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Vulvar Vestibulitis Syndrome: An Ultrastructural and Epidemiological Investigation

by

Penelope Sargeant BSc (Hons)

A thesis submitted to the University of Plymouth in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

(July 1996)

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Vulvar Vestibulitis Syndrome: An Ultrastructural and Epidemiological Investigation

Penelope Sargeant BSc (Hons)

Abstract

Vulvar Vestibulitis Syndrome (VVS) is a chronic inflammatory condition affecting the vestibular epithelium of the vulva, which has been estimated to affect 15% of the female population (Goetsch, 1991). Many studies have attempted unsuccessfully, to elucidate the cause of this condition, and few advances have been made towards the understanding of the associated inflammatory response. The initial, and principal aim of this investigation was to characterise normal vestibular epithelium using electron microscopy. The ultrastructural characteristics of normal vestibular epithelium were compared with closely related epithelia, and with vestibular epithelia from VVS patients. Other aims included an investigation of the epidemiological characteristics of VVS; an assessment of vulvar sensitivity over several months, and an evaluation of ketoconazole as a non-invasive treatment for VVS.

Transmission electron microscopy, confirmed that vestibular epithelium was non-keratinised, and closely resembled oral and vaginal mucosae. Intermediate cells were predominant, characterised by pale staining cytokeratin filaments and glycogen deposits. Leukocytes were present in small numbers. Using SEM, superficial cells were characterised by an interlacing network of rounded microridges.

By comparison, vestibular epithelium from VVS patients demonstrated the presence of numerous, intensely staining, apoptotic-like cells.These cells were associated with membrane bound cytoplasmic lobules and leukocytes of varying types. A similar ultrastructural appearance was observed in post-treatment biopsies. However, apoptotic-like cells appeared heavily vacuolated, and the number of cytoplasmic bodies present was increased. Mature plasma cells, NK-like cells and macrophages were common in the dermis. Leukocyte counts, demonstrated a significantly greater number of leukocytes in the VVS biopsies compared with the controls, however, there was no statistical difference in the number of leukocytes in pre and post-treatment samples. The presence of apoptotic-like cells accompanied by a significant inflammatory cell infiltrate, may suggest a cell signalling defect, resulting in the pain associated with VVS. Treatment with ketoconazole cream was found to have very little effect on either the number of leukocytes or the frequency of apoptotic-like cells as quantified using image analysis.

The epidemiological characteristics of VVS patients were investigated using a structured questionnaire interview. All of the VVS patients interviewed fulfilled the diagnostic criteria established by Friedrich (1987), and epidemiological findings were generally consistent with previous epidemiological reports. Unique to this study, HPV infections were rare, however recurrent *Candida* infections and cystitis were commonly reported. The 'Vulvar Algesiometer', was designed and developed in Plymouth, to assist diagnosis and assessment of VVS patients. Using this equipment, VVS patients demonstrate heightened vestibular sensitivity when compared with control patients.

The utilisation of a pain measuring device the 'Vulvar Algesiometer', in accordance with the questionnaire and ultrastructural investigation has formed a novel and balanced approach to the study of VVS. This study has demonstrated several distinct features of VVS which have not previously been described, features which may be important in elucidating the cause of this condition. These features centre around the presence of apoptotic-like cells and associated cytoplasmic bodies which have not previously been described in association with VVS.

List of Contents

Figure list

List of Tables

Chapter 7

List of Plates

I dedicate this thesis to my parents, Vivienne and Derek Sargeant for their continual love, support and encouragement over the last 25 years, without which I would not have achieved what I have today.

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Authors Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award. This study was partially funded the Plymouth Post Graduate Medical School, and was carried out in collaboration with Dr.G.D. Morrison, Plymouth Health Trust, Freedom Fields, Plymouth.

A programme of advanced study was undertaken, which included courses in biological electron microscopy, ultrastructural pathology, immunocytochemistry for TEM and SEM, low temperature processing techniques for TEM, and statistics.

A number of scientific seminars and conferences were attended at which work was presented. External institutions visited for consultation purposes include: Addenbrookes Hospital, Cambridge; Dudley Road Hospital, Birmingham; Royal Victoria Hospital, Belfast; Royal Marsden, Cancer Research Centre, Sutton.

List of publications and conference contributions

Courses

- Electron Microscopy in Pathological Diagnosis , RMS course - Queens University, Belfast, 6-10th September, 1993.
- Ultrastructural Immunocytochemistry - Royal Marsden, November, 1994

Papers

P. Sargeant, R.M. Moate, J.E.Harris, and G.D. Morrison,'Ultrastructural study of the epithelium of the normal human vulva', 1996, Journal of Submicroscopic Cytology and Pathology, 28 (2), 161-170.

J.S.H.Curnow, L. Barron, G.D. Morrison and P.Sargeant, 'Vulval Algesiometer', 1996, Medical & Biological Engineering and Computing, 34, 266-268

G.D.Morrison, J.Adams, Parson, P.Sargeant, 'The treatment of VVS using ketoconazole', accepted by the British Journal of Investigative Dermatology, May 1996.

P.Sargeant and G.D. Morrison, 'Vulvar Vestibulitis Syndrome: A review of the current literature' submitted to the Journal of STD & AIDS, July 1996.

P.Sargeant, R.M. Moate, J.E. Harris, G.D. Morrison and C.Sonnex, Ultrastructural characteristics of vestibular epithelium from women with Vulvar Vestibulitis Syndrome', submitted to the Journal of Submicroscopical Cytology and Pathology, August 1996.

Oral Presentation

P.Sargeant, 'Ultrastructural characteristics of vestibular epithelium from women with Vulvar Vestibulitis Syndrome', 2nd biannual meeting of the British Society for the Study of Vulvar Disease, Plymouth, June 1996, oral presentation.

Poster Presentations

P. Sargeant, R.M. Moate and J.E. Harris, ' Ultrastructural characteristics of the epithelium of the normal human vulva', Plymouth Post Graduate Medical School Open Day, 1995, poster presentation.

P.Sargeant, 'Ultrastructural characteristics of the epithelium of the normal human vestibule: A comparison of tissue from women with VVS', Regional GPs Meeting, Exeter, 1995, poster presentation.

P.Sargeant, G.D. Morrison, J.S.H. Curnow, and L.Barron, 'The Vulvar Algesiometer', 3rd International Symposium on Vaginosis / Vaginitis, Funchal, Madiera, January 25th - 29th, 1994, poster presentation.

Curnow, J.S.H., L.Barron, G.D. Morrison, Parsons, and P.Sargeant, 'The vulvar pain stimulator', meeting of the Medical Physics Society, Bath, 1994, poster presentation.

Other conferences attended:-

- The Pathology of the Vulva, Royal College of Obstetricians and Gynaecologists, Cardiff, 1996
- 3rd European Course on Genital HPV Associated Pathology, Stockholm, Sweden, 19th - 22nd June, 1993.
- Immunological Aspects of Cutaneous Warts - Royal Society of Medicine, London, 5th April 1993

Signed .....

Date 25/9/96

Hyperaesthesia of the Vulva

"It consists in an excessive sensibiltiy of the nerves supplying the mucous membrane of some portions of the vulva; sometimes the area of tenderness is confined to the vestibule, at other times to the labia minus, at others to the meatus urinarius; and again a number of these parts may be simultaneously affected. It is a condition of the vulva closely resembling that hyperesthetic state of the remainder of the hymen which constitutes one form of vaginismus....

.....No inflammation affects the tender surface, no pruritus attends the condition, and physical examination reveals nothing except occasional spots of erythematous redness scattered here and there.....The slightest friction excites intolerable pain and nervousness; even a cold and unexpected current of air produces discomfort; and any degree of pressure is absolutely intolerable....

....The treatment I would recommend from my experience is this: to send the patient away from home where, in addition to enjoying change of air, scene, and surroundings, she would live <u>absque marito</u>: to put her upon the use of general tonics, as arsenic, strychnine, quinine, and iron; and having cured any local exalting disease, like vulvitis or urethral vegetations or tumours, to make frequent ablution with warm water and apply sedative and calmative substances in the form of lotions or ointments. As examples of these, I would mention opium or its salts, carbolic acid, chloroform, bellatonin, and idoform..... My observations of the results of caustics and the knife is not such as to inspire me with confidence in them."

Thomas, T.G., 'A Practical Treatise on the Diseases of Women', 5th Edition, 1880.

