence of yeasts to human epithelial cells can differ from day to day with one donor's cells and between donors (King et al 1980). For these reasons one strain of candida was used throughout the experiments. Whilst it was not possible to assess the variability of response between strains of fungus, this reduced the number of experimental variables in a system already susceptible to wide variation. Stationary phase yeasts adhered much better than yeasts in the logarithmic growth (King et al., 1980; McCourtie and Douglas 1981) so cultures of yeasts were used after a 24 hour incubation in Sabouraud's

medium. Preliminary attachment experiments were carried out in phosphate buffered saline but sometimes attachment was at such a low and variable level in the controls it was difficult to measure.

Attachment of yeasts to human buccal epithelial cells from mouth scrapings has been shown to be significantly greater in human saliva at 37°C than in phosphate buffered saline. (Kimura and Pearsall 1978). Enhanced adherence was associated with germination of the yeast cells. Maximum adherence of yeasts to exfoliated human vaginal epithelial cells was obtained after 2 hours incubation in Williams E medium rather than in phosphate buffered saline. This was again thought to be because William E medium enhanced germination of the yeasts and factors which enhance germination also enhance attachment (Sobel et al.,1981)

Our criteria for our attachment assays were based on providing the optimum conditions for attachment to occur based on the results obtained by previous workers (Kimura and Pearsall 1978; King et al.,1980; Sobel et al.,1981; Sobel et al 1982; Lee and King 1983;) so that the action of the azoles would be demonstrated under conditions of maximum adherence.

The vagina has a pH of 4.5 to 5, but the pH at the cervix is 5.6 to 5.7 and within the cervical canal is 6.1 to 6.8 (Singer 1975). Candida albicans can grow over a wide pH range (Ryley 1986). Vaginal pH rises with infection (Gleeson personal communication), although this study was made on pregnant women. In another study no rise in vaginal pH was found in women with vaginal infection (Cohen 1969). Yeast cells in histological biopsies of vagina have shown that they can be found up to 15 cell layers deep, (Schnell, 1982), therefore the pH at which the experiments were carried out probably has little effect on the yeasts. Low pH has been shown to increase the solubility of azoles (Haller,1985) and some treatment pessaries contain citric and lactic

acid. We studied the effect of the pure drug it was considered that this was easier to control during the experiments since it is the pH adopted by the media in the CO₂ incubator and because it again measured efficacy of the azoles under in vivo physiological conditions.

It has been pointed out that usually before an organism can invade target tissues it has to adhere to the cells of the relevant epithelium (Ryley 1986). The association between attachment and virulence has been demonstrated with a comparison of a mutant strain of candida which has a reduced ability to adhere and a wild strain in a murine vaginal infection model (Lehrer et al., 1986). Also, strains of candida isolated from active buccal infections could be induced to show greater adherence and to produce a more virulent infection in mice after growth in medium containing a high sucrose or glucose concentration. In the same experiment two strains isolated from asymptomatic carriers were induced to adhere and to produce infection in the mice, albeit of a lesser severity than strains isolated from infections (McCourtie and Douglas 1984). Degree of adherence may be an indicator for virulence potential, and attachment or predisposition for attachment in the yeast may be linked to pathogenicity.

On the other hand it has been reported that enhanced susceptibility to adherence does not appear to be a factor responsible for recurrent candidal infection (Trumbore and Sobel 1985). In our cultures very little germ tube production was noted during the two hour incubation period with Williams E medium and it is thought that serum in the medium provides the optimal growth milieu for germ tube production.

Whether or not germ tube and hyphal production in yeasts is the deciding factor in yeast pathogenicity is controversial, but a study by Sobel et al. (1984) shows that the capacity of C. albicans to produce hyphae appears to be important but not essential because mutant strains of Candida albicans which were unable to produce hyphae were able to produce signs of infection (Sobel et

al., 1984). Factors which enhance attachment have been said to also encourage the production of germ tubes (Sobel et al.,1981).

We did not find any difference in our control cultures between the number of yeasts and germ tubes attached at two hours and those attached at four hours. A certain number of yeasts attach to the epithelial cells during the first 2 hour incubation period. The cultures were incubated for a further 2 hours and it can be assumed that in these further 2 hours the majority of the unattached yeasts in the medium would produce germ tubes. If germ tubes are supposed to attach more readily than yeast cells then it is likely that these later produced germ tubes would attach and the resulting number of germ tubes per mm area of epithelial monolayer would be higher after 4 hours. Similar results were also obtained by Anderson and Odds (1985). The exact time of the start of germ tube production is not known and the germ tubes we counted had started to grow before the addition of the azoles. Action of the azoles on complete inhibition of germ tube formation in this model system has yet to be examined and would require a time controlled study in which the drugs were added at short intervals over the critical period for germ tube production (which is 2 hours from inoculation). However from our results it appears that adherence of yeast cells occurs before germ tubes are produced and unattached yeasts which produce germ tubes do not attach after germ tube production.

Anderson and Odds (1985) have reported in their scanning electron microscopy study that the penetrating germ tube tip or hyphal tip is sometimes the only part of the yeast attached to the epithelial cells. This could give the impression that germ tubes do attach but it may be that yeast cells to attach first to the epithelial cells, then they produce germ tubes and hypha, which start to invade the epithelial cells and the yeast cell then detaches.

In the experiments by Anderson and Odds (1985) they demonstrated a dose-response relationship with ketoconazole at various concentrations ranging

from 0 - 10^{-7} M ketoconazole for both yeast cells and germ tubes. These results are comparable with ours for the yeast cells, but at higher concentrations of drug than used in our experiments. Although ketoconazole is an azole which can be taken orally and therefore is more soluble than topical agents it was dissolved in acetone for the experiments. The failure by us to produce a dose-response relationship in the germ tube growth studies may be because we added the drug 2 hours after inoculation of the yeast, but Anderson and Odds apparently added the drug at the same time as the germ tubes to the epithelial cells.

Some experiments in which yeasts were pretreated with antifungal drug were carried out. The yeasts were cultured for 30 minutes in various concentrations of the drugs, they were then harvested by centrifugation washed and added to the epithelial cells. No attached yeasts were found on the epithelial cells. Maximum uptake of the drug by yeasts occurs in the first 30 minutes (Berg et al., 1986; Rumler, 1984) and it appears to be sufficient to interfere with subsequent attachment.

In some experiments the epithelial cells were pretreated with various concentrations of the drugs and preliminary results suggest that they may be preventing attachment of yeasts even when the drug is removed and the cells washed before the addition of the yeasts.

The experimental system we have used to study the action of azoles on pathogenesis of candida infections in epithelial cells is susceptible to wide variation between cultures - a common problem in attachment experiments. Nonetheless we were able to demonstrate a significant dose-response relationship with the antifungal azoles. Even the limited concentrations of azole antifungal obtainable in neutral solution can interfere with adhesion of yeasts to epithelial cells. Adhesion is likely to be not only the first phase

of infection but also a mechanism involved in sustaining continuing infection, new yeast cells formed by the organism attaching to cells before producing germ tubes. For therapeutic effect it is therefore unimportant that an effective concentration of the antifungal agent be maintained in the vagina for at least several days.

CHAPTER 6

CHLAMYDIA

Chlamydia are intracellular micro-organisms which grow and divide within inclusion bodies in cells. They are classified into two subgroups, A and B, on the basis of differences in the way they respond to antibiotics, although both groups are sensitive to tetracyclines. The inclusion bodies of subgroup A contain glycogen. Both families of chlamydia exist in two forms, a small nucleoid structure called an 'elementary body' about 300 nm in diameter, which is extracellular and highly infectious and an intracellular, non-infectious, reticulate 'initial body', 800 to 1200 nm in diameter, which does not have a dense nucleoid body and is the dividing form. Division takes place within the inclusion body and is by binary fission, as with rickettsiae and bacteria. The dividing bodies then mature into elementary bodies which eventually burst out of the inclusion ready to infect another cell. Classification of chlamydia as either a virus or bacteria is difficult because they have characteristics of both. For example chlamydia is incapable of surviving outside a cell, which is comparable to an intracellular virus, but divides in the same way as a bacterium.

Subgroup A chlamydia are called Chlamydia trachomatis and are the more common of the two groups which are infective in humans. Several types of eye disease are caused by Chlamydia trachomatis and the organism also causes infections of both the male and female genito-urinary tracts. These include Lymphogranuloma venereum (LGV) in males and females and chlamydial (non-specific) urethritis in the male. In females in addition it is believed that chlamydial infection plays a part in pelvic inflammatory disease, including inflammation of the fallopian tubes.

The serotypes of Chlamydia trachomatis isolated from humans are

trachoma inclusion conjunctivitis (TRIC agents) serotypes A-C, serotypes D-K which are isolated from genital tract and are also associated with eye disease in neonates, and lymphogranuloma venereum agents 1, 2 and 3.

The subgroup B are called Chlamydia psittaci. They cause the diseases psittacosis and ornithosis in humans and similar diseases in other animals.

In 1907, Halberstaedter and von Prowazek first discovered inclusion bodies of chlamydia in material from the conjunctival sac of trachoma patients in Java. Then in 1957 Bedson and Bland described the life cycle of Chlamydia psittaci in the mouse and in tissue culture. Thygeson (1934) first drew attention to the morphological resemblances between Chlamydia trachomatis and Chlamydia psittaci. The first successful isolation of the latter organism was by T'ang et al., (1957) using the chick embryo. It was not until 1964 that Moulder put forward evidence that the chlamydial organisms were not atypical viruses but could also be regarded as bacteria. Reeve et al., (1957) published a laboratory procedure for the isolation of Chlamydia trachomatis. This method involved centrifugation of clinical specimens onto McCoy cell monolayers. It came to light in the development of this procedure that certain pre- and post-treatment regimes for the cultures were necessary before maximum isolation is achieved.

Since the first suggestion that there was a relationship between infertility in females and chlamydial infections, suitable laboratory models have been sought to examine the action of chlamydia on the different sections of the mammalian female genital tract. Hutchinson et al., (1979) centrifuged chlamydia onto bovine oviduct in culture and reported that no extensive epithelial cell damage was observed but there was no loss of ciliary activity detected in cultures inoculated with either Chlamydia trachomatis or Chlamydia psittaci. They therefore proposed that where salpingitis is caused by chlamydial infection, it is probably immunologically mediated.

The first report of successful inoculation of human ectocervical epithelial cells in monolayer culture with Chlamydia trachomatis was from a group working in the United States. Wyrick et al.,(1982) pretreated human cervical cell monolayers with diethylaminoethyl(DEAE)-dextran or with Neisseria gonorrhoeae to obtain the highest numbers of inclusions compared to non or other pretreatment regimes. In 1986 the same group published the successful inoculation of endocervical cells in tissue culture with Chlamydia trachomatis. Centrifugation and exposure of the cells to DEAE-dextran was again used to aid inoculation. The addition of cyclohexamide to the post-inoculation culture medium also slightly enhanced the numbers of inclusion bearing cells. They found adherence of chlamydiae to genital cell cultures not as uniform as to McCoy cells (Moorman et al.,1986).

We wished to investigate inoculation of endocervical cells in culture with chlamydia and to examine the cultures under the scanning electron microscope, because tissues of the endocervix are the natural target cells for chlamydial infection. We also wished to compare the relative infectivity of different strains of C.trachomatis.

METHODS

Chlamydia strains: Human urogenital isolates of C.trachomatis serotypes D and E and Lymphogranuloma venereum serotype 2 (supplied by the Department of Virology, Hammersmith Hospital) were tested. The inocula were the supernatants from McCoy cell cultures in which 90% of the culture had positive inclusions.

Serotyping was performed at the Institute of Ophthalmology. Chlamydia were maintained in the following medium:

Chlamydia maintenance medium:

10% Minimal essential medium (Eagle)

5% fetal calf serum

0.05% glucose

1 ml vitamins in 100 ml of medium

1% glutamine

100µg/ml vancomycin

50 µg/ml streptomycin

Culture of endocervical cells: Endocervical tissue was set up in culture as described on page. On the 4th day in culture the medium was changed and replaced with a penicillin and streptomycin-free medium. Eagle's minimal essential medium with D-valine was used in the early experiments to try to eliminate fibroblast growth. Following chlamydial inoculation, cells were maintained in Dulbecco's modified Eagle's medium containing 0.12% sodium pyruvate, 15% heat inactivated fetal calf serum, 2.4% streptomycin, 0.3% amphotericin and 5% glutamine.

McCoy cell cultures: McCoy cell cultures (supplied by the Virology Department, Hammersmith Hospital) were used as positive control cultures for the viability of the chlamydia inocula. They were obtained in plastic tissue culture flasks. They were maintained for 72 hours in epithelial cell culture medium containing 10µg gentamicin and 2.5µg of amphotericin per ml following which they were

passaged using a trypsin/versene (0.25% and 0.02% respectively) mixture on to 15 mm diameter coverslips and incubated in a humidified 5% CO₂ incubator at 37°C for 24 hours.

Chlamydial infection of endocervical cells and McCoy cells

Coverslips with cultures of endocervical cells or McCoy cells uppermost were placed in 5 ml plastic vials. Chlamydial inoculum in maintenance medium, 0.2 ml, was added to each coverslip. The vials were centrifuged at 2,800 revolutions per minute and 37°C for 90 minutes in a Mistral 6L centrifuge (G-force equivalent to 2500g).

Following centrifugation inocula were aspirated and a fresh 1 ml of culture media was added to each infected monolayer. Cultures were further incubated for 72 hours, before termination of the cultures by fixing.

Some cultures were incubated for a further two hours following centrifugation before removal of the inoculum.

1 and 2 µg of cyclohexamide per ml of medium was added to some cultures after centrifugation before the addition of fresh medium.

Preparation of cultures for scanning electron microscopy.

The

cell cultures were fixed in 3% buffered glutaraldehyde and prepared for scanning electron microscopy as described previously.

Staining of cell cultures with Giemsa and Iodine.

Cell cultures were fixed in 100% methanol for at least 10 minutes, then stained with buffered Giemsa, pH 6.8, for 30 minutes. The cultures were washed twice and permanently maintained with 'Univert' mountant, then they were examined under

the microscope by dark ground illumination.

Three slides were stained with Giemsa after viewing in the scanning electron microscope. The aluminium stub was dissolved away from the glass coverslip using concentrated hydrochloric acid. The cultures were then stained. The iodine stain used was Lugol's 10% iodine. The coverslips were dipped in iodine for 1 minute then in potassium iodide for 12 minutes and finally in distilled water for 12 minutes.

Monoclonal antibody stain.

The monoclonal antibody stain used was in the SIVA Microtrak Kit; Chlamydia trachomatis Test. The monoclonal antibody was raised in a mouse and was fluorescein labeled.

The cultures were fixed in ethanol for at least 10 minutes. 30 µl of monoclonal reagent with conjugated fluorescein label, was pipetted on to each monolayer, care was taken to keep the monolayer moist at all times. The coverslips were then incubated at 37°C for at least 30 minutes in moist chambers. The slides were then rinsed in deionized water and the coverslips were mounted on slides using the mounting medium supplied with the kit.

RESULTS

No detectable difference in infection rate was noticed with pre-treatment of cells with DEAE-dextran or post-inoculation treatment with cyclohexamide.

Light microscopy

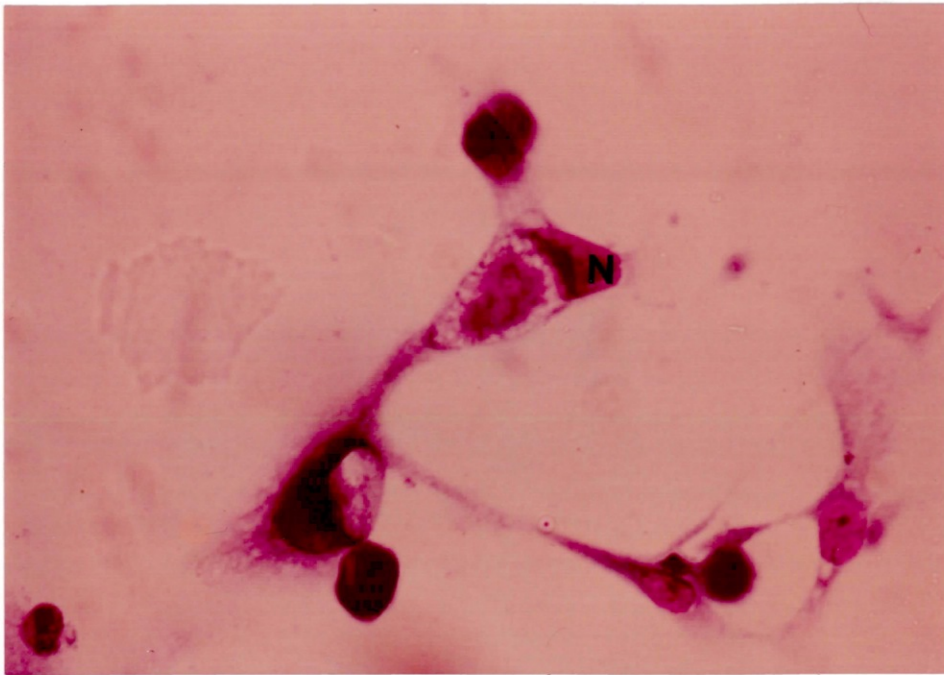
Positive results were obtained in endocervical cells with Lymphogranuloma venereum serotype 2 and C.trachomatis serotype D cultures infected with chlamydia had inclusion bodies in approximately 1 in 30 cells.

Giemsa stained cells viewed under light microscopy show the inclusion body as a vacuole, which can take up the entire cell cytoplasm and displace the nucleus to one side. Sometimes a dense mass is seen in the middle of the vacuole surrounded by a fainter, granular area between the mass and the edges of the vacuole (Figure 1).

The same cell as seen in (Figure 1) seen under dark ground illumination in (Figure 2) was confirmatory of chlamydia present in the inclusion. The purple mass in the centre are the dividing or reticulate bodies. Numerous green specks on the glass coverslip are individual elementary bodies. The nucleus can be seen at one side.

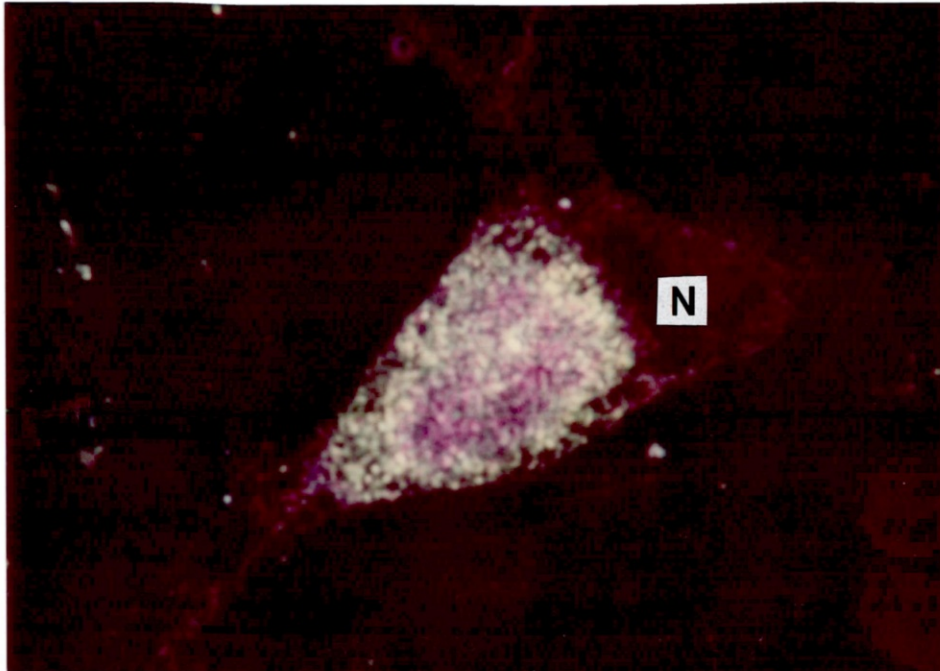
The presence of chlamydial inclusions in endocervical cell cultures was also demonstrated with iodine stains and the monoclonal antibody against C.trachomatis culture confirmation test. Both tests are standard laboratory procedures for the identification of chlamydia.

Figure 1.



An epithelial cell from the cervical canal in culture after infection with Chlamydia trachomatis viewed under light microscopy with Giemsa stain. The nucleus (N) has been pushed to one side by the inclusion body.
Magnification (x100)

Figure 1



The same cell as seen in Figure 2 viewed under dark ground microscopy showing laterally displaced nucleus (N), a central inclusion nucleoid in blue representing the dividing initial body and the numerous elementary bodies to the periphery of the inclusion in a characteristic apple green colour. The minute green dots on the slide surrounding the cell are elementary bodies. Magnification (X120)

Scanning electron microscopy

Endocervical cells without chlamydia infection under scanning electron microscopy have been described in chapter 3.

A similar picture was obtained with both serotypes of chlamydia. Inclusions are 10-20 μm in diameter. The cell in Figure 3 is the same cell as in Figures 1 and 2. The inclusion appears to be collapsed, in the middle leaving a thick rim. The position of the reticulate body mass in the centre of the inclusion can be seen. A partially inflated inclusion is seen in Figure 4. Microvilli can be seen on the rest of the cell but not over the inclusion. The area of the cell around an inclusion always appears to be unharmed. The nucleus is often pushed to one side and this displacement may interfere with cell division.

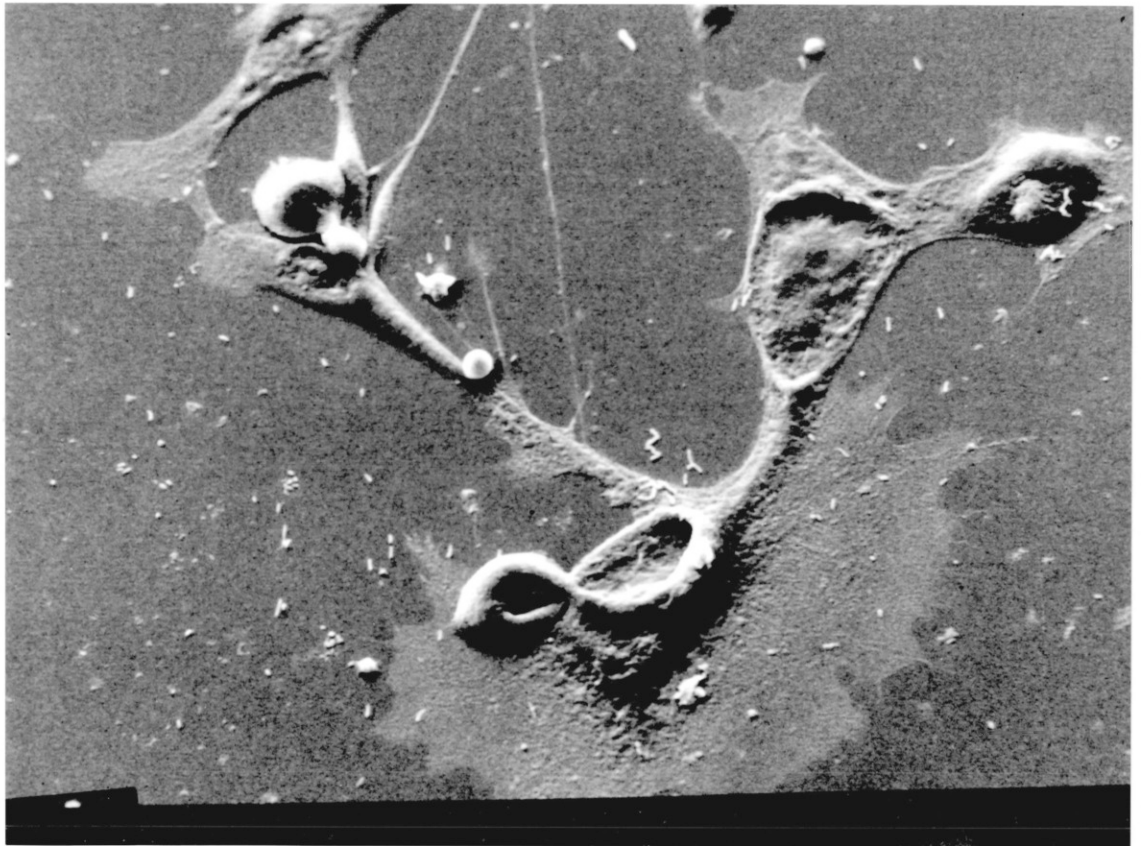
Examples of burst inclusions are seen in Figures 5 and 6. These are characterised by a raised lip, with cell debris in the middle of the crater. Elementary bodies have presumably been shed. (Inclusions in these cells were confirmed by Giemsa staining after scanning electron microscopy).

Empty craters can also be seen in some cells (Figure 7), which do not appear to be burst inclusions because the surface of the crater is intact and they do not appear to contain dividing bodies as in Figure 1. Next to these empty are intact inclusions. Cells with just one large inclusion were more commonly observed. Phagocytosis of an elementary body into a cell has not yet been observed.

DISCUSSION

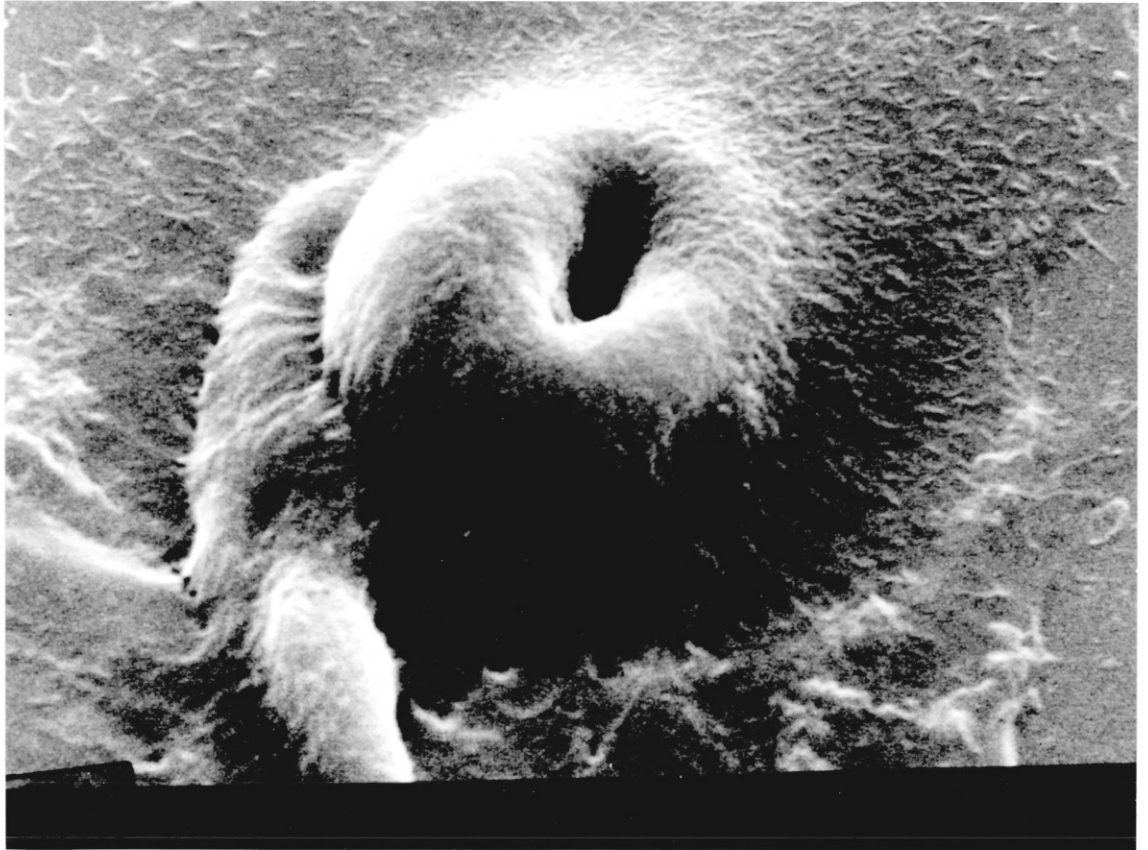
Chlamydia cannot synthesize ATP and therefore depend on the host cell to provide energy for their reproduction and maturation (Becker 1978). The