Abbreviations

D - Darkened cell
Bc - Basal cell
Bm - Basement membrane
C - Connective tissue
Ce - Centriole
F - Cytokeratin filaments
Cf - Cytoplasmic folds
De - Desmosome
G - Glycogen
Ic - Intermediate cell
M - Mitochondria
R - Ribosomes
Su - Superficial cell
Der - Dermis
F - Cytokeratin filaments
Mu - Mucus
V - Vacuole
Lc - Langerhans cell
Mc - Mast cell
He - Hemidesmosome
Pmn - Polymorphonuclear cell
La - Lymphocyte arm
Mr - Microvillous ridge
Mi - Mitotic cell
HPV - Human papillomavirus
OC - Oral contraceptive
HSV - *Herpes simplex* virus
PCR - Polymerase chain reaction
LTFS - Low temperature freeze substitution
AFS - Automated freeze substitution
SEM - Scanning electron microscopy
µm - Micrometer
mV - millivolts

Ap - Apoptotic cell
Le - Leukocyte
Cj - Cytoplasmic junction
No - Nodule
Kg - Keratohyalin granules
Mel - Melanin granules
Sg - Sebaceous gland
Er - Endoplasmic reticulum
Gc - Granular cell
L - Lymphocyte
Mv - Microvillous projections
Sp - Spiny cell
Sq - Squame of keratin
Mcg - Membrane coating granule
N - Nuclear material
Lb - Lobule of cytoplasm
Me - Membrane
Ca - Capillary
Ve - Vesicle
Gr - Granule
Pl - Plasma cell
Ma - Macrophage
Fl - Flattened area
VVS - Vulvar Vestibulitis Syndrome
TEM - Transmission electron microscopy
OTC - Over the counter
LT - Leukotriene
ROS - Reactive oxygen species
ISSVD - International Society for the Study of Vulvar Disease
iu - International units
kV - Kilo volts
M - Molar
^{0}C - degrees Centigrade

Glossary

- **Acantholysis:**loosening of spines, loss of cohesion between cells that leads to the formation of intra epithelial clefts, vesicles or blisters.

- **Atrophy:** a decrease in the overall skin thickness usually due to decreased thickness of the dermis and less commonly of the subcutaneous fat. Atrophy may occur due to the thinning of the collagen bundles due to ageing. Skin appears thin, shiny, whitened, with the loss of surface markings.

- **Dyskeratosis:** premature, and abnormally cornified cells which have eosinophillic cytoplasm and small darkly staining nuclei. Difficult to distinguish from necrotic keratinocytes. Dyskeratotic cells occur in inflammatory and neoplastic processes.

- **Dyspareunia**: difficult or painful coitus in women.

- **Dysplasia**: an abnormality of development in size, shape, and in organisation of adult cells.

- **Erythema**: redness of the skin around caused by congestion of the capillaries in the lower layers of the skin, which may occur with any skin injury, infection or inflammation.

- **Hyperkeratosis**: increased thickness of the cornified layer. Hyperkeratosis may be absolute in that the is an actual increase in the cornified layer, or the change may be relative, an apparent increase in comparison with the spinous cell layer.

- **Hyperplasia:** increased number of cells that results in a thick epidermis. The degree of thickening may vary, there are four main forms of hyperplasia; psoriasiform, irregular, papillated and pseudocarcinomatous

- **Hypertrophy:**increase in size of the spiny cells which results in a thickened epidermis. This may be due to hyperplasia (an increased number of cells) or hypertrophy (an increase in the size of the cells).

- **Hypoplasia:** decreased number of cells which results in a thin epidermis.

- **Koilocytosis**: characterised by cytoplasmic vacuolation and nuclear pynknosis within squamous epithelium.

- **Lichenfication:** a papular thickening of the skin associated with accentuation of the normal skin markings. Usually results from chronic rubbing of the skin.

- **Necrosis:** local death of cells or tissue that occurs within a living organism. Characterised histologically by nuclear fragmentation, nuclear shrinkage, and swollen eosinophilic cytoplasm.

- **Papilloma:** finger-like projections above the skin surface due to upward extensions of the dermal papillae which are usually covered by hyperplastic epidermis.

- **Papillomatosis:** projections of dermal papillae above the skin surface.

- **Papillomaviruses:** any of a group of papoviruses causing papillomata in humans and various other animals. Double stranded DNA viruses that cause epithelial proliferations of skin and mucosa. Human papillomaviruses cause warts, particularly planar and genital warts on the skin and mucous membranes. These viruses may be transmitted by direct and indirect contact.

- **Parakeratosis:** departure from the normal cornification. Hyperkeratosis where in which pyknotic nuclei are retained in cells or squames of the cornified layer. Often occurs in inflammatory processes such as psoriasis, and in necrotic conditions.

- **Priuitus vulvae:** condition associated with chronic itching.

- **Scleroses:** a circumscribed area of hardened skin only detectable by touch. Sclerosis signifies hyalinization of collagen bundles with decreased fibroblasts.

- **Vestibule:** space or cavity at the entrance to another structure. The vulvar vestibule is the space between the labia minora into which the urethra and vagina open.

- **Vestibulitis:** chronic inflammation of the mucous glands of the vulvar vestibule. Persistent vulvar burning and discomfort especially during intercourse are characteristic symptoms.

- **Vulva:** two pairs of skin folds protect the vaginal opening. The larger folds are the labia majora, and the inner smaller folds are the labia minor. The upper or forward ends of the labia join around the clitoris. The opening of the urethra, which empties from the bladder, lies between the clitoris and the vagina.

- **Vulvectomy**: excision of the vulva.

- **Vulvitis**: inflammation of the vulva. Erosive vulvitis may be due to a variety of microbial infections, in this case gangrenous ulcerations similar to the lesions seen in the oral tissues affect the labia majora.

- **Vulvodynia**: chronic vulvar discomfort, especially that characterised by the patients complaint of burning and sometimes rawness, irritation, and stinging. Vulvodynia should be differentiated from pruritus vulvae. Vulvodynia is a symptom which is agreed to have a variety of causes.

- **Vulvovaginitis**: inflammation of the vulva and vagina.

CHAPTER ONE

General Introduction

Vulvar discomfort with dyspareunia was first recognised over a century ago by Skene (1889), and was described a few years later by Thomas and Munde as excessive sensitiveness or hypersensitivity of the vulva (1891). In 1928, Kelly described a reddening of the vestibule which made intercourse difficult, but it was not until 1976 that Pelisse and Hewitt associated the pain experienced during intercourse with erythemous vulvitis. The history and potential causes of these symptoms have been reviewed by many researchers who have given the syndrome a variety of names including, focal vulvitis, burning vulva syndrome, vestibular adenitis, and the minor vestibular gland syndrome (Young, 1984; Peckham *et al.*, 1986; Friedrich, 1987; McKay, 1991; Mc Kay *et al.*, 1989; Marinoff & Turner, 1991, 1992).This distinct clinical condition has received very little attention in the literature in the last 20 years. However, renewed interest in vulvar conditions, and in particular unidentified vulvar pain, has coincided with an apparent increase in prevalence of VVS (Friedrich, 1987; Reid *et al.*, 1988).

Vulvar Vestibulitis Syndrome is a chronic inflammatory condition, which affects the vestibule of the vulva (Friedrich, 1987; Marinoff & Turner, 1991, 1992). The vestibule is that part of the vulva which extends from the hymenal ring outwards, blending with the more keratinised skin of the labia minora, upward to the frenulum of the clitoris and downward to include the posterior fourchette. Embryonically the vestibule represents the only part of the female genitalia which is endodermal in origin, originating from the urogenital sinus of the embryo (Woodruff & Friedrich, 1985; Wilkinson, 1992). The vestibule contains a number of glands termed minor vestibular glands, which are believed to secrete mucus. These glands are small and shallow, are variable in number, being numerous, or completely absent in some individuals. Generally however, 2-10 identifiable glands are present. The epithelium of this

area is not characterised by true mucus secreting glands, but is bathed in mucous from the vagina and cervix. The epithelium of the vestibule has been described as non-keratinised having a similar appearance as mucosal-like surfaces, however, some reports suggest that the whole of the vulva is keratinised (Friedrich, 1983; Woodruff & Friedrich, 1985; Friedrich, 1987; Wilkinson, 1992).