Figure 3



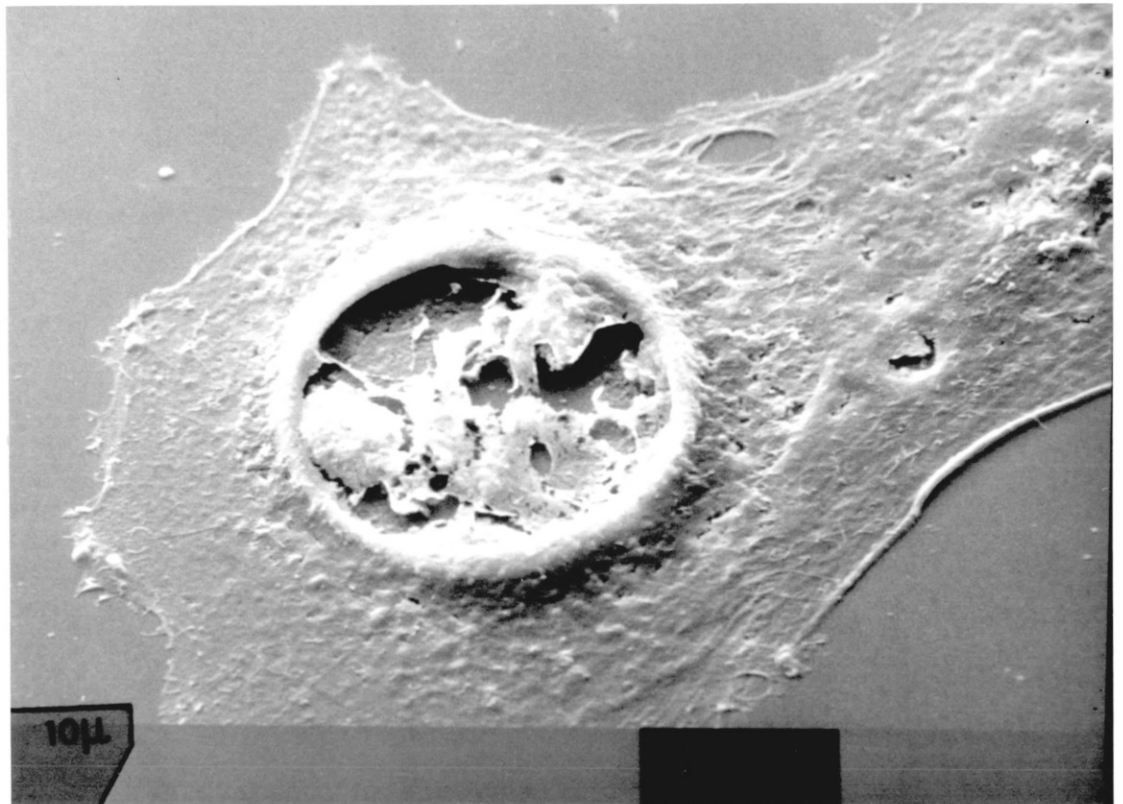
The same cell as in Figures 2 and 3 viewed under
scanning electron microscopy.
Bar marker 40 μm

Figure 4



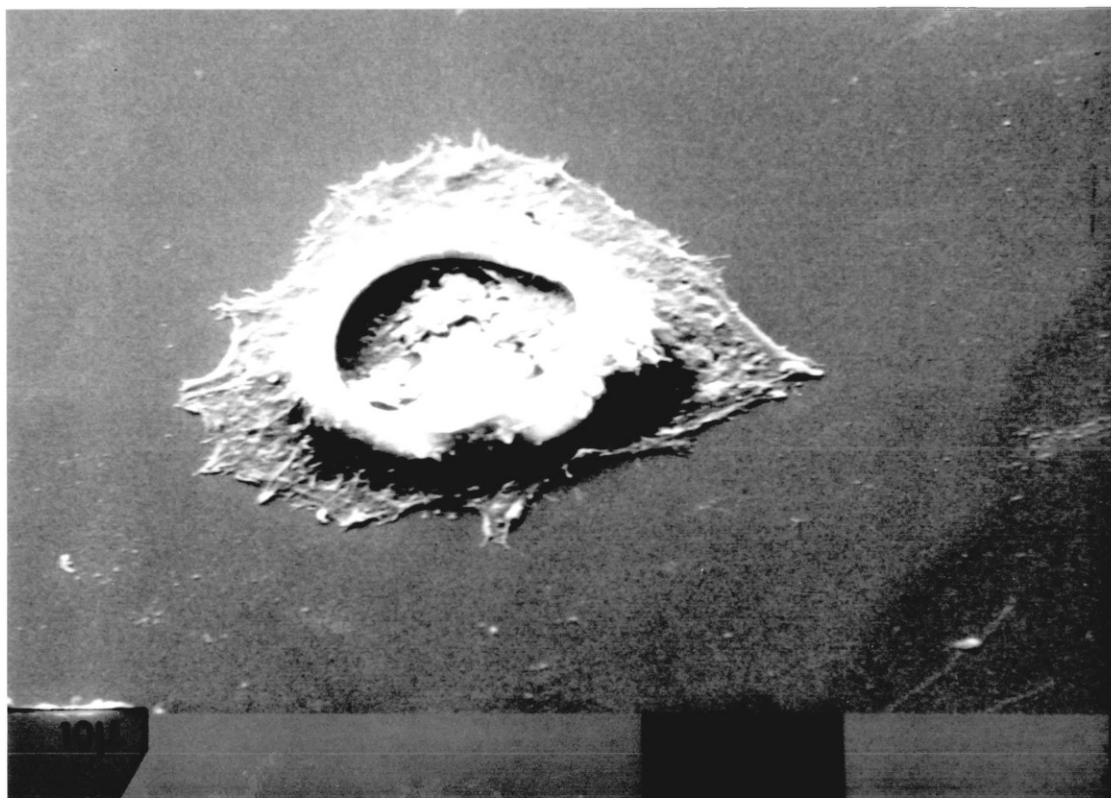
An endocervical cell in culture infected with Chlamydia trachomatis. The inclusion body appears collapsed in the centre.
Bar marker 4 μ m

Figure 5



An endocervical cell in culture infected with Chlamydia trachomatis. The inclusion body has burst only debris remains. The elementary bodies have been shed into the culture medium. Bar marker 10 μ m

Figure 6



Another example of an endocervical cell in culture
with a burst inclusion from Chlamydia infection.
Bar marker 10 μm

Figure 7



An endocervical cell in monolayer culture after inoculation with Chlamydia. Two vacuoles around the nucleus (N) appear to be intact inclusions, but there are eight other vacuoles which appear empty.

Bar marker 20 μ m

infectious form is the elementary body which attaches to the cell and enters by phagocytosis. Within 16 hours it has reorganised into an initial body that divides by binary fission. At 26-48 hours after infection the initial bodies differentiate into elementary bodies, the inclusion bursts and releases the infectious elementary bodies (Becker et al., 1969, Byrne 1976, Friis 1972, Kuo et al., 1973). Our cultures were incubated for 72 hours after inoculation with C. trachomatis. This presumably allowed inclusions formed by chlamydia when they were centrifuged onto the cultures to grow to maturity and burst. These burst inclusions were seen in the scanning electron micrographs. The elementary bodies released must have infected the monolayer again, because intact inclusions were seen in the same monolayers.

This is interesting because it is a point of discussion as to why pretreatment regimes and centrifugation are required to obtain infections in cells in vitro but in vivo infection occurs apparently unaided. It is possible that some change in the physiological state in vivo does occur at the time of infection.

HeLa cells have a net negative surface charge as do chlamydia, so polycationic DEAE-dextran was used as a pre-treatment regime of HeLa cells by some researchers in the hope that it would change the surface charge of the cells sufficiently to allow the chlamydia to attach and thereby enhance infection (Kuo et al., 1973). DEAE-dextran did not improve or enhance the number of inclusions obtained in our endothelial cell cultures and this finding was in agreement with that found by Moorman et al., (1986). It is possible that centrifugation overcomes the electronegative forces, but Moorman et al., claim that if the chlamydia elementary bodies are suspended in a very small volume of inoculum in the presence of 0.1M-salt it is enough to allow for adequate adsorption of the chlamydia onto the epithelial cells by Brownian motion (Moorman et al., 1986). This may explain why the elementary bodies released during incubation of the

cells after inoculation and after the original inocula had been removed were able to infect the cells in the monolayer and form new inclusions. We have not as yet tried passive inoculation of the cultures as a method for the initial infection.

In our early experiments we were using Eagle's minimal essential medium with D-valine to try to eliminate fibroblast growth, because fibroblasts do not grow without a supply of L-valine as unlike epithelial cells they are unable to convert the D-form to the L-form. During the time we used this medium we failed to obtain inclusions. This may be because chlamydia require D-valine for growth, and they do not grow if there is an essential amino acid missing (Hatch 1975). Restoration of cells positive for chlamydia is only possible with the addition of cyclohexamide or the exogenous addition of the missing amino acid.

The cells with the large apparently empty craters may be cells dying or they may be the early stage in the formation of an inclusion body in which there were only elementary bodies (which are much smaller than the dividing bodies). Chlamydia are protected from lysosomal bodies in the host cell in the initial stages of infection because of some surface antigens on the live replicating chlamydia. In the later stages of infection they become susceptible to host cell lysosomes (Friis, 1972; Kellogg et al., 1977). It is possible the craters in (Figure 7) are vacuoles full of lysosomes being produced by the epithelial cells. It may be that host cell lysosomes are involved in the mechanism of rupturing the inclusion body once all the chlamydia have differentiated into elementary bodies and it would explain why these vacuoles were not often seen next to inclusions, because they would probably only be there for a short time.

CHAPTER 7

HAEMOPHILUS VAGINALIS

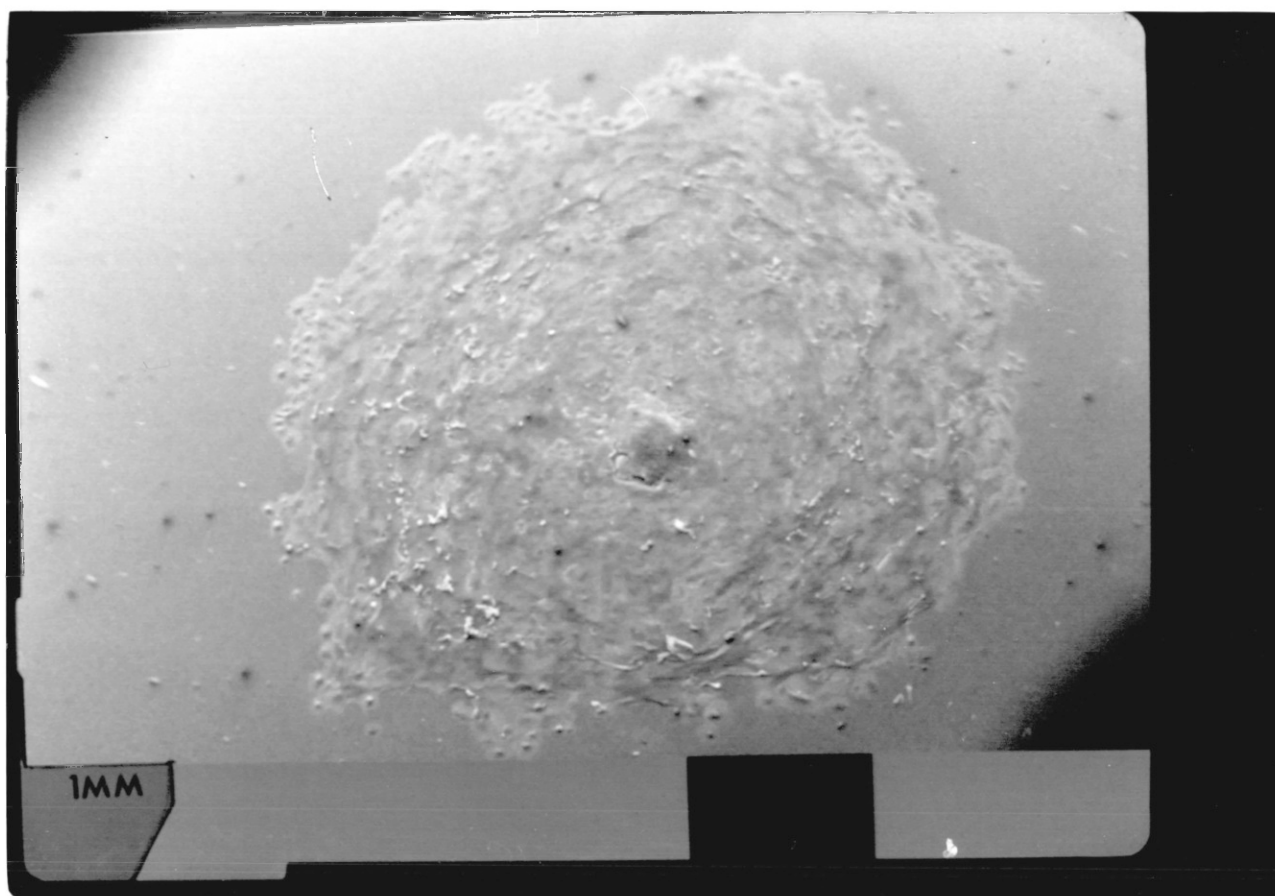
Haemophilus vaginalis has been described under two other names : Gardnerella vaginalis or Corynebacterium vaginale. The first of these names was applied because Gardner (1955) was the first to define the organism and its characteristics and the second because its growth is coryneform on blood agar plates. There has been considerable dispute about the degree of pathogenicity of the organism in the vagina.