The term VVS was suggested by Friedrich (1987), and was incorporated into the current terminology at the 1987 meeting of the International Society for the Study of Vulvar Disease (ISSVD), to replace inaccurate terms such as focal vulvitis, vestibular adenitis and the burning vulva syndrome (Ridley, 1989). Friedrich, suggested that the term VVS should be applied only to those women who present with the following constellation of symptoms: a) sensitivity to pressure located on the vestibule, b) erythema of the periductal tissue of varying degrees, and c) pain during sexual intercourse or on attempted vaginal entry (Friedrich, 1987). These diagnostic criteria have been widely accepted and are now commonly used for the assessment of patients with unidentified vulvar discomfort. McKay (1985, 1988, 1989), suggested that a distinction between pruritus and the pain associated with VVS should be made. Patients who complain of itching, but do not have a burning sensation should not be diagnosed as having VVS.

The etiology of the syndrome remains unknown. Associations have been made with: recurrent *Candida* infection; hypersensitivity to *Candida* antigens (Marinoff & Turner, 1986); sub-clinical HPV infection, autoimmunity (Ashmann & Ott, 1989); IgA deficiency (Scrimin *et al.*, 1991), allergic and, or irritant reactions to topical therapies (Friedrich, 1987); psychosexual problems (Koblenzer, 1983; Lynch, 1986; Schover *et al.*, 1992, Abramov *et al.*, 1994, Kehoe & Luesley, 1995); and interstitial cystitis or urinogenital sinus syndromes (Fitzpatrick *et al.*,1993; Kozios *et al.*, 1993; Foster *et al.*,1993). Histopathological investigations have been

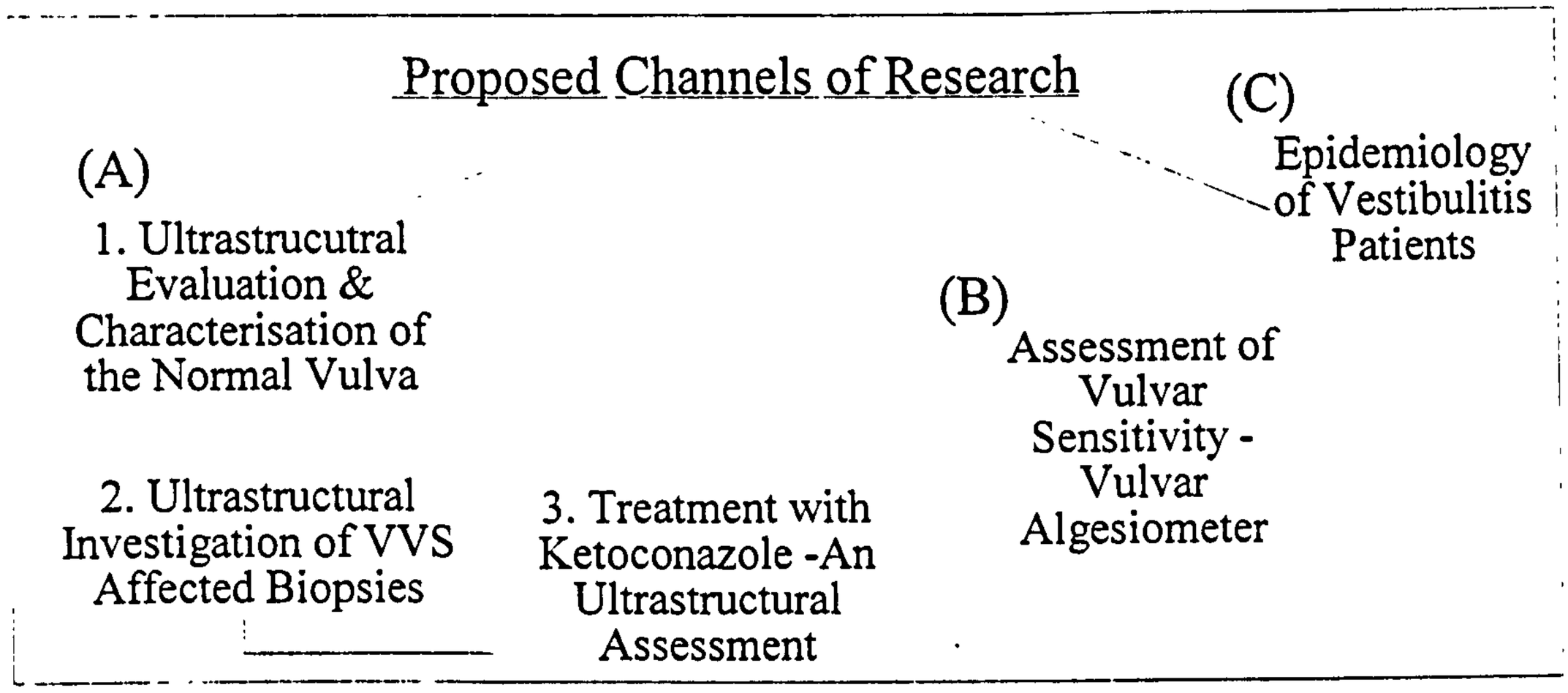
of limited help in elucidating the pathogenic cause of the syndrome. Several authors have completed basic histological examination of biopsies from VVS patients (Turner & Marinoff, 1988; Furlonge *et al.*, 1991), however, the most comprehensive investigation was completed by Pyka *et al.*(1988). A similar investigation was completed recently by Prayson *et al.* (1995), this investigation involved the study of 36 VVS samples from vestibulectomies, using microbiological and immunological tests to characterise the type of inflammatory reaction or to detect the presence of an etiological agent. In common with a majority of work published on VVS, Prayson *et al.* (1995) concentrated on the role of HPV in the development of vestibulitis syndrome. Despite numerous reports of HPV associated VVS very little work has been completed on the histology of VVS since the landmark paper by Pyka *et al.*(1988).

Research has concentrated on treating the symptoms of vulvar vestibulitis syndrome, usually with interferon, rather than investigating the source of the problem (Hatch, 1988, 1991, 1992; Horowitz, 1989; Kent, 1990; Bornstein *et al.*, 1991, 1993; Umpierre *et al.*, 1991; Waltzman & Wade, 1991; Larsen *et al.*, 1993). Associations with HPV were originally founded on the presence of koilocytes in the epithelium of VVS samples, which are considered indicative of HPV infection. Despite only a small percentage of cases being proven to contain HPV using the polymerase chain reaction (PCR), Southern blotting or *in situ* hybridisation, research has continued in this area (Wilkinson *et al.*, 1993; Bergeron *et al.*, 1994). Although interferon has been used for the treatment of VVS in numerous studies, the results are inconsistent (Wilkinson *et al.*, 1993; Bergeron *et al.*, 1994). Surgical removal of the affected area remains the most successful method for the treatment of VVS, however vestibulectomy is invasive and also the most expensive means of treatment (Woodruff & Parmley, 1983; Marinoff *et al.*, 1993; Bornstein *et al.*, 1995). Other treatments for VVS have included: carbon dioxide laser (Davis, 1989), local alcohol injection (Clowser & Friedrich, 1986), steroid and anti-*candida* therapies (Friedrich, 1988), pelvic floor musculature development (Glazer *et al.*, 1995) and

calcium citrate (Solomons *et al.*, 1991).

One of the initial aims of this study was to investigate any possible link between HPV and Vulvar Vestibulitis Syndrome using transmission electron microscopy and associated immuno-gold labelling techniques. However, when reviewing the literature on the VVS it became apparent that a large volume of research had already been completed on HPV associated VVS, and that very little advance had been made towards understanding or even proving this association. In comparison, there was a large area which had not received any attention. The vestibule of the vulva had been neglected histologically for many years, with a majority of work concentrating on cervical and vaginal disease. The normal histology, and the ultrastructural appearance of the vagina and cervix had already been characterised, however, the vulva, and in particular the unique epithelia of the vestibule had been overlooked (Ferenczy & Richart, 1974). The ultrastructural characteristics of normal vulvar tissue were thus considered essential to any investigation of VVS. A detailed investigation of the vestibular epithelium was required in order to establish a baseline for the ultrastructural investigation of VVS. Very little information was available on the normal appearance of this tissue, and consequently, normal skin and keratinised areas of the vulva were the only similar tissues available for comparison. Electron microscopy was chosen to investigate the ultrastructural characteristics of the vestibule, and the pathology of vestibulitis syndrome. The project then followed three main avenues: firstly, an ultrastructural investigation of normal, and VVS affected vestibular epithelium, followed by the assessment of ketoconazole as a non-invasive treatment for VVS (A); secondly, the assessment, diagnosis and monitoring of vulvar sensitivity in VVS patients (B); and finally an investigation of the epidemiological characteristics of the study cohort (C). The channels of research are perhaps best exemplified in Figure 1.0.