The clinical symptoms of women with H.vaginalis have been described as a gray odorous vaginal discharge of pH 5.0 to 5.5. The bacteria can be observed adhering to 'clue cells' (vaginal epithelial cells), if a sample of the discharge is viewed under light microscopy (Gardner, 1980).

Two facts in particular may give some support to the theory that H. vaginalis causes vaginitis, the first is that most normal women develop an offensive vaginal discharge if inoculated with material from an infected woman (Gardner and Dukes, 1955). Secondly newborn girls from infected mothers do not develop vaginitis. This is consistent with the fact that newborn girls do not have any glycogen in the vagina from about 5 days after birth until puberty. The five days are not sufficiently long enough to develop vaginitis from H vaginalis as the incubation period is said to be 7 days (Cruickshank and Sherman, 1934).

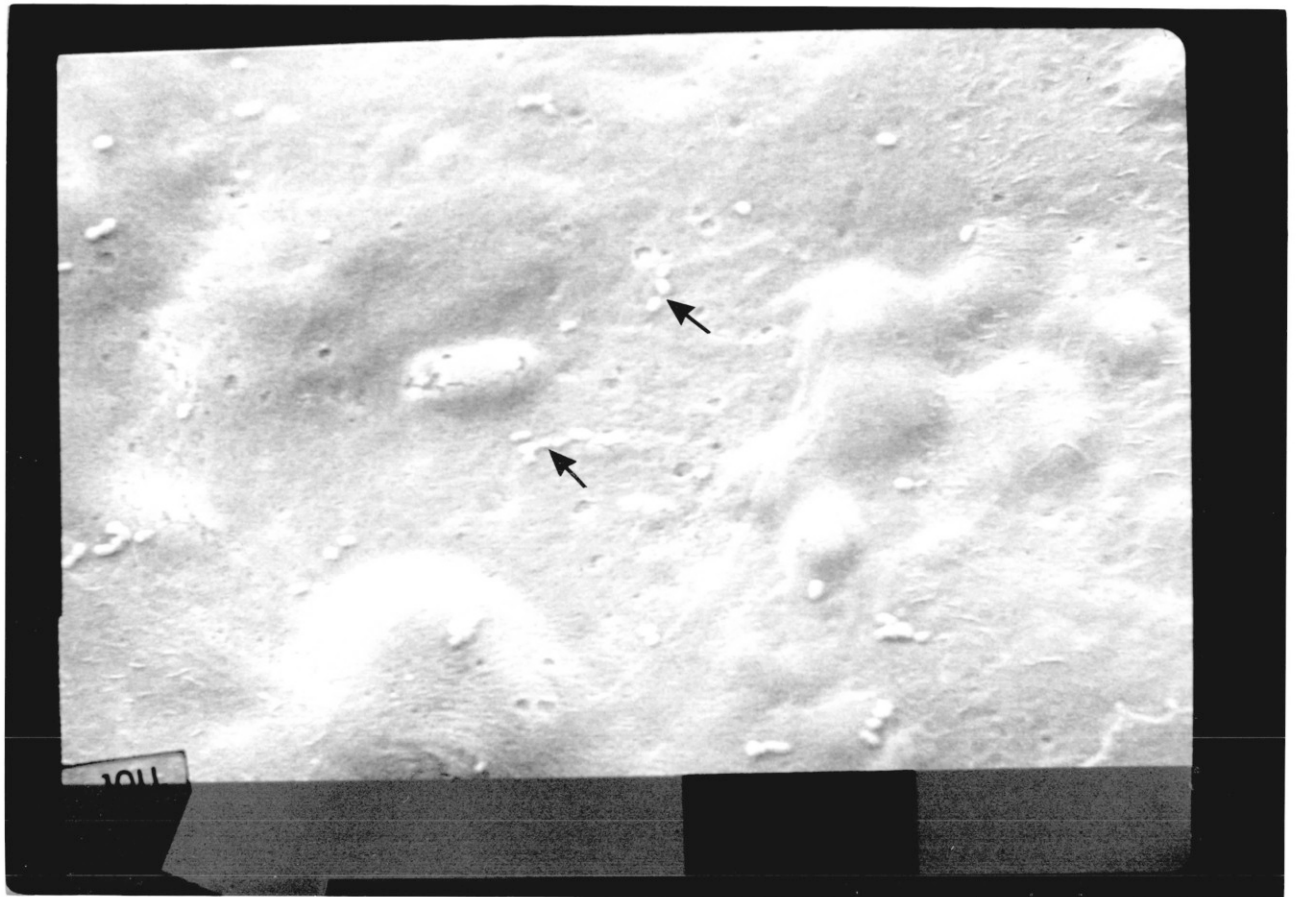
These facts prompted us to make some preliminary studies with H.vaginalis on cultures of cervical epithelial cells, although cervicitis does not seem to be associated with this infection.

Figure 1



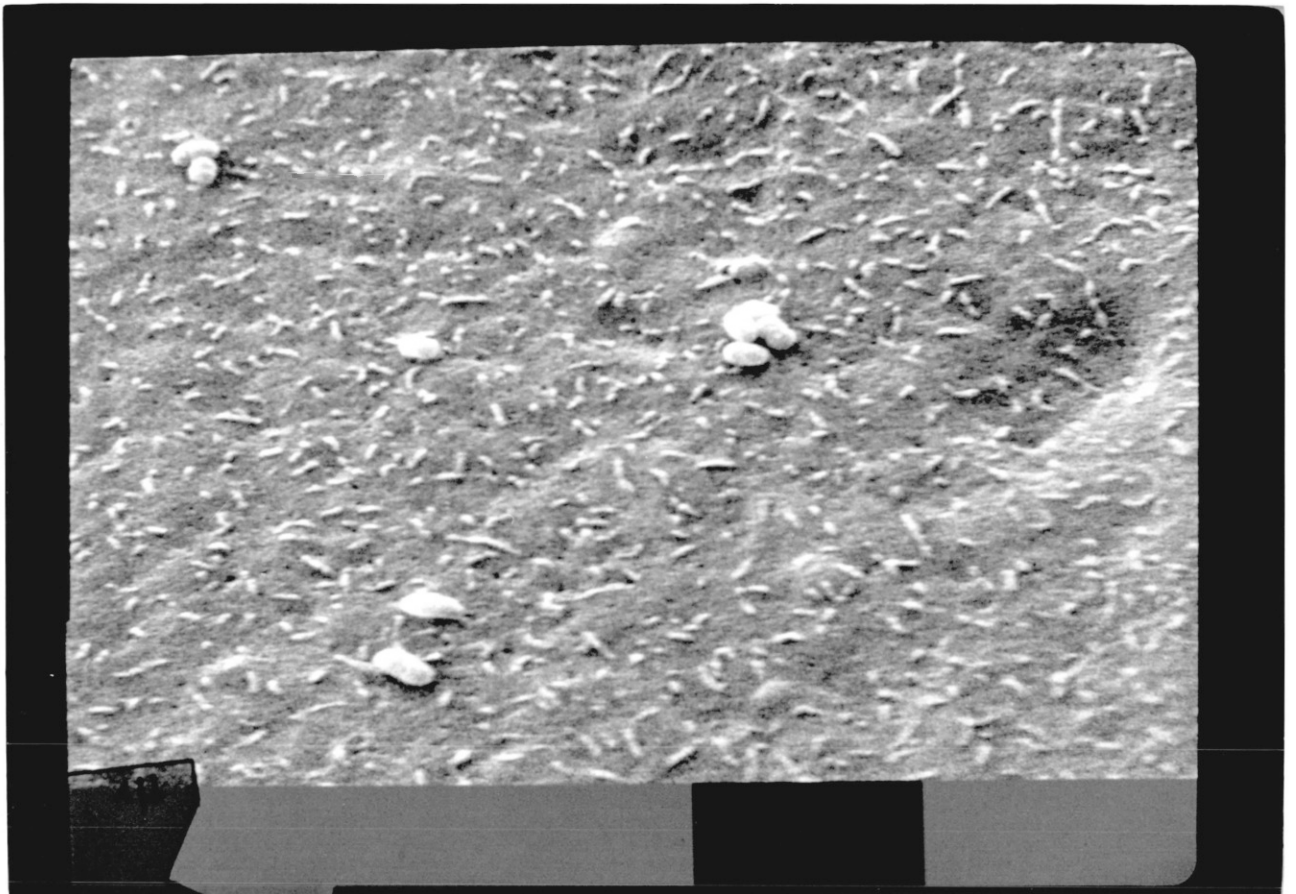
A scanning electron micrograph of an entire culture of epithelial cells from the ectocervix after 24 hours incubation with Haemophilus vaginalis. The monolayer is intact and no damage by the bacteria is apparent.
Bar marker 1 mm

Figure 2



The surface of the epithelial cells remain intact
in the presence of Haemophilus vaginalis (arrows)
Bar marker 10 μm

Figure 3



The bacteria scatter randomly over the surface of
the cell lying individually or in groups of 3 to 5.
Bar marker 10 μm

METHODS

Two blood agar plates with colonies of H. vaginalis were obtained on separate occasions from the Bacteriology Department of Queen Charlotte's Hospital. Four cultures from two donors were infected on the 8th day using a sterile platinum wire that had been scraped over the colonies on the blood agar, to introduce the bacteria into the culture medium over the cell culture. The controls had a sterile platinum wire dipped into the medium. The cultures were incubated for 24 hours in a 5% carbon dioxide humidified atmosphere at 37°C. Then the epithelial cell cultures were prepared for scanning electron microscopy as previously described.

RESULTS

The cultures of squamous epithelial cells under low magnification looked healthy and intact (Figure 1). The bacteria were seen lying scattered at random over the surface of the cells, individually or in groups of 3 to 5 (Figure 2). Under higher magnification there was no damage to the cells, they did not appear to have been invaded, there were microvilli and microrugae present as with untreated squamous cells (Chapter 3). The bacteria were not obviously attached to the surface of the cells, so it is not known how they survived the many washings in preparation for scanning electron microscopy. It is not possible to know how many settled on the cultures, but the monolayers did appear almost densely speckled with bacteria. Under the scanning electron microscope the organisms are coccoid in shape, about 1 μm long and 0.5 μm wide, smooth and white in colour.

CONCLUSIONS

In these preliminary experiments H.vaginalis did not appear to cause any damage to a monolayer of squamous cervical epithelial cells after 24 hours in culture.

CHAPTER 8

QUANTITATIVE ANALYSIS OF DEGREE OF ABNORMALITY
IN NEOPLASTIC CELLS IN TISSUE CULTURE

Studies on the behaviour of cells from normal tissue have shown that they grow as a monolayer, moving as they divide, away from the explant. If two cultures growing side by side meet, the number of directions the cells can move in as a monolayer is decreased. The alternative movement is for cells to move over each other, that is to overlap. Two cells can be said to overlap if one cell is wholly or partly superimposed on another cell. It is difficult, sometimes, to see whole cells under the light microscope, so overlapping cells are defined as overlapping nuclei.

It has been observed that neoplastic tissue in culture grows in a haphazard form, either showing areas of no cell cohesion or areas of tightly packed cells. The pattern of growth of the cultures may differ from culture to culture and from donor to donor. Richart (1964) suggested the following criteria for deciding on the degree of abnormality of the cells: the degree of cell cohesion, the differences in the number of cell processes; marginal fibrils and microvilli and differences in the ability of the cell to move.

Attempts have been made to quantitate the degree of abnormality of growth in tissue cultures of malignant cells in the hope that tissue cultures could be used in diagnosis and grading of tumours. Currently grading of neoplasias is cytological and histological, and with the employment of certain stains or monoclonal antibodies. There is a problem in obtaining a representative sample of tissue to section. It has been suggested that part of the biopsy might be set up in tissue culture simultaneously as an aid to diagnosis, and for clarification in ambiguous cases. Tissue cultures were at first assessed qualitatively (Mellgren et al., 1967) and then quantitative

methods were sought (Ebling and Tanneberger, 1979).

One of the methods is derived from a hypothesis which stated that cells from normal tissue tend to grow as a monolayer. To prove this a quantitative comparison between the actual distribution of cells, with the distribution which might be expected if there was no restriction on overlapping of cells was made, this was expressed as an overlap ratio. (Abercrombie and Heaysman 1954). A higher overlap ratio for cultivated sarcoma cells than for cultivated normal fibroblasts was found (Abercrombie and Heaysman 1957). This was also the case in another study in which overlap ratios for normal cervical epithelium, dysplasia and carcinoma of the cervix, invasive carcinoma of the cervix and ovarian carcinoma were compared (Ebling and Tanneberger, 1979). Overlap ratios in cultures from normal cervical epithelium were significantly lower than the overlap ratio of all the other tissue examined, and lower in mild dysplasia than in frank carcinoma in situ. There were no significant differences between overlap ratios in carcinoma in situ or dysplasia and invasive cervical carcinoma. They found a high standard deviation of overlap ratios for all culture groups, normal and neoplastic (Ebling and Tanneberger, 1979).

The method of quantifying the degree of neoplasia used by Abercrombie and Heaysman and by Ebling and Tanneberger tested in our cell culture system for abnormal cells. Two other methods were also tested. One of these methods was derived from a method devised to quantitate the degree of abnormality of cell arrangement in histological sections of neoplastic tissue (Chalkey, 1943). The third method involved counting the number of overlapping nuclei per thousand cells. The three methods were used on the same cultures and the results obtained were compared and evaluated for their possible use in quantitating grade of dysplasia in tissue cultures of cells from neoplasms.

Methods

Cell cultures

Ten monolayers from 9 donors with histologically normal cervixes were used for the controls and were cultured as for normal epithelium, stained for light microscopy using haematoxylin and eosin.

Two donors with abnormal cervical tissue were studied. This tissue was cultured and stained in the same way as for the controls. These donors were diagnosed histologically at the time of culture as having the following cervical pathology:

Donor code: WI Squamous carcinoma in situ of the cervix but no evidence of invasion.

Donor CU: Carcinoma of the cervix stage III B.

Methods for calculating overlap ratios.