Figure 1.0 Proposed channels of research



Studies investigating both the basic histology of VVS, and the presence of HPV using DNA probes often lacked controls. As very little information was available on the normal appearance of the vestibule, an investigation of the normal vestibular tissue using EM was considered essential as a control for the study of VVS affected epithelium. However, obtaining suitable control samples for the ultrastructural investigation proved to be one of the greatest challenges of the project. Samples from cadavers and vulvectomy operations were considered inappropriate controls for the EM investigation, as changes in ultrastructure due to autolysis or tumours would significantly complicate the ultrastructural investigation. Biopsies of vestibular epithelium are not removed routinely during any gynaecological procedure, and therefore obtaining samples from women between the ages of 20-50 years, who had not suffered with vulvar pain proved problematic. After several months, a group of women in this age range, scheduled for gynaecological repair surgery were identified. Ethical committee approval was required, and was obtained in February, 1994.

Initially the Genitourinary Medicine (G.U.M.) clinic at Freedom fields Hospital, Plymouth, was the sole centre for the study of VVS patients. In the years 1990-1993 forty patients had been diagnosed as having VVS, and were now being treated at this centre. Patients already being treated for VVS were not suitable for this study, as it was intended that the ultrastructure of the vestibular epithelium would be investigated pre and post treatment. However, in true scientific fashion, soon after ethical committee approval was received, no new patients became available to participate in the study. Other sources of VVS patients were explored, and Addenbrookes G.U.M. clinic (Cambridge) agreed to provide vestibular samples from women with VVS. Dudley Road Hospital (Birmingham), is one of the only hospitals in the country carrying out vestibulectomies for VVS. Permission was given to use archival material from VVS patients, embedded in paraffin wax for the light microscopy part of the investigation. Contacts made at both Birmingham and Cambridge proved invaluable to the success of the study, both centres providing additional biopsies for the EM investigation.

Biopsies of periductal tissue from VVS patients, were taken from beside the right Skenes' gland and processed for TEM. Vestibulitis patients were then treated for four months with ketoconazole, after which a second biopsy was removed from the lateral border of the left Skenes' gland. The objectives of the ultrastructural investigations were to a) describe the overall structure of the epithelium, b) characterise the different cell types present, c) investigate the inter-relationships of the epidermal and non-epidermal cells present, d) describe differences in ultrastructural appearance in VVS samples, and e) indicate any change in the ultrastructural appearance of VVS affected epithelia after treatment with ketoconazole.

As an addition to the ultrastructural investigation, low temperature (LT) processing for TEM was expected to be completed on several control and VVS samples. Low temperature freeze substitution (LTFS), maintains the antigenicity of the tissues and provides good ultrastructural

preservation. This was essential, as immunocytochemical labelling of VVS tissues was planned if sufficient samples became available. It was expected that differences in ultrastructure would be evident using this LT method of processing, giving a more accurate picture of both the control and the VVS samples. However, due to limited sample numbers, only a small number of control samples were processed using this technique. Scanning electron microscopy (SEM) was completed on several samples of vestibular tissue from control and VVS patients, the surface characteristics were compared with keratinised perineal epithelium, and vaginal epithelium. Finally, it was expected that the results obtained from both the TEM and SEM investigations would be quantified using image analysis. Various parameters would be compared in control, VVS and post treatment samples, as well as differences between vestibular and perineal samples.

Measurement of vulvar sensitivity in VVS patients is notoriously problematic. A number of studies have used a cotton tipped swab to assess the sensitivity of the vestibular epithelium. However, this means of assessment is highly subjective, not reproducible, and does not provide a measurement of the degree of sensitivity. Vulvar discomfort was monitored in VVS patients throughout the study using a pain measuring device, the 'Vulval Algesiometer', which was designed specifically for the investigation. Measurements were taken at monthly intervals from the time of initial diagnosis, and for four months whilst the patients were treated with ketoconazole.

Women with VVS have been studied on several occasions using self administered questionaries. One large investigation of 210 women in the USA has dominated the literature (Goetsch, 1991). Several characteristics of the syndrome were established during this investigation, and have since been confirmed by other authors (Furlonge *et al.*, 1991). The study by Goetsch (1991), although large, involved the assessment of vulvar pain in a group

of women attending a vulvar clinic, and consequently, the proposed incidence of VVS in the population studied may be inaccurate. In the present pilot investigation, epidemiological data was obtained from 19 women with VVS attending the G.U.M. clinic in Plymouth. A similar group of women without VVS were used as controls for the epidemiological investigation. The aim of the epidemiological study was to test several of the hypotheses reported in previous studies (Friedrich, 1987; Goetsch, 1991; Furlonge *et al.*, 1991; Marinoff & Turner, 1992), and to establish whether our study group of VVS patients met the criteria suggested by Friedrich (1987). Areas of interest included; biographical details, gynaecological history, self treatment by patients, treatment by their GP, patients perception and description of pain, and their assessment of the treatment received at the G.U.M. clinic.

CHAPTER TWO

Vulvodynia : A review of the current literature

2.0 The anatomy of the vulva

The female vulva is defined as that area of the genitalia bounded laterally by the genitocrural folds, anteriorly by the mons pubis, and posteriorly by the perineal body (Fig.2.0). The components of the vulva are the labia minora, labia majora, urethra, clitoris, mons pubis, the vestibule, the vaginal orifice and the openings of Bartholins' and Skenes' glands.

The labia majora and labia minora

The labia majora, which are composed of adipose and fibrous tissue, form the lateral extent of the vulva. These fuse with the labia minora where, posteriorly, they flatten and become narrower towards the fourchette, where they are seen to terminate. The labia majora develop as a secondary sexual characteristic to completely cover and protect the labia minora, and are consequently different in appearance. The skin of the labia majora is notably pigmented, giving this area a darker appearance than the remainder of the vulvar epithelium. The structure of the epithelium covering the labia majora is the same stratified, squamous epithelium as that found covering other parts of the body. This epithelium has all of the characteristics of normal skin; the underlying dermis, is characterised by sebaceous, apocrine and sweat glands.

The labia minora consist of flat folds of connective tissue with a small amount of adipose tissue. The labia minora are partially covered with skin, but on the medial aspect the epithelium is mucosal-like. Hart (1893) described this junction of keratinised and mucosal epithelium, now termed Harts' Line; "if the genitals can be looked at carefully, we can trace a line of demarcation between the skin and mucous membrane as running along the base of