The overlap ratio for the cultures is obtained in method 1 and 2 by dividing the expected number of overlaps by the actual number of overlaps multiplied by 100. The expected number of overlaps is calculated from counting various parameters on the cell cultures and then putting the data obtained into a formula. To obtain data on the cell cultures a microscope graticule was used.

The cell culture area is divided by a grid on the graticule, the area covered by the grid is called a field. The area of the field is 100 μm and the number of overlapping nuclei in each field was counted to give the number of actual overlaps per mm area of monolayer.

The formula in the Chalkley method for the calculation of expected number of overlaps requires the number of nuclei sitting under grid intersections (P) per field; the number of cells per field (N) and a constant which is the number of grid intersections (Q), which for the graticule used in this study was 121.

Method 1 (Chalkley 1943)

$$\text{Expected overlaps} = \frac{2 P(N-1)}{Q}$$

The formula in the Abercrombie and Heaysman method requires the mean area of nuclei per field, which is calculated from assuming the nuclei are circular and thereby calculating their areas from the formula for the area of a circle: πr^2 . The radius is mean radius of the nuclei per field, (r); the total number of cells per field (N) and a constant which is the area of the field, which for the graticule used in this study was 0.9mm².

Method 2 (Abercrombie and Heaysman, 1954)

$$\text{Expected overlaps} = \frac{2 \pi r^2 N (N-1)}{A}$$

The third method does not calculate an expected number of overlaps, but simply expresses the overlaps as the ratio of the actual number of overlaps per 1000 cells.

RESULTS

The overlap ratios obtained with the three methods are presented for the normal cell cultures in Table I, for the dysplastic cell cultures in Table II, and for the invasive carcinoma cell cultures in Table III. All three methods are measures of the degree of overlapping in the cell cultures, but since they are calculated from different formulae they do not give similar numerical values.

The mean overlap ratio for the normal cells obtained with methods 1, 2 and 3 were 46.2%, 15.1%, and 56% respectively and the medians were 48.8%, 6.9% and 22.7%. The coefficients of variation between donors were high, 40 - 45 per cent for the means and 50 - 57 per cent for the medians.

The overlap ratio means obtained for the 10 cultures from one donor with carcinoma in situ for methods 1, 2 and 3 were 18.7%, 6.7% and 12.9% respectively, the medians were similar for all three methods (Table II). These results were lower than the results obtained for the normal cell cultures. The coefficient of variation of the means was 60 per cent for methods 1 and 2 and very high at 100 per cent for method 3; the coefficients of variation of the medians were lower at 70 to 80 per cent for all three methods. This variation was between cultures from the same donor.

The overlap ratio means obtained for 5 cultures from one donor with invasive carcinoma for methods 1, 2 and 3 were 130.4%, 18.5% and 58.0% respectively; the coefficients for the medians were considerably lower (Table III). This was probably due to the result obtained with slide: CU2, which had a very low score compared to the results of the other four slides and due to the small number of slides counted levied a significant bias in the median results.

Table I

Normal cervical epithelium. Overlap ratios in 10 monolayers from 9 donors (Method 1 Chalkley 1943; Method 2 Abercrombie et al. 1954)

Culture	OVERLAP RATIO		
	Method 1	Method 2	Nuclear overlaps/1000 cells
AX5	40.2	12.5	33.5
DI23	57.8	14.2	69.9
DV6	52.2	21.0	34.0
EB4	51.4	19.5	58.9
EB7	17.9	24.9	74.8
EH14	72.6	21.1	71.0
FH19	33.8	8.7	36.0
DZ7	83.2	17.4	97.0
BG4	29.0	7.4	28.0
BN8	23.8	3.9	56.7
mean	46.2	15.1	56
s.d. of mean	21.2	6.9	22.7
median	45.8	15.	57.8
s.d. of median	26.5	8.6	28.4
co. of var. means	45.9%	45.7%	40.5%
co. of var. medians	57.4%	57.0%	50.7%

Table II

Carcinoma-in-situ. Overlap ratios on 10 cultures from the same donor (Method 1. Chalkley 1943; Abercrombie et al. 1954
METHOD 2

OVERLAP RATIO

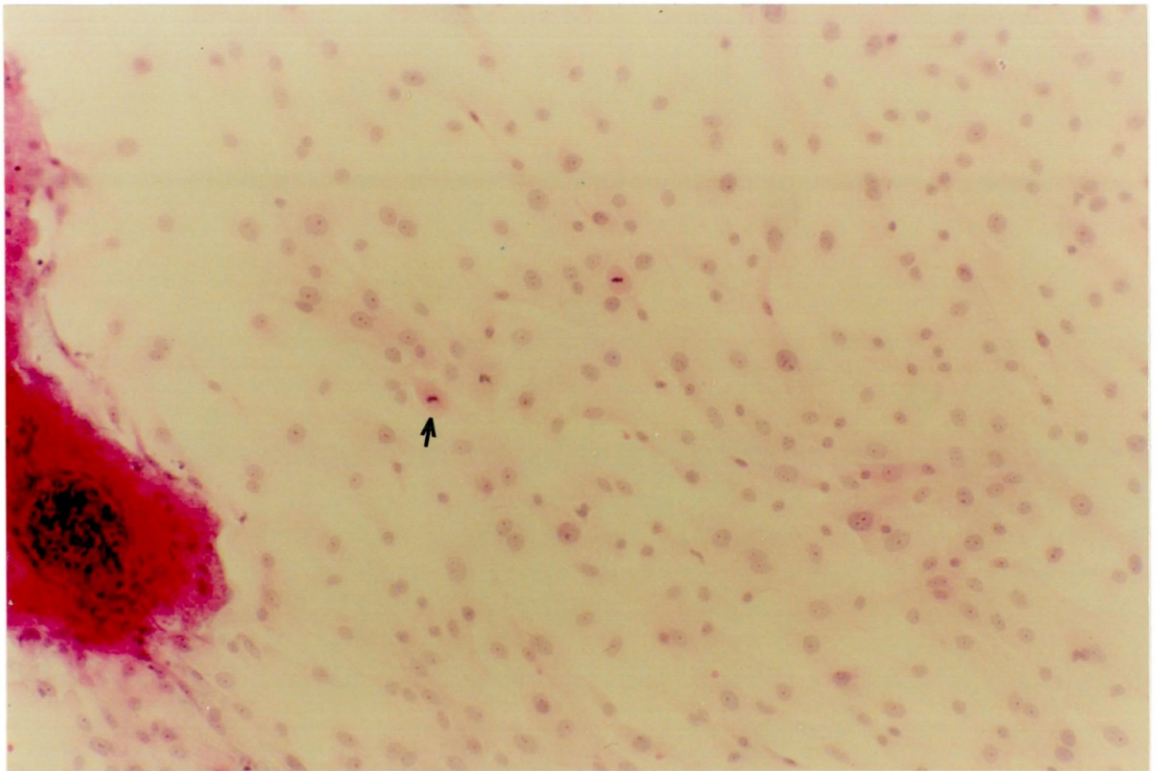
Donor Code/Slide No.	Method1	Method2	Nuclear overlaps/1000cells
WlFF 1	10.4	3.8	5.9
2	26.7	10.0	20.7
3	3.6	0.4	1.5
4	38.0	13.3	14.7
5	28.2	12.3	22.5
6	14.0	4.1	16.9
7	10.3	3.5	8.2
8	11.2	4.9	7.8
9	30.4	11.3	22.3
10	14.0	3.6	8.5
mean	18.7	6.7	12.9
s.d. of mean	11.2	4.5	7.5
median	14.0	4.5	11.6
s.d. of median	14.0	5.6	9.4
co. of var. means	59.9%	67.2%	104.3%
co. of var. medians	74.9%	83.6%	72.9%

Table III

Invasive carcinoma of the cervix. Overlap ratios on 5 cultures from the same donor (Method 1 Chalkley 1943; 2 Abercrombie et al. 1954)

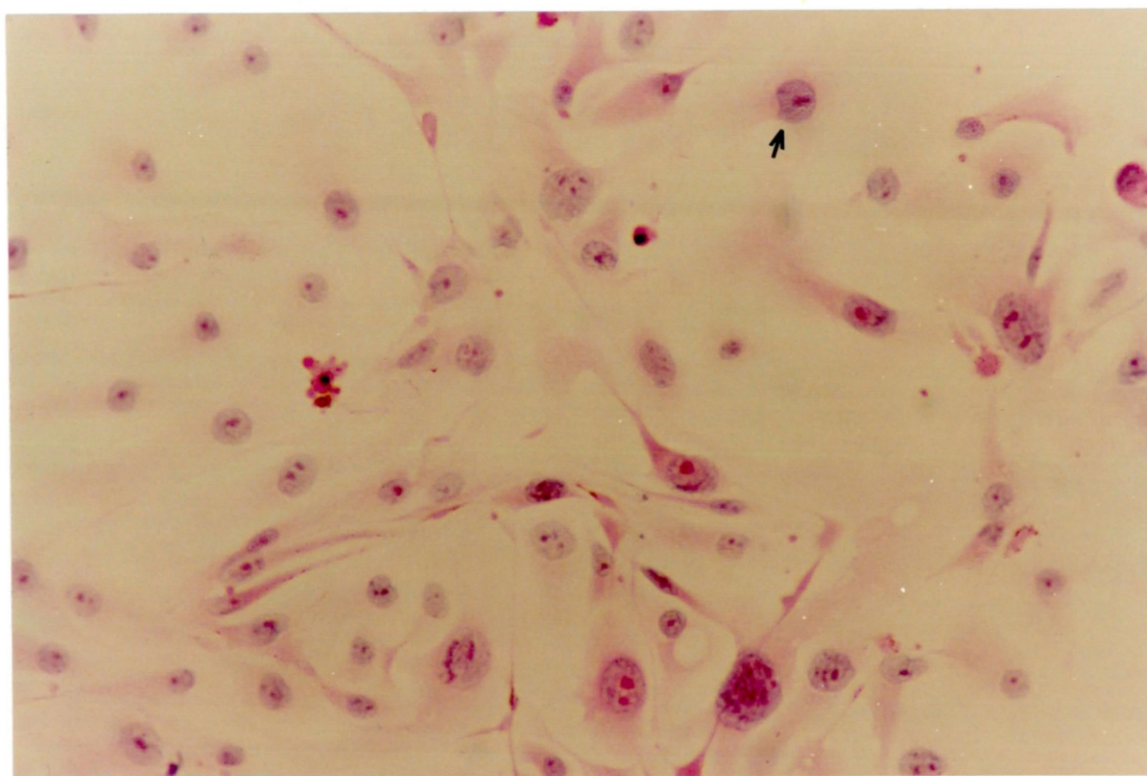
Donor code/slide no.	Method 1	Method 2	Nuclear overlaps/1000 cells
CU2	15.4	2.8	8.4
CU5	182.0	21.9	21.3
CU7	183.5	16.7	27.4
CU8	121.4	25.2	155.4
CU9	149.7	25.7	77.7
mean	130.4	18.5	58.0
s.d. of mean	17.3	9.5	60.5
median	182.0	21.9	27.4
s.d. of median	21.6	11.9	75.6
co. of var. means	13.3%	51.4%	104.3%
co. of var. medians	16.2%	64.2%	130.3%

Figure 1



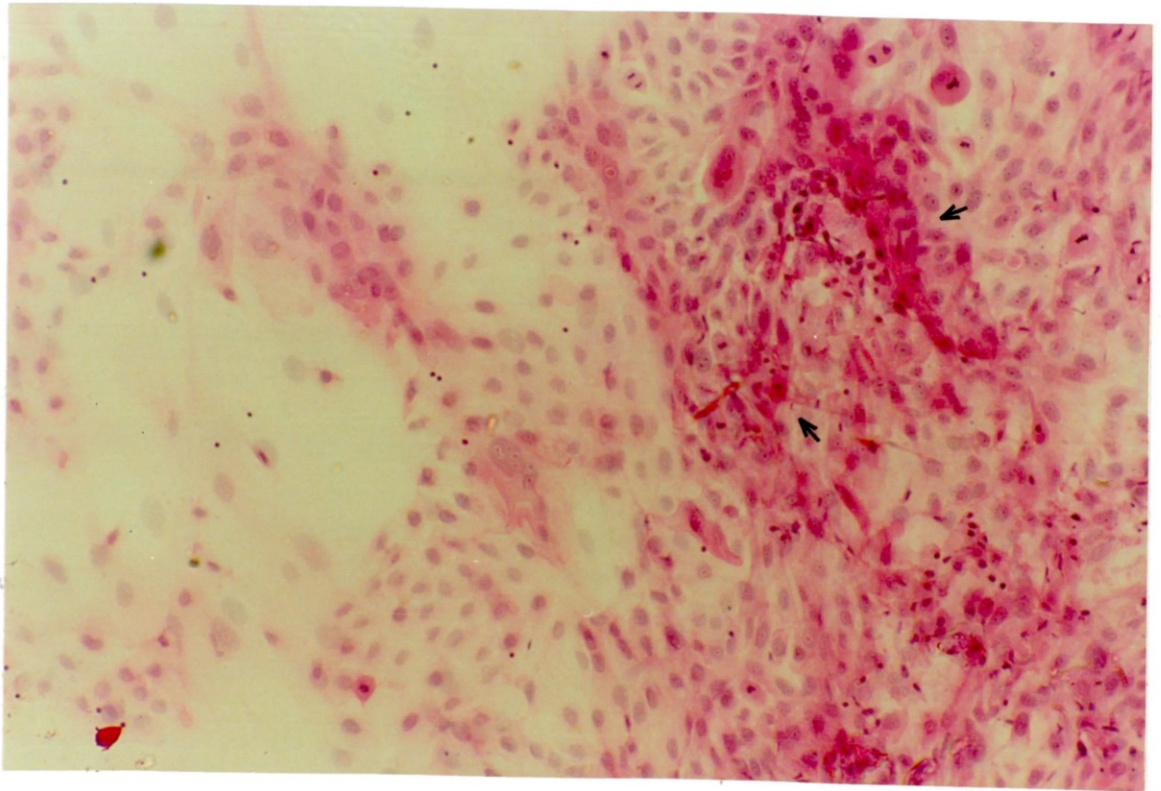
Tissue culture of cells from donor: WI with grade III carcinoma-in-situ. Most of the cells appear normal, there are some cells in mitosis (arrow). The cells do not form a cohesive sheet.
Magnification (x50)