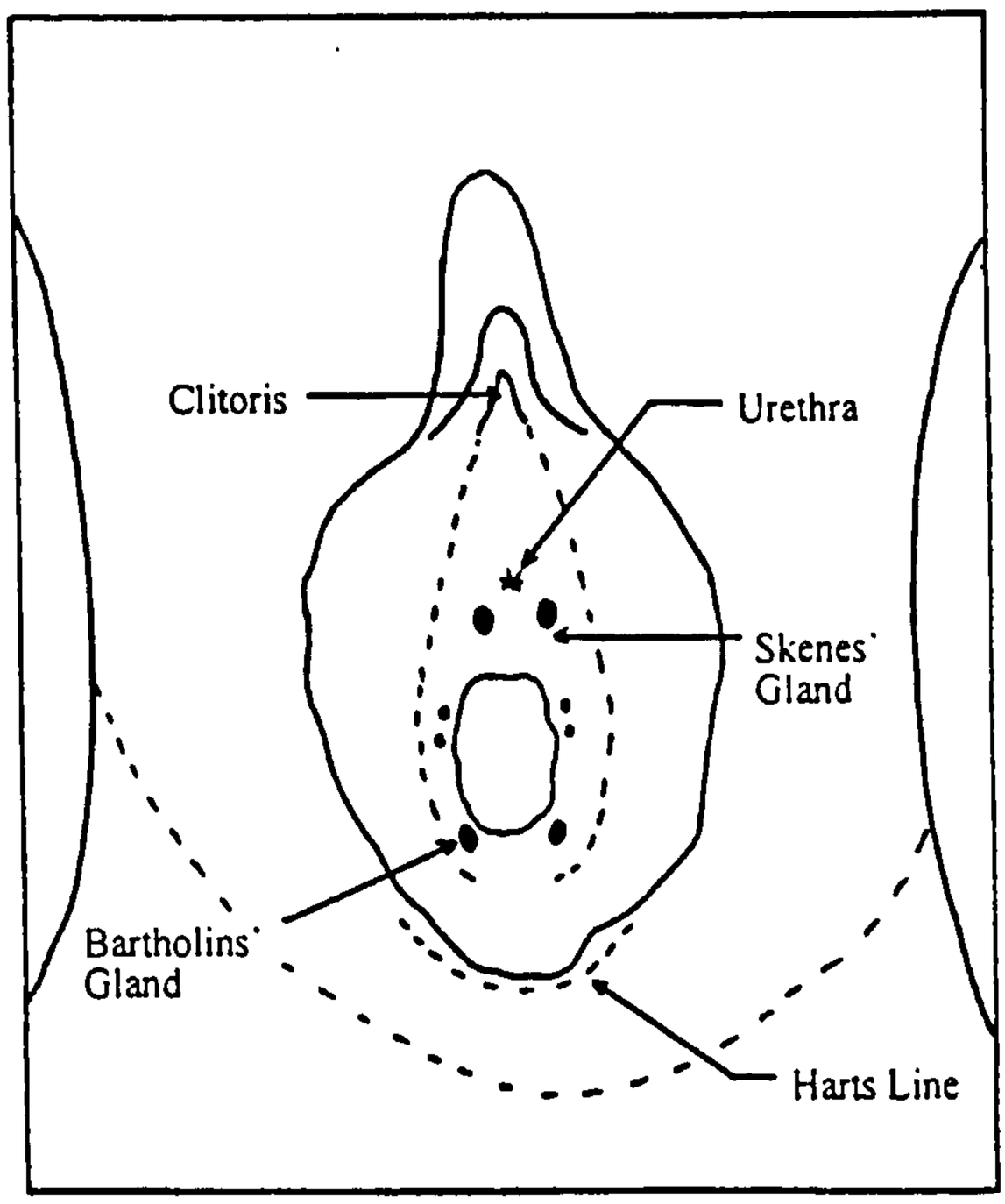
the inner aspect of each labia minora." Anteriorly the labia minora form the prepuce and frenulum of the clitoris, and posteriorly, they blend with the labia majora to form the posterior fourchette (Friedrich, 1983) (Fig.2.0). The cleft between the two labia minora is termed the vestibule, into which, open the vagina, urethra, and vestibular glands. The epithelium of this area is not characterised by mucus secreting glands, but is bathed in mucous from the vagina and cervix. However, the epithelium is mainly non-keratinised having a similar appearance as mucosal-like surfaces. In comparison with the epithelium covering the labia majora, the labia minora is covered by a lightly pigmented, non-keratinised epithelium, with a thin or absent granular cell layer and few of the appendages seen in skin. There are no hair follicles on the medial aspect of the labia minora, but sebaceous glands are abundant. The dermis is highly vascular, with loose connective tissue rich in elastic fibres. Enlargement of the labia minora has been reported as a congenital disease, or as a secondary response to trauma or infection. Post menopausal women frequently show shrinkage of the labia minora due to hormonal changes which affects the storage of fat in the labia, giving a similar appearance to that seen in pre-pubescent girls (Wilkinson, 1992).

The vestibule and vestibular glands

The vestibule, along with the urethra and the trigone of the bladder, represent the remains of the urogenital sinus of the embryo, and is analogous with the penile urethra of the male. The vestibule contains a number of mucous secreting glands termed minor vestibular glands. These glands are small and shallow, are variable in number, being numerous or completely absent in some individuals, however, generally 2-10 identifiable glands are present (Woodruff & Friedrich, 1985). The orifices of these glands, which drain via ducts, are visible as pits in the vestibular surface. The columnar epithelium lining these ducts often undergoes metaplasia, squamous epithelium replacing columnar cells. Metaplasia of the glands often occurs due to a condition termed vulvar vestibulitis syndrome. As a result of this condition, the columnar

cells are largely replaced by squamous cells, however, in the base of the vestibular cleft some columnar cells are visible (Friedrich, 1987).

Figure 2.0 The anatomy of the vulva



Other glands which open onto the vestibular surface of the vulva are Bartholins' and Skenes' glands. Bartholins' glands lie beneath the fascia on each side of the vestibule, these glands are homologous to Cowpers' glands in the male. Lobulate, racemose, glandular structures, these glands contain multiple acini grouped around the termination of each of their many branching ducts. Lining these ducts are cuboidal epithelial cells, which contain mucin to be secreted during sexual activity. The main ducts of Bartholins' glands reach the vestibular surface at 5 and 7 o'clock outside the hymenal ring (Figure 2.0). On each side of the vaginal opening are bulbocavernosus muscles situated beneath the floor of the vestibule. Within these muscles are the bulbi vestibuli which are homologous to the bulbi penis in the male. Bartholins' glands and the associated structures are rarely involved in vulvar disease. The Skenes' ducts or paraurethral ducts open into the urethral canal external to the urethral meatus. Paraurethral

glands are branched tubular structures which empty into paraurethral ducts. These ducts are inconsistent in number and location, they form an extensive network throughout the tissues of the distal urethra, extending close to the bladder. These ducts terminate in tubular glands which are lined mainly with columnar epithelium, and have a limited secretory activity (Huffman, 1948).

Using light microscopy, the vestibule of the vulva has a similar appearance to that of vaginal epithelium, and oral epithelium. At electron microscopy level, vestibular epithelium has features in common with both of these epithelia, but also the epithelia of the cornea and the bladder (Sargeant *et al.*, 1996). Vaginal, bladder, oral and vestibular epithelia store glycogen in intermediate and surface cells, and often appear non-keratinised (Kurman, 1987). The surface of the oral cavity and the esophagus is particularly interesting, and has many features in common with vestibular epithelium (Rhodin, 1974, Landay & Shroeder, 1977, 1979). Oral epithelia may be non keratinised, para-keratinised or fully keratinised; a similar range of keratinisation may occur in the vulva. Moving from the vestibule towards Harts' line the epithelium appears to change from non-keratinised to keratinised epithelium (see Chapter 5). Other similarities with oral epithelium include the presence of pale staining cytokeratin filaments, interdigitating cytoplasmic processes, and leukocytes in all cell layers. Both oral, vaginal and vestibular epithelium are stratified, squamous epithelia, however these mucosal-like epithelial surfaces share some characteristics with other epithelia. The bladder is composed of transitional epithelium, which is a modification of cuboidal type epithelium. In common with bladder epithelium, cells of vestibular epithelium are glycogenated, have few desmosomes and numerous cytoplasmic processes (Richter & Moize, 1963 as cited by Rhodin, 1974). Surface cells of bladder, soft palate, cornea and esophagus are characterised by the presence of microvillous-like processes, which are believed to maintain a layer of mucus or moisture on the surface of these epithelia, and which are evident on surface cells of

vestibular epithelium (Sargeant *et al.*, 1996).

2.1 Non-neoplastic and neoplastic epithelial disorders of the vulva

The earliest reports of 'itching, white lesions' of the vulva were written by Wier in 1875 (as cited by Friedrich, 1985). Since this observation, many authors have concentrated on these lesions which appeared to be pre-cancerous. Progress was hampered for many years by the lack of uniform nomenclature (Table 2.0), until the landmark paper by Jeffcoate and Woodcock in 1961 (as cited by Lawrence, 1993), which suggested the replacement of non-specific terminology. They suggested that "all skin changes for which a specific cause is not clear are best given clinically an all embracing and non committal title such as 'chronic epithelial dystrophy'". Since this major change in terminology, the nomenclature of vulvar disease has been modified extensively, especially over the last twenty years. Current terminology classifies conditions of the vulva into neoplastic and non-neoplastic, a classification which has been widely accepted (Lawrence, 1993; Laverly, 1994).

The incidence of non-neoplastic epithelial disorders (NNEDs) is unknown. Generally, it is believed that disorders of this type are under diagnosed, as the primary characteristic of the condition is either minor or non existent pruritus, which is difficult to demonstrate and to assess. The local environment of the vulvar epithelium effects the appearance of non-neoplastic epithelial disorders. It has been reported that vulvar skin sweats more than any other epithelial surface of the body except the axilla (Lawrence, 1993). The moist, warm environment, frequently results in the modification of lesions so that they are often indistinguishable from the same dermatological lesion on another areas of the body, and similar dermatological lesions on other parts of the vulva. The presence of an infectious agent as well as dermatological vulvar lesions, is a further complicating factor in the accurate diagnosis of vulvar dermatoses, as the infectious agent may further modify the epithelium. It

is these factors which have lead to complicated and often conflicting terminology over the last century. This may now have been resolved due to the introduction of less ambiguous and universal terms by the International Society for the Study of Vulvar Disease (ISSVD).

Table 2.0 History of nomenclature associated with vulvar disease

Author / Date	Description / Nomenclature
Weir 1875	Persistent, pruritic, benign vulvar lesion in association with leukoplakia of the tongue - Greek "leukos" white and "plax" plate.
Breiski 1885	White lesion of the vulva designated as leukoplakia - probably represented lichen sclerosus.
Berkeley & Bonney 1909	Leukoplakia vulvitis added to literature to describe white lesions of the labia, perineum, thigh and perianal regions, thought to be premalignant.
Taussig 1929	Leukoplakic vulvitis described as involving any part of the vulval or perianal skin. Leukoplakia implied a precancerous lesion.
McAdams & Kistner	Leukoplakia used to describe any lesion with histological atypia regardless of gross appearance.
Jeffcoate 1961	General white lesions of the vulva are benign. Excessive use of the term leukoplakia to describe atypia of the vulva.
Jeffcoate & Woodcock 1961	Introduced term 'chronic epithelial dystrophy' to include intractable vulval diseases of unknown etiology previously termed leukoplakia, or leukoplakic vulvitis.
Jeffcoate 1966	Altered terminology to 'chronic vulval dystrophy', included any vulvar skin change of uncertain etiology, including red and white lesions

(Adapted from Lawrence, 1993)

During the next decade, the ISSVD adopted its first classification of vulvar dystrophies using microscopic criteria (Ridley, 1989). The first challenge put to the ISSVD was to form a system of nomenclature for vulvar disease which would be accepted by gynaecologists, doctors, GU physicians, pathologists and dermatologists alike. The term dystrophy was not popular, as it suggested abnormal, or defective nutrition and is commonly used to describe musculoskeletal disorders. Other arguments against the term dystrophy have suggested that the term is non-specific, non-diagnostic and imprecise. Despite this, the term dystrophy is still widely used for the description of vulvovaginal disorders (Friedrich, 1983a, 1985; Carcio, 1992; Mahmud *et al.*, 1992; Laverly, 1994). In 1982, Sanchez and Mihm (as cited by Lawrence, 1993) suggested the term hyperplasia to replace dystrophy; vulvar epithelial hyperplasia would

include categories with and without atypia. Hyperplasia is defined as an abnormal increase in volume of a tissue or organ caused by the formation and growth of new normal cells. Another classification was also included, that of benign dermatoses, which included all dermatoses commonly affecting the vulva. In 1987 the ISSVD suggested that the term squamous cell hyperplasia should only be used for conditions where hyperplasia can not be attributed to particular tissue processes, specific lesions, infections or dermatoses, e.g., lichen planus, psoriasis, candidiasis, or condylomata. The subgroups of squamous atypia have been removed from the non-neoplastic epithelial disorders to be covered by the umbrella term 'vulvar intra epithelial neoplasia' (VIN) (Table 2.1). If non-neoplastic and neoplastic disorders are present together, they should be reported individually (Carcio, 1992; Mahmud *et al.*,1992).

Itch vs. Burn

Vulvar pain may manifest itself in many different ways, and may be associated with infectious agents such as Human Papillomavirus (HPV), *Candida*; or with other factors, for example cosmetic irritants, therapeutic agents, psychological factors, or nerve damage. It is important that itching and burning, two symptoms common in the study of vulvar disease, can be differentiated by both the physician and also by the patient as these symptoms suggest very different causes and therefore require different treatments. It is essential that the patient is encouraged to describe her symptoms fully and accurately, to enable a complete assessment by the physician, who should be prepared to believe the patient. Patients with itching generally show skin changes associated with such conditions, such as excoriation, lichenfication, erythema and oedema. In comparison, the patient with vulvodynia complaining of burning rarely exhibits such changes (McKay, 1985, 1992). The perception of burning and itching often causes confusion, as treatments prescribed for infections which cause itching, may in fact result in contact dermatitis and burning. However, if burning and itching are reported together an infectious agent such as *Candida* may be complicating the diagnosis.

Table 2.1 Non-neoplastic and neoplastic epithelial disorders

Non-neoplastic epithelial disorders of skin and mucosa (ISSVD 1990)
1. Lichen sclerosus
2. Squamous cell hyperplasia (not otherwise specified) e.g., Lichen planus, candidiasis, condylomata
3. Other dermatoses - distinct clinicopathological features or known etiological agent
Vulvar intraepithelial neoplasia (VIN)
1. Squamous - VIN I mild dysplasia formerly mild atypia - VIN II moderate dysplasia; formerly moderate atypia - VIN III severe dysplasia / carcinoma *in situ*; formerly severe atypia
2. Non squamous Padgets disease Melanoma *in situ*

(Adapted from Lawrence, 1993, [ISSVD 1990])

The sensations associated with itching and burning are very similar in their initiation (Herndon, 1975). These sensations are perceived by the same nerve endings, receptors for which are situated in the dermo-epidermal region of the epidermis. It has been suggested that burning and itching sensations are in fact the same entity, one being a continuation of the other as the intensity of the stimulation increases. As itching escalates and scratching occurs, the sensation of itching is often replaced by burning. The nerve fibres for itching may be affected by the phenomenon of sensitisation, which during prolonged stimulation causes random background firing of the nerves which persists when the stimulant is removed (e.g., the infection causing itching). During the itch response, scratching is initiated, which involves larger nerve fibres being stimulated resulting in a negative feedback phenomenon which inhibits the itch fibre stimulus. In contrast during burning there is no scratching, which may be due to a protective mechanism to help prevent any further tissue damage, and to allow damage caused by itching to be repaired (Mc Kay, 1985).

2.2 Vulvodynia

The gynaecological problem of vulvar burning with no documentation of a causative agent has proved a long term problem for gynaecologists, dermatologists and GU physicians. Many clinicians have misdiagnosed such patients as having a psychological problem. It has only been in the last 15 years that research has indicated that many of the reported psychological cases are in fact due to infective agents such as HPV and *Candida* infections (Marinoff &Turner, 1991, 1993; Ashman & Ott, 1989; McKay, 1991; Sobel, 1992, 1993; Sharp, 1993). Vulvodynia is an umbrella term which covers a variety of NNEDs which cause vulvar discomfort, and is defined as a syndrome of unexplained vulvar pain, sexual dysfunction and psychological disability (McKay, 1985, 1988, 1991, 1992). The incidence, or prevalence of the condition is unknown, however it is understood that there are various subsets of vulvodynia, recognition of which is important in the treatment of such conditions (McKay, 1988; Paavonen, 1995a, 1995b). With the exception of *Candida*, *Herpes simplex*, and rarely HPV, disorders associated with vulvodynia are not contagious. It is important the patient be made aware that their condition is treatable in the long term, as often they harbour fears of malignancy and sexually transmitted diseases.

Tovel and Young (1978, as cited by McKay, 1989) first suggested the term vulvodynia, from the Greek "odynia" meaning pain, in replace of the term 'burning vulva syndrome'. Later at the ISSVD meeting in 1983, McKay suggested the same term, vulvodynia, which she suggested was analogous with glossodynia (painful tongue).The ISSVD accepted this terminology, and defined vulvodynia as "chronic vulvar discomfort, especially that characterised by the patients complaint of burning, stinging, rawness or irritation" a condition which should be differentiated from the itching associated with pruritus vulvae (Young , 1984; McKay, 1989; Ridley, 1989). Since the introduction of this term, there have been several reviews on the subject, particularly describing etiological agents, subsets of vulvodynia and

vulvovaginal conditions associated with the development of vulvodynia (McKay, 1985; Lynch., 1986; McKay, 1985, 1988, 1989, 1991, 1992, 1993 and Sonnendecker *et al.*, 1993; Paavonen, 1995a & b; Sonni *et al.*, 1995). As described by McKay (1988), and supported by other authors (Foster *et al.*, 1993; Sharp 1993) there are five main subgroups of vulvodynia; vulvar dermatoses, essential vulvodynia, cyclic vulvodynia, vestibulitis syndrome and HPV associated vulvodynia.

The vulva and vagina are embryonically different, the vestibule of the vulva being endodermal in origin. However, the closeness of these two tissues often results in inflammatory conditions which affect both areas. Vaginitis, caused by infective agents such as *Candida*, *Trichomonas* or *Gardnerella* are therefore important in the study of vulvar disease, as these agents may initiate the onset of symptoms associated with vulvodynia. Redness and oedema, characteristic of vulvodynia usually occur due to inflammation. A reddened vulva with no infection present may suggest the presence of a vaginal infection which is irritating to the vulva. However, patients with vulvodynia characterised by vulvar burning, rarely have any physical findings associated with disease such as exorcisation, scratching or rubbing (McKay, 1989, 1992). This is essentially why the diagnosis of psychological disease is so frequent, especially when physical findings are subtle and dyspareunia is the major problem. It is essential that all possible causes are investigated as often more than one factor may be evident in the development of the condition (Table 2.3).