Figure 2



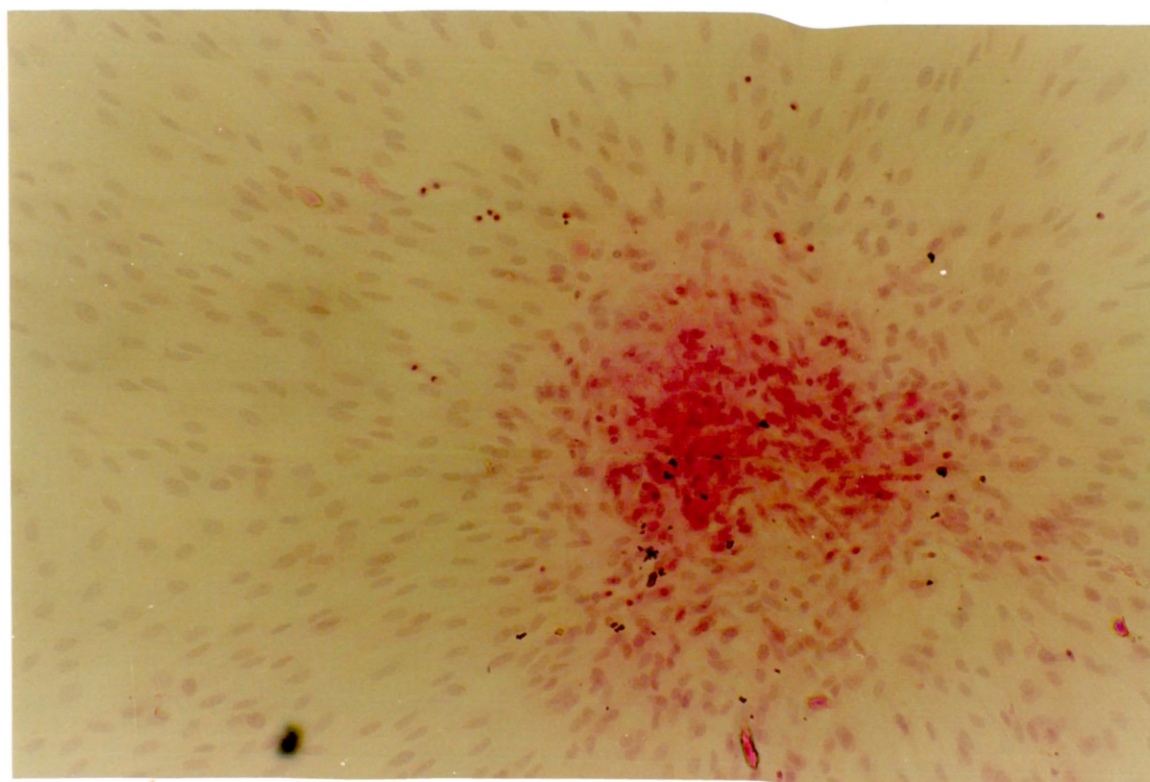
Another view of the same monolayer as in Figure 1. Cell growth is in a fibroblast-like form. One cell has a double nuclei (arrow). Magnification (x100)

Figure 3



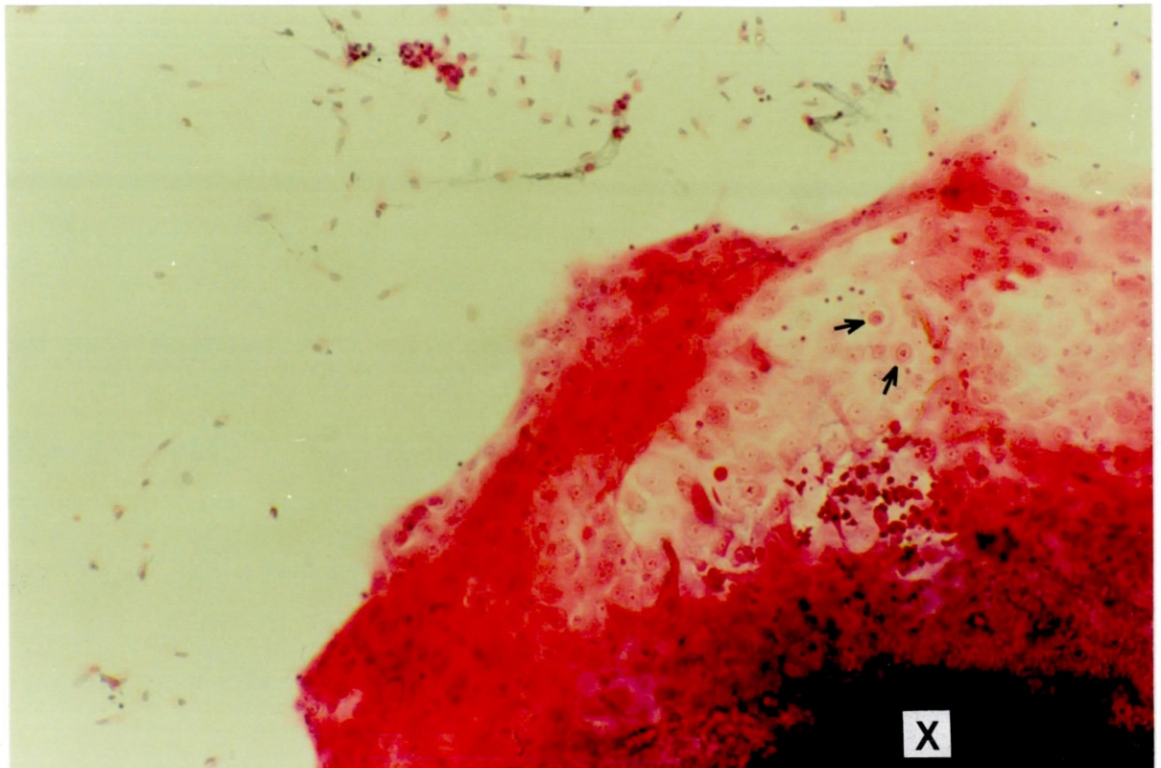
Cells from carcinoma-in-situ in tissue culture.
Donnor: LA. Areas of a high degree of cell
overlap (small arrows) and areas of a dispersed
cell growth form in the same culture, within the
same microscopic field.
Magnification (x 50)

Figure 4



Cells from an invasive carcinoma in tissue culture.
Donor: CU An uneven pattern of growth, the dense
mass of overlapping cells are surrounded by elongated
cells radiating out and showing a very low incidence
of overlapping.
Magnification (x50)

Figure 5



Cell culture and explant (X) from invasive carcinoma the same donor as in Figure 4. A different growth pattern is observed. The culture is condensed with many of the cells piled on top of each other, but with small areas of monolayer growth. Abnormal nuclei are visible in some cells (arrows). Magnification (x50)

This low score also raised the coefficient of variation on method 1 without which it would have been very low. The coefficients of variation for the other two methods were high at about 55 per cent for method 2 and 100 for method 3. The overlap ratios obtained were three-fold higher than the normal cells with method 1 but not significantly different with methods 2 and 3.

The cells in culture from donor WI with carcinoma in situ show a dispersed growth pattern over most of the monolayer and very little overlap of cells is seen (Figure 1). Under higher magnification the cells do show abnormalities in their nuclei; some of them are double and in parts of the monolayer the cells have a fibroblastic appearance (Figure 2). Cells in culture from another donor, with the same carcinoma in situ grade III lesion as donor WI, show in one part of the culture a very high degree of overlap and in a neighbouring part of the culture a very dispersed cell growth form with very little overlap (Figure 3).

Two completely different growth patterns were obtained with the invasive carcinoma cells in culture. Slide: CU2 showed foci of cells piled on top of each other and dispersed cells growing in a radiating pattern away from this foci (Figure 4). On another slide CU8 the growth pattern was different and almost the reverse in that there were large areas of overlapping cells and small areas with no overlapping, but the culture was not dispersed (Figure 5).

DISCUSSION

A large number of controls were counted to assess what variation in overlap if any there might be in cultures from normal tissue. The results obtained with all three methods indicate there is a high degree of variation between donors. Two cultures from one donor (EB) also differed in overlap.

The "lower than the normal" overlap ratios for the carcinoma in situ cells was unexpected, because Ebling and Tanneberger (1979) reported dysplasias as having a higher overlap ratio than the normals.

Method 1 gave a three-fold higher than the normals overlap ratio for invasive carcinoma and there was little variation between four of the five cultures counted. This result was similar to that obtained by Ebling and Tanneberger for invasive carcinoma, but they obtained the result with method 2.

Our results seem to indicate that method 1 gives the best estimate of the degree of overlap because it has the lowest coefficients of variation. If the number of cultures per donor and the number of donors counted were larger the variability in the results may be reduced. Method 2 and 3 gave highly variable results. In the case of method 2 this was also found by Ebling and Tanneberger.

A possible explanation for the differing values for overlap ratios and the large standard deviations, may lie in a sampling error. The photographs of the dysplastic and invasive carcinoma cells in culture show the wide variation in growth pattern from piled-up cells to dispersed cells. With such a divergent growth pattern it is not possible to express quantitatively in one ratio two extremes of cell behaviour. Differences in the patterns of cell growth could also arise from sampling of the lesion on the cervix when the biopsies are taken for culture.

In view of the large amount of work required in setting up tissue cultures and the need for several cultures from each donor it is unlikely that measuring overlap ratios is a useful diagnostic technique. However it may be useful in terms of a research technique to quantitatively describe cell behaviour in toxicity, mutagen or carcinogen tests. Particularly in cell lines where cells from the same culture can be divided and used as control and test simultaneously while different experiments performed.

CHAPTER 9

METAL IONS

Metal ions are an important part of dietary intake as they are incorporated into enzymes and proteins used in a wide number of metabolic pathways. Metal transporting proteins such as blood proteins and transferrin and the metal-storage protein ferritin, are some examples. The metal binding is reversible, because the metal ion is only a temporary component of the protein. For example, iron containing proteins including the cytochromes and respiratory pigments; haemoglobin; and enzymes, for example catalase. Zinc is present in insulin and carbonic anhydrase; the glycolytic enzymes and proteases sometimes contain manganese; copper is present in some oxoreductases and some enzymes require specific cations for activity, for example the sodium dependent membrane ATPases.

The way in which the body copes with an excess of metal ions started to be elucidated in 1960 with the discovery of metal ion proteins in horse kidney cortex. These proteins were found to contain of nitrogen (13.2%), a high sulphur content (4.1%), about 2.9% cadmium, 0.6% of zinc of the dry weight of protein. Since the proteins consisted mainly of metal ions and sulphur they were termed metallothioneins (Kagi and Vallee, 1960). In 1966 a metallothionein was isolated from human renal cortex (Pulido *et al.*, 1966). This protein was found to contain 4.2% cadmium, 2.6% zinc, 0.5% mercury and 0.3% copper. The presence of mercury was explained by the fact that patients whose kidneys were removed post-mortum for these experiments had been treated with mercurial

diuretics. In 1974, two zinc containing forms of metallothioneins were isolated from human liver (Buhler and Kagi, 1974)

The general properties of metallothioneins are demonstrated by these two liver proteins. They have molecular weights around 6 600 Daltons (some metal proteins have molecular weights around 10 000 Daltons.) About 30-35% of the protein is cysteine which accounts for the high sulphur content. Aromatic amino acid residues and histidine are absent. There are three mercapto groups for each metal atom bound. In contrast to the renal metallothioneins the liver proteins have a higher zinc content.

The biological function of the metallothioneins is not known although there is some evidence to suggest that they are involved in heavy metal ion detoxification, transport and perhaps storage. Rats infected with zinc 24 hours before the administration of cadmium are partially protected against the toxic effects of cadmium which causes acute destruction of the rat testes. (Parizek 1957, Webb, 1972) The suggested mechanism of action is that the zinc induces synthesis of the metallothioneins so that when the cadmium is introduced enough metal protein is available and is manufactured to cope. (Webb, 1972b). An interrelationship between zinc and copper also exists. Symptoms of zinc toxicity have been shown to be reversed by incorporating added copper into the diet of rats on a high zinc diet (Smith and Larson 1946, Van Reen, 1953).

The protective effect of one metal ion over high levels of another is not just restricted to liver and kidney cells. Cadmium resistant human skin epithelial cells have been shown to have a cadmium binding protein (Rugstad and Norseth, 1975) whereas the non-resistant parent strain cells do not. When these cells were exposed to 386 μM zinc the cadmium resistant cells showed diminished but still good growth, but the non-resistant cells showed no growth at all by cell protein determination. When exposed to 77 μM mercury there was apparently good growth by both cell lines but at a concentration of 120 μM mercury cells in

both cell lines ceased to grow. In this study it was shown that human epithelial skin cells resistant to cadmium can tolerate higher doses of zinc than the non-resistant cell, but that they did not show a cross tolerance to mercury (Haffner and Rugstad, 1976)

In 1969, Zipper et al., found that incorporation of a small amount of copper wire on to an intra-uterine contraceptive device increased the efficacy of the device. Since then investigations into the effect of metal ions on the cells of the female genital tract have been carried out. (Zipper et al., 1969; Hagenfeldt, 1972; a,b,c,d.; Jones et al., 1973) One study dealt with the effect of copper sulphate on growth of cultures of human cervical epithelium (Hounsell and Hawkins, 1974). At concentrations of 10^{-4} M they found the area of outgrowth of cells from the explant was significantly reduced and growth was prevented at 4×10^{-4} M. The mitotic index of the cells was also significantly reduced by exposures of up to 2×10^{-3} M of copper for only two hours. If growth did occur in the presence of the copper the gross morphology of the cells did not differ from the controls (Hounsell and Hawkins, 1974).

This finding led to an interest in the interaction of zinc with copper to see if there were any accumulative or antagonistic effects of one ion against another, as had been demonstrated in the study of Smith and Larson (1946) and Van Reen (1953). If skin epithelial cells have a metal binding protein and could develop resistance to cadmium, it may be that cervical epithelial cells may have a similar protein. From the results of Hounsell and Hawkins (1974) if such a protein exists in cervical epithelial cells it was either only present in sufficient quantities or was not active. Perhaps the addition of another metal ion would initiate the production of a metal protein or activate one already there.

The work of Webb (1972 a and b) which showed that zinc induced the synthesis of metallothioneins to protect against cadmium, and we decided to test

the action of zinc alone on our cultures and then the action of zinc with copper to see if one metal ion had a protective effect against the action of the other.

The present experiments were undertaken to see what effect a concentration of zinc which did not affect growth would have on the action of a concentration of copper which reduced growth by 50%.

An attempt was made to look at the profile of proteins present in the epithelial cells to see if any proteins of around 10000 Daltons appeared on gel electrophoresis after adding copper and zinc ions to the cells.

Stress proteins synthesised by human endometrium and decidua have been described (Ron and Birkenfield, 1987) They have a range of molecular weights from 68 k Daltons to 94 k Daltons and an isoelectric point of approximately 6.0. They can be induced by heat and have therefore also been referred to as heatshock proteins.

These proteins were also looked for on the electrophoresis gels to see if they were induced or expressed in cells exposed to copper and zinc ions.

METHODS

Sterile stock solutions of copper and zinc were incorporated into the tissue culture media to give final concentrations of $3 \times 10^{-5}\text{M}$ and $3 \times 10^{-4}\text{M}$ for copper alone and $3 \times 10^{-4}\text{M}$ of copper with $3 \times 10^{-4}\text{M}$ zinc. The metal ions were incorporated into epithelial cell medium when it was changed on the fourth day of culture and the cultures were incubated for another four days before fixing. All stock solutions and epithelial cell medium was made up with deionised water.

The areas of the cultures were measured by colour television

quantitative image analysis.

Method For The Induction of Stress Proteins

Explant cell cultures grown to confluency in microtitre well plates were incubated in Ham's F10 medium (an amino acid deficient medium, Gibco) with copper at $3 \times 10^{-4}\text{M}$ and copper ($3 \times 10^{-4}\text{M}$) and zinc ($3 \times 10^{-4}\text{M}$) for 2 hours. The medium was then replaced with fresh Ham's F10 medium containing ^{35}S -methionine at $50\mu\text{Ci/ml}$ per culture dish. Incubation was at 37°C for 6 hours after which the cells were washed with phosphate buffered saline and the cellular proteins extracted with $100\mu\text{l}$ of lysis buffer.

Determination of the amount of ^{35}S -methionine incorporated into the total protein was determined by trichloroacetic acid precipitation. The amount in counts per minute then used for electrophoresis was adjusted to 100 000.

Two dimensional SDS-Polyacrylamide gel electrophoresis

Isoelectric focussing was performed on a Pharmacia flat apparatus with cooling at 15°C . Gels were cast in 8M urea, 13.2 per cent acrylamide, 4 per cent ampholine pH 5-7, 1 per cent ampholine pH 3-10 and 0.5 per cent nonodete-P40. Focussing was at 600 volts for 8 hours, SDS-PAGE was performed according to the method of Laemmli (1970). The gels were then fixed and impregnated with phosphorous pentoxide according to the method of Bonner and Lasky (1974), dried onto filter paper and placed at -70°C with Kodak X-omat-S film. A mixture of methylated C-labelled proteins of known molecular weight (Amersham) was used as markers. This mixture contained lysozyme (14300^{D}) carbonic anhydrase (30000^{D}) ovalbumin (46000^{D}), bovine serum albumin (69000^{D}) phosphorylase B (92500^{D}) and myosin ($200\ 000^{\text{D}}$) (Personal communication J. Croxtall).

RESULTS

Area growth measurements

Results are presented graphically as the geometric means of the areas (Figure 1). A Mann-Whitney test on these results indicates that both concentrations of copper inhibited growth of the monolayers, compared with that of the controls ($P < 0.01$). There was no statistical difference in growth in epithelial cell cultures treated with $3 \times 10^{-4}M$ of zinc added in conjunction with copper.

Gel electrophoresis

The gels for protein separation are presented in (Figure 2). No spot appeared on the gels in the position equivalent to 10 000 Daltons which would correspond to a metallothionein. Spots are present on gels B, C and D are present with some degree of staining at 69 kDaltons (arrows), corresponding to stress proteins. In A at this position there is only a very faint spot.

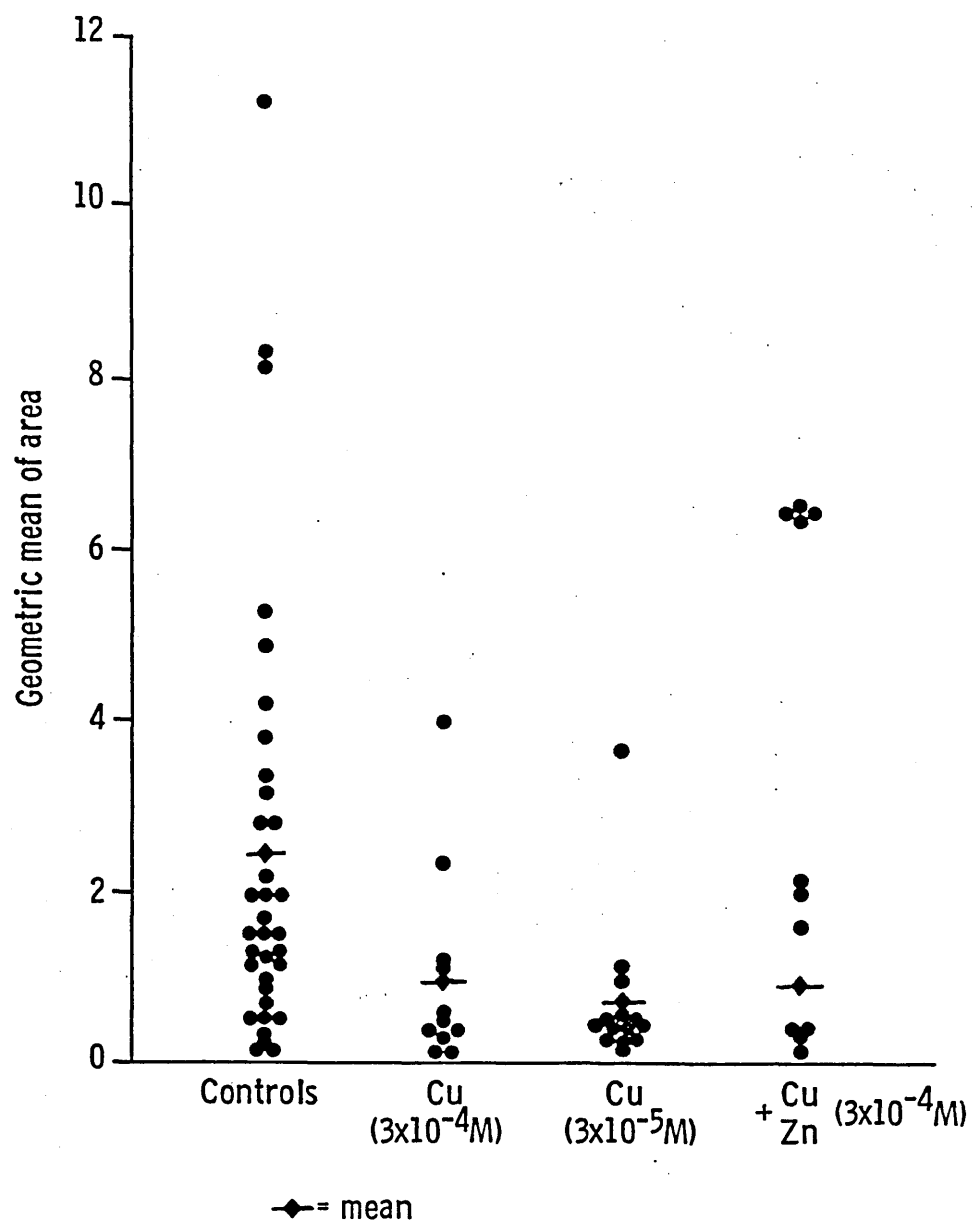
A is the gel run on control cells, that is cells without any copper or zinc.

B is the gel for copper ($3 \times 10^{-4}M$) alone, and it has the strongest colouration marking two intensities.

C is the gel for zinc ($3 \times 10^{-4}M$) alone, a faint mark of one spot can be seen.

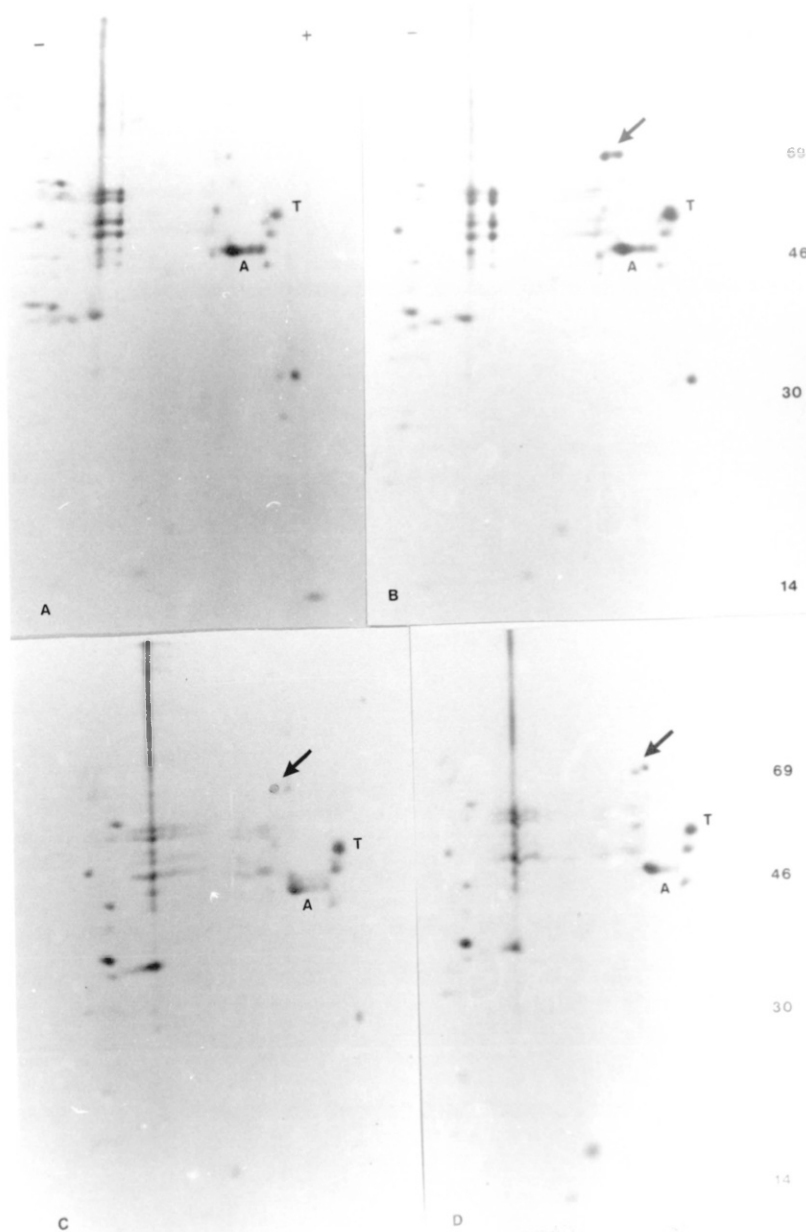
D is the gel for zinc ($3 \times 10^{-4}M$) and copper ($3 \times 10^{-4}M$). It shows an intensity in the middle of that shown for copper alone and zinc alone.

Figure 1



Geometric means of areas for control cultures
copper treated and copper and zinc treated
cultures.

Figure 2



Electrophoresis gels. Positions relating to molecular weights: 14,30,46 and 69kDaltons are marked at the right side of the photograph. A is the gel for the control, B is the gel for copper alone,C is the gel for zinc alone and D is the gel for copper and zinc. Marker proteins, actin (A) and the tubulins (T) are marked.

DISCUSSION

We were unable to detect a protective effect of zinc against the inhibitory action of copper on growth of epithelial cells in the cultures directly, at a concentration of zinc which had been shown previously to have no effect on the growth of cells. (Cameron and Hawkins, personal communication).

A protein of molecular weight 69 kDaltons pK 6 did appear with strong intensity on the gels in the copper treated cultures and to some extent on the copper and zinc gel. However only a very faint spot was seen on the zinc alone gel. There was no evidence on the gels for the production of a protein produced at 10 000 Daltons which means that it is unlikely that the epithelial cells produce metallothioneins. However low molecular weight proteins are sometimes difficult to extract on this gel. Copper seems to have two effects on epithelial cells in culture. It can inhibit cell growth by as much as 90 per cent of the controls and, in the cells that grow, stress protein synthesis is induced.

Basal levels of stress proteins may exist as part of the repair system in restoring the structure of naturally denatured proteins or as part of a system that is responsible for protein folding immediately after (or during) synthesis of the ribosome. (Pelham, 1986). This explains the faint spot on the control gels.

The low level of spot intensity was also seen in the zinc only treated culture, but was greatly intensified in the presence of copper. It is possible that the zinc does have some small inhibitory or protective effect on the cells against the action of copper as the intensity of the spots on the gel was less than in the copper alone gel, indicating that the proteins were only partially present.

CHAPTER 10

CONCLUSIONS

The cervix is an internal organ which can be exposed to a wide range of pathology-inducing microorganisms and chemical agents. An investigation was carried out to develop an in vitro model to study different aspects of cervical pathology.