Remission or exacerbation of symptoms may occur when a treatment for one particular problem affects the onset of another, such as spread of HPV infection, or sensitivity to medication. Interaction of such factors results in a complicated picture of vulvodynia, for example, long term *Candida* or HPV infection may suggest an auto-immune problem with antigen sensitivity causing local inflammation. Thorough diagnosis and patient evaluation are

therefore essential, firstly in the recognition of vulvodynia, which frequently manifests as a multifactorial problem, and secondly in the treatment of patients with this distressing condition.

Table 2.3 Subsets of vulvodynia

Subset of Vulvodynia	Characteristics of Subset
Dermatoses	Acute and linked to skin damage due to excoriation. Irritant or allergic responses may occur.
Lichen sclerosus	Rarely seen associated with vulvodynia. Flattening of rete pegs, chronic inflammatory infiltrate in dermis.
Inflammatory dermatoses	Examples include *Herpes simplex*, lupus erythematosus, bullous dermatoses, and aphthosis.
Periorifical Dermatitis	Over use of potent steroid creams causing secondary dermatitis.
Other Dermatoses	Lichen planus, Lichen simplex, psoriasis, all of which are termed papulosquamous disorders. Thickening of the epidermis occurs in plaques (Maibach & Mathias, 1985)
Infections	
Candida albicans	Not associated with vulvar burning but with itching - over use of antibiotics may result in super *Candida* infection and vulvodynia.
Human Papillomavirus	Clinical or subclinical infections rarely itch or burn. Vestibular papillomatosis may be seen associated with subclinical and clinical infections. Acetowhitening used to detect dysplastic areas.
Herpes simplex virus (HSV)	Cyclic or recurrent infection common. Papules or vesicles erode to form sore patches. Subclinical infection possible.
Essential Vulvodynia	No physical findings associated with disease.
Cyclic Vulvovaginitis	Freedom from symptoms between recurrences. Redness and irritation are common, skin splits easily due to underlying oedema. Often linked to menstrual cycle, some associated with recurrent *Candida* infection.
Vulvar Vestibulitis Syndrome	Chronic and continuous pain elicited when pressure is applied to the vestibule. HPV and *Candida* may be of etiological importance.

2.21 Diagnosis and treatment of vulvodynia

Management, rather than cure of the condition is the goal in the treatment of most dermatological conditions, as very few recurring skin infections are easily corrected. McKay and others, stress the need for a complete patient history before treatment of any kind (Carcio, 1992; McKay, 1992; Sharp, 1993 and Sonnendecker *et al.*, 1993). 'Over the counter' (OTC) preparations, and doctors prescribing treatments 'over the phone' are other complicating factors which should be avoided. OTC preparations frequently contain benzocaine for vulvar itching, however, this is a well known sensitizer which commonly initiates contact dermatitis (McKay, 1992). Often, by the time the patient sees a clinician she probably will already have tried several treatments, which may have altered the original problem, preventing an accurate diagnosis. Bland soothing preparations are often required to allow the inflammation to subside and the vulvar mucosa to heal before more potent therapeutic chemicals are prescribed. Before treatment of any kind, details such as gynaecological history, past surgery, number of children, exposure to potential sensitisers and demographic details should be obtained. An account of pre-existing dermatoses should be taken, and any current treatment regimes clearly defined. This should be followed by a complete and careful physical examination of the vestibule, vulva, introitus and finally the vagina. Diagnostic tests for the exclusion of infections should include a vaginal smear, culture for *Candida*, and acetowhitening to detect dysplastic changes in the vulvar epithelia which may indicate HPV infection. Acetowhitening, describes the reaction of the epithelium to the application of dilute acetic acid to detect thickening of the epithelium. Hyperkeratosis, indicated by whitening of the surface on the application of acetic acid, may be indicative of HPV infection. If itching is the primary complaint, in addition to tests for infectious vaginitis the possibility of dermatoses, general pruritus, carcinoma in situ, and cervicitis should be investigated. If burning is also evident, urinary tract infections, post inflammatory changes and dysesthesia should be eliminated as diagnoses (McKay, 1985, 1989, 1991, 1992; Carcio, 1992; Sharp, 1993).

The nerve threshold determining the pain experienced may be manipulated using analgesics or local anaesthetics, which alter the mediators of the nerve response (McKay, 1985). Other means of relieving the pain associated with vulvodynia involves destruction of the nerve endings by cryo or laser therapy, alcohol injection or vulvectomy (Woodruff & Parmley, 1983; Clowser & Friedrich, 1986; Davis, 1989). Alcohol injection for pruritus was originally carried out by Stone (1926, as cited by Clowser & Friedrich, 1986). Findings reported by Clowser & Friedrich (1986), were variable with patients reporting remission of symptoms for 6 -12 months or more. Other authors have shown similar findings; Wilson (1949), described 49 patients, 35% of which achieved complete recovery, and Woodruff & Thompson (1972), reported similar results on 30 women with anogenital pruritus. Woodruff &Babankia (1979), achieved a 90% recovery rate, but emphasised that careful patient selection was particularly important as burning was not relieved, and the treatment was unsuitable for patients with acute inflammation of the vulva, or diabetic candidiasis. Other drugs with properties useful in the treatment of vulvodynia include tranquillizers and tricyclic anti-depressants, which have pain killing properties, and may alter the patients perception of pain. It is essential that the physician aims to locate the principal cause of the pain rather than treating the symptoms of the condition with topical and systemic drugs, as certain methods of management are more successful for vulvodynia than others (McKay, 1989).

2.3 Dermatoses

Dermatoses may occur on all areas of the body, however, factors such as environmental conditions for example; vaginal discharge and a moist epithelial surface, complicate the diagnosis of vulvar dermatoses. Vulvar dermatoses were originally a subset of a group of conditions termed vulvar dystrophies. There are several recognised types of vulvar dermatoses including lichen sclerosus, squamous cell hyperplasia, psoriasis, lichen planus and lichen simplex (Maibach & Mathias, 1985; Carcio, 1992; McKay ,1992; Lawrence, 1993) (Table 2.4). Visualisation of the epithelium using colposcopy is particularly important as the condition of the epithelium may improve during treatment despite continued itching and burning (McKay, 1989). Several authors have stressed the importance of biopsying affected areas of the vulva, as the moist environment may complicate an otherwise straightforward diagnosis (McKay, 1989; Carcio, 1992; Lawrence, 1993).

Table 2.4 Subsets of Vulvar Dermatoses

Subset of Vulvar Dermatitis	Treatment	Characteristics of Dermatitis
Psoriasis	Topical hydrocortisone or fluorinated steroids.	Common in areas such as knees and elbows. On the vulvar mucosa, moist greyish opaque plaques form which may appear glossy and red in skin folds.
Lichen planus	Topical steroids	5 P's - purplish, pruritic, polygonal, papules and plaques. Generally white patches or erosions on mucous membranes.
Lichen Sclerosus	Topical steroids Testosterone	Sclerotic dermis with thin atrophic epidermis, which may be finely wrinkled or scaly. Blisters or lacerations may develop. Lack of architecture of the vulva due to the labia minora resorbing.
Tinea	Antifungal e.g., clotrimazole	Fungal infection of the skin, many forms, classically scaly eruptions due to dermatophyte infection

(after Carcio, 1992)

Irritant reactions of the vulva are fairly common in comparison with allergic reactions. A response such as itching and burning immediately after application of a topical therapy is consistent with irritant reactions; however the delayed onset of itching and burning, with a longer duration of symptoms suggests a cell mediated allergic reaction. Common irritants include soaps, cosmetics, medications and components of topical therapeutic chemicals such as alcohol or propylene glycol (McKay, 1992).

Imidazole derivatives are often reported by patients to induce irritation. The imidazoles are probably the widest used of all anti-fungals. Miconazole, econazole, tioconazole and isoconazole are commonly reported allergens, however, these are the derivatives which are most frequently prescribed. Reports have suggested that there is an increase in the sensitivity to certain imidazole derivatives, which may be capable of causing contact allergy. Often however, it is the treatment vehicle (base cream) which is causing the allergic reaction and not the prescribed drug. In these cases it is important that alternative vehicles are used, or that fungal infections are treated with clotrimazole or ketoconazole which do not cause contact irritation (Dooms-Goosens *et al.*,1995).

Itching, associated with irritant or contact reactions often causes lichenfication, resulting in a leathery, thickened epithelial surface with prominent skin markings. The term 'lichen' is used to denote several dermatologic conditions seen to affect the vulva e.g., lichen sclerosus, lichen planus and lichen simplex (Lawrence, 1993). Lesions are usually pruritic, characterised by thickened plaques, and often erosions in lichen planus and lichen sclerosus, however, these conditions are all histologically distinct. Topical steroid creams are generally used for the treatment of lichenfication to thin the thickened plaques and to reduce the inflammatory response to break the scratch / itch cycle. Although topical steroids are essential for the treatment of lichenfication their use should be limited as thinning of the skin seen after

prolonged use, results in skin fragility and easy bruising (McKay, 1989).

The use of topical steroids on the vulvar epithelium can result in periorifcal dermatitis. Periorifical dermatitis, common on the face, generally occurs due to overuse of potent steroid creams, resulting in a secondary dermatitis which is easily confused with the original condition. This is a common problem seen with the overuse of clotrimazole, commonly found in topical anti-fungals such as Canestan (Bayer). Due to repeated and prolonged use, a rebound inflammatory reaction, characterised by burning and oedema, may occur which is seen to recur each time the cream is withdrawn (Mc Kay, 1989). The problem is exacerbated by patients continued use of the topical treatment which is in fact causing the dermatitis, in these cases the use of potent steroids should be tapered gradually and not withdrawn hastily (Britz & Maibach, 1979).

2.4 Infectious Agents

Infectious agents have frequently been implicated in the initiation of vulvodynia. Commonly however, vulvodynia is not associated with the presence of an etiological agent, despite this many researchers have studied the relationship between HPV, *Herpes simplex* virus, and *Candida* species with different subsets of vulvodynia. One of the most frequently investigated relationships being that between vulvar vestibulitis syndrome and HPV infection (Hatch, 1991, 1992; Kent, 1991; Kelly *et al.*, 1992; Marks *et al.*, 1995).

2.41 Herpes simplex Associated Vulvodynia

If vulvar discomfort is described as burning, localised or a deep ache, *Herpes simplex* virus (HSV) should be suspected. Generally patients report aching followed by itching and the formation of vesicles or papules which erode to form sore patches. Subclinical HSV causes the patient to complain of episodic discomfort and pain in the groin and buttocks. HSV

infection of this type affecting the lower back and thighs may act as a trigger to pudendal neuralgia (Turner & Marinoff, 1991). Positive cultures are difficult to obtain, the main diagnostic criteria of HSV infection is the pattern of recurrence. Constant or episodic pain lasting for two weeks or less is not likely to be associated with HSV infection (McKay, 1992). It is possible that HSV infection may be confused with cyclic vulvodynia especially if the pain is episodic and, or, linked to the menstrual cycle. However, the pain associated with cyclic vulvodynia is generally more diffuse then the site specific pain described with HSV.

2.42 Papillomatosis - HPV Associated Vulvodynia

The link between HPV and vulvar pain was initiated by the use of anti viral preparations of interferon for the treatment of VVS, a subset of vulvodynia. HPV exists as clinical and subclinical infections, the recognition of subclinical infections has fuelled speculation and research to establish a link between HPV and vulvodynia, and particularly between vestibulitis syndrome and HPV (Boden *et al.*, 1989; Moyal-Baracco, 1990; Potkul *et al.*, 1990; Cechinni *et al.*, 1991; Hatch, 1991; McKay, 1991; Fimiani *et al.*, 1993; Welch *et al.*, 1993; Marks *et al.*, 1995). Although it is generally agreed that HPV is involved in a small sub group of patients with vulvodynia, long term antiviral treatments are generally used only in patients who have shown an improvement with use of these treatments in the past (McKay 1988).

Application of dilute acetic acid to the epithelium is commonly used to detect acetowhitening, considered to be evidence of HPV infection. This technique was used originally to detect dysplastic changes on the surface of the cervix and is consequently of more use in this area than when examining the vulva, where false positive results are common (Brucks & Jacobs, 1992). Although acetowhitening is effective for the detection of dysplastic changes, it has been shown that all acetowhitening is not due to the presence of HPV. DNA hybridisation studies have not linked all cases of acetowhitening to HPV suggesting that a multifactorial

association is probably present (McKay, 1989; McKay *et al.*, 1991). Papillomatosis is characterised by the presence of small cutaneous papillae on the labia minora, vestibule, and posterior area of the introitus. Epithelia affected by papillomatosis may have a mosaic or punctate vascular appearance, with capillaries entering individual papillae which are visible using colposcopy. These papillae have been considered to be a natural variant of the epithelium (Friedrich, 1987; Bergeron *et al.*, 1994; Welch *et al.*, 1993), however, other reports suggest that HPV, particularly subclinical HPV, may be important in the formation of these structures (Reid *et al.*, 1987, 1988). Other authors described patients with unidentified vulvar burning and superficial dyspareunia who also have vulvar papillomatosis (Growden *et al.*, 1985; di Paola and Rueda 1986). A more recent study of 295 women estimated the natural incidence of vulvar papillomatosis to be 1% of the study group (Welch *et al.*,1993). Other women in this investigation who had been diagnosed as having papillomatosis, showed a variety of symptoms; 9 of 18 were asymptomatic, the remaining 9 had intermittent or mild symptoms. Of these 18 patients, 94% showed histological evidence of HPV. Using DNA in-situ hybridization, 39% were shown to be HPV positive, and 72% had a history of genital warts. In summary this study showed a definite link between papillomatosis and HPV infection, contrasting with results from previous investigations which suggested that only 7% of patients with papillomatosis harboured HPV using southern Blot analysis (Moyal-Baracco, 1990). De Deus *et al.* (1995), have also investigated the incidence of papillomatosis, and whether HPV has a role in this condition. Using *in situ* hybridisation and PCR, these authors concluded that papillomatosis of the vestibule is not associated with HPV, and should be considered a 'para-physiologic' formation of the vulvar vestibule. They add, that a diagnosis of HPV should be avoided in the absence of undisputed histological evidence, as a positive report of HPV commonly leads to unnecessary biopsies and deleterious treatment regimes (De Deus et *al.*,1995). There were several possible differences in these studies which may affect the incidence of reported HPV; detection technique, frequency of false positives or negatives,

and the use of controls for the detection rate. These factors can not be discussed further as insufficient details are available.

Dennerstein *et al.* (1994), pose the question of whether HPV vulvitis is a new disease or an unfortunate mistake ? They studied 71 women who had been diagnosed as having HPV, to determine whether HPV was responsible for the symptoms of burning and irritation associated with vulvar pain and pruritus. The results of the study are consistent with the findings of other researchers, in reporting that a diagnosis of HPV is incorrect. Dennerstein *et al.* (1994), suggest that the term HPV vulvitis is not suitable, as it has resulted in often destructive treatments for HPV which are unfounded. In particular the authors were not happy with the use of histology and colposcopy for the diagnosis of HPV infection which are commonly used in pathology departments, which have frequently been reported to give false positive results. They concluded from this study that there was no evidence that HPV caused 'conditions such as vulvodynia, VVS, and pruritus, and that destructive treatments aimed at eradicating the virus should be abandoned and research concentrated on other more likely causes of these difficult clinical problems' (Dennerstein *et al.*, 1994).

Extremely sensitive detection techniques such as Southern Blotting, PCR, and *in situ* hybridization have resulted in a higher detection rate of HPV in specimens than would normally occur using routine histology. This is consistent with the findings of McKay (1988) who suggested that HPV infection of the female genital tract could be much more common than first anticipated. These techniques have demonstrated HPV in tissues subsequent to treatment and after eradication of condylomata. Several researchers have suggested that these detection techniques are too sensitive, and the costs prohibitive, however the use of these techniques for the localisation and typing of virus in suspected vulvar carcinomas is invaluable.

2.43 Dysesthetic or Essential Vulvodynia

The term dysesthetic vulvodynia was originally used to describe a neurological condition which may occur due to damage of the nerve endings. Patients in this category show no physical findings on examination, and complain of continuous burning, with a diffuse pattern localised to the vulva. When treated with low dose, tricyclic anti-depressants, patients with dysesthetic vulvodynia respond in a similar way to patients with glossodynia or neuralgia (McKay, 1985, 1992). Neuralgia is defined as pain in the nerve, or along the course of one or more nerves. Depicted by a sharp spasm like pain which may recur at intervals, neuralgia is caused by inflammation of, or injury