The study was performed as follows:

1. Primary cultures of human ecto- and endocervical cells were examined by light and scanning electron microscopy and by the use of cell kinetic measurement techniques to obtain basic data on the growth of cell cultures.
2. The cell cultures were used as an in vitro model to study by scanning electron microscopy the development of fungal infections due to Candida albicans and four other species of yeasts (Saccharomyces cerevisiae, Candida krusei, Candida tropicalis, and Torulopsis glabrata) which can cause vulvovaginitis.
3. Three azole antifungal agents, clotrimazole, bifonazole and an oral agent (vibunazole) were tested for their action on two stages of infection of the ectocervical cell cultures by Candida albicans.
4. Endocervical cells in culture were used to study by light and scanning electron microscopy the course of infection with Chlamydia trachomatis.
5. A scanning electron microscopic study of Haemophilus vaginalis on ectocervical cells in culture was made.
6. Two methods of estimating the degree of loss of contact inhibition in neoplastic cells in culture by calculating the ratio of overlapping nuclei was compared to a simple ratio of number of nuclear overlaps per 1000 cells in the

monolayer.

7. The action of zinc as a possible antagonist to concentrations of copper which have been found to inhibit ectocervical epithelial cell growth was assessed.

Cell growth as a monolayer begins after 4 days of incubation of the cervical tissue explants. Initial studies on epithelial cell growth revealed that exponential growth continues up to eight days in culture, after which the cell production rate begins to slow down.

This data led to establishing criteria for using the cultures in experiments. For example, concentrations of metal ions were incorporated into the growth medium on day 4 of culture when the growth of a bud of epithelial cells had proved explant viability. The Candida experiments were carried out on day 8 of the cells in culture because then the monolayers were at their greatest area and the number of cells dividing was in proportion to the number of cells dying. From the anticytokeratin staining experiments it also appears that in eight day old cultures there is a degree of keratinization in the cells, which may be indicative of cell maturation and differentiation.

The time course study of the growth of C.albicans on the epithelial cell cultures indicated three stages of infection. (Farrell et al., 1983)
Firstly the yeast cells attach to the epithelial cells, then there is germ tube production and finally invasion of the cells by germ tubes or by fully formed hyphae. Inoculation of C.albicans on to the epithelial cells on the eighth day of culture demonstrated that yeasts will attach to cells in an apparently random distribution. They were seen in higher numbers associated with cells strongly positive in cytokeratin. Whether or not this is indicative of higher numbers of

yeast cells attaching in these areas or attached yeasts at these sites are stimulated to bud more cells would be interesting to investigate. The keratophilic nature of C.albicans has been demonstrated by Kapica and Blank (1957) and there have been reports from other studies that attachment of yeast cells to desquamating vaginal epithelial cells is higher than to dividing cells. (Sobel et al., 1982) Trichophyto mentagrophytes is a biphasic fungi in which morphological changes from yeast cell mycelial form can be induced by growing the organism on nutrient media containing animal hair or skin keratin as the sole nitrogen source (Abbink et al., 1986). It may be that growth of C.albicans on keratin induces the formation of mycelia and that those mycelia may be more pathogenic. It would be interesting to grow the yeast cells to the hyphal stage on the epithelial cell cultures and then stain with anticytokeratin monoclonal.

The action of three azole antifungals was assessed at the first two of the stages of cell infection with C.albicans, those of attachment and germ tube growth. Experiments with yeast cells treated with azoles resulted in lack of any detectable yeast cell attachment. Attachment of yeast cells was prevented by 80 percent of the number of yeasts attached in the untreated control cultures at maximum concentrations of the three drugs achieved in aqueous solution. There was some decrease in the attachment of yeast cells at concentrations as low as 1 ng per ml for clotrimazole and bifonazole. The oral drug, vibunazole had the same action, but at much higher concentrations.

Germ tube growth was reduced in cultures with doses as low as 1 ng per ml of clotrimazole and bifonazole and 0.1 µg per ml of vibunazole. To investigate the action of the drugs on the initiation of germ tube production a time course study could be carried out by fixing the yeast epithelial cell co-culture with and without drugs at fixed intervals between 1 and 2 hours after inoculation of

the yeasts.

It is proposed to study the action of azoles on stage of infection of the monolayers, that of invasion of the epithelial cells by germ tubes and hyphae, by using scanning electron microscopy.

Infecting cells with Chlamydia trachomatis was difficult at first and several methods were used to obtain inclusions. The centrifugation method reported in this thesis was succesful for the two strains of Chlamydia used. It would be interesting to study the other serotypes of Chlamydia which have caused infections in humans. Inclusion bodies in endocervical cells seen with the scanning electron microscope were confirmed by giemsa staining and dark ground microscopy.

The results from the study of C.albicans, C.trachomatis and H.vaginalis and from a study on Trichomonas vaginalis in this cell culture system (Cameron et al.) show the use of our cell culture model system for examining the pathogenicity of microorganisms of the human female genital tract. The model has been further developed to look at the action of antifungal drugs on yeast cell invasion.

Cells from neoplastic lesions of the uterine cervix can be grown by this method of short term monolayer tissue culture. Three methods were used to define the degree of neoplasia by quantitatively assessing loss of contact inhibition. Results showed high standard deviations about the mean for overlap ratios with normal, carcinoma in situ and invasive carcinoma with all three methods used. Observations on culture growth patterns reveal areas of high overlap and areas of "normal" cell growth can occur in the same culture, or the

two growth forms can be seen on different cultures from the same donor. Loss of contact inhibition in some areas was demonstrated by the cells piling on top of each other. These differences in growth pattern suggest the reason for the wide variation in overlap ratios due to sampling errors, both at the time of counting the number of overlapping nuclei by not obtaining a representative sample from both growth patterns; on the other hand it could be a sampling error at the time of taking the original biopsy for tissue culture, in that normal tissue was sampled as well as abnormal tissue.

The study of the action of copper and zinc demonstrated the potential for the use of these cells in preliminary toxicology studies. Substances can be assessed for harmful effects on cell growth and for the production of heat shock (stress) protein.

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SHORT COMMUNICATION

SCANNING ELECTRON MICROSCOPE STUDY OF *CANDIDA ALBICANS* INVASION OF CULTURED HUMAN CERVICAL EPITHELIAL CELLS

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The invasion of monolayer cultures of epithelial cells from the human uterine cervix by clinical isolates of *Candida albicans* was observed. Blastospores settle on the epithelial cells and produce germ tubes within 2 h. Hyphae penetrate the epithelial cell walls and destroy the cells, weaving in and out of the cytoplasm. No phagocytosis of yeast cells by epithelial cells was seen.

Candida albicans may cause cell damage and inflammation by direct hyphal invasion of epithelial tissues. Light and electron microscope views of exfoliated cells and cells in tissue culture from the mouth and vagina have shown the hyphae passing through cell membranes [3, 5, 10]. In some tissues candidal spores can be phagocytosed by macrophages and once inside the cell, the hyphae from the yeast blastospores stretch the cell membrane, eventually bursting through, so destroying the cell from within [V. C. Stanley, 1968; Ph.D. thesis, University of London]. We have examined by scanning electron microscopy the way in which *Candida* invades the intact epithelial surface provided by short-term monolayer cultures of cells from the human uterine cervix.

Minute biopsies of cervical squamous epithelium were taken to the depth of the basement membrane of the cervix of women undergoing gynaecological operations and they were prepared for tissue culture as described previously [1, 2, 4]. After 8 days monolayers containing an average of 5000 cells have grown from the explants. Fifty monolayers from 13 donors were cultured. Ten different isolates of *C. albicans* were obtained from the Bacteriology Department at Queen Charlotte's Maternity Hospital and subcultured once before they were inoculated on the 8th or 9th day of the epithelial cell culture to give 300-600 cells ml⁻¹ in each culture dish. The *C. albicans* was always in the yeast cell stage when inoculated.

The infected cultures were fixed 2-24 h after inoculation, with at least one culture fixed every 0.5 h. For light microscopy, the cultures were fixed in 0.3 M trichloroacetic acid and stained with haematoxylin and eosin. For electron microscopy the cultures were fixed in buffered glutaraldehyde, critical-point dried and sputter-coated with gold. The specimens were then examined with a Cambridge Stereoscan 600 electron microscope at 15 kV.

The *C. albicans* blastospores settled on the epithelial cells and, when placed in an incubator at 37°C, they produced germ tubes within 2 h of inoculation.

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Control samples of *C. albicans* in Eagle's medium alone also produced germ tubes within 2 h, developing to hyphae and forming a matrix.

As hyphae were produced they either started to invade the epithelium, passing through the cell and re-emerging within the same cell or in an adjacent cell (Fig. 1 a), or they grew longer before penetrating the upper surface of the wall (Fig. 1 b). Near the point of invasion, deposits of floccular material were sometimes seen on the surface of the epithelial cell, lying next to the hyphae (Fig. 1 b).

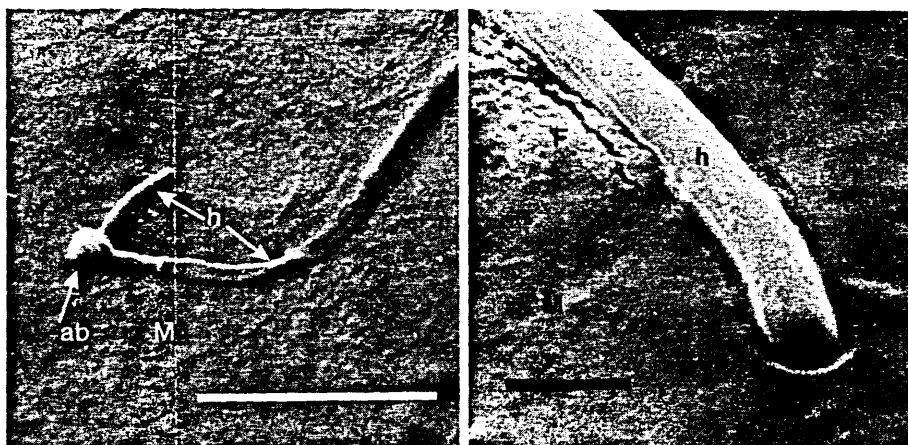


Figure 1 a (left). Hyphae (h) growing out of yeast cell (ab) into cytoplasm of epithelial cell. (M). (Bar marker 20 μ m.)

Figure 1 b (right). Parallel-stranded true hypha (h) of *C. albicans* at point of invasion into the upper surface of the epithelial monolayer (M). Deposit of floccular material (F) on cell surface. (Bar marker 2 μ m.)

After 4 h the hyphal threads could be seen weaving into and out of the cytoplasm (Fig. 2 a) causing wrinkles or folds in the flat surface of the monolayer. Within 6 h of inoculation the hyphae appeared to have cracked open the upper surfaces of some of the epithelial cells (Fig. 2 b), leaving hyphae laced between the two edges of the split. Under light microscopy the damage was such that small lacunae appeared in the monolayer of cells.

There was no evidence of phagocytosis of *C. albicans* by the epithelial cells.

Not only is *C. albicans* the most common of the human fungal commensals, but it is also the most pathogenic, both *in vivo* and in laboratory experiments [9]. One of the factors affecting pathogenicity is attachment of the *Candida* to the epithelial cells. In our cultures, cells that remained in the yeast form did not invade epithelial cells, and their attachment must have been minimal as they were easily washed off the monolayer. The fact that *C. albicans* yeast cells production of germ tubes in Eagle's medium at 37°C is not dependent on the presence of epithelial cells has been described in another electron microscope study [5]. It has been reported that when not epithelial cell associated [6], germ tube production is slower.

The floccular material sometimes seen next to the point of invasion of the epithelial cell has been described in another electron microscope study [5]. It is not known if it comes from the fungus or is a breakdown product of epithelial cell cytoplasm due to the action of phospholipases, which have been isolated from the

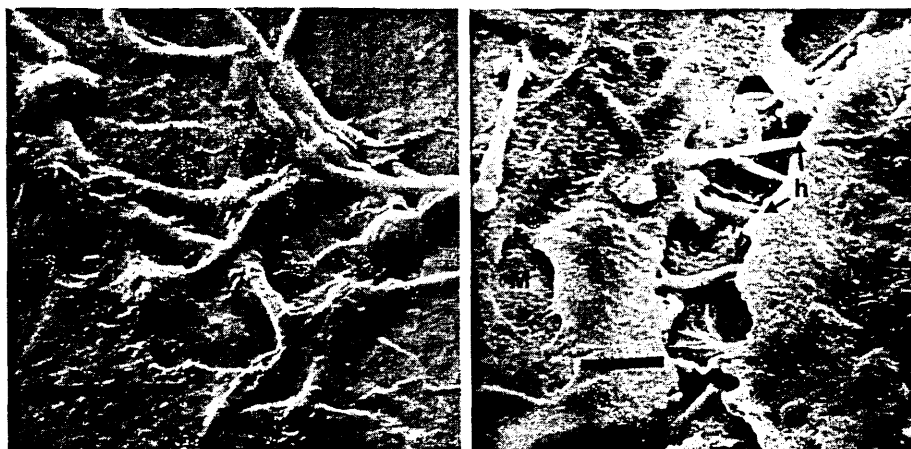


Figure 2 a (left). Apical bud (ab) can be seen partially under the cytoplasm (C) of the epithelial cell. The hyphae (h) are threading into and out of the cytoplasm. (Bar marker 10 μ m.)

Figure 2 b (right). The upper surface of the cells in the monolayer (M) has cracked open to show the lower surface of the cell cytoplasm (C) and branches of hyphae (h) of *C. albicans*. (Bar marker 10 μ m.)

developing hyphal buds and the membranes and walls of non-dividing *C. albicans* cells [7, 8]. The floccular material was not seen on *C. albicans* hyphae that were not on epithelial cells, so it may be that the deposits are epithelial in origin.

RÉSUMÉ

Des cultures monocouches de cellules épithéliales de cols utérins humains sont observées après inoculation par *Candida albicans*. Les blastospores se déposent sur les cellules épithéliales et produisent des tubes germinatifs dans les deux heures. Les hyphes pénètrent à travers la paroi des cellules épithéliales qui sont détruites et s'entremêlent tant dans le cytoplasme qu'à l'extérieur. Les levures ne sont pas phagocytées par les cellules épithéliales.

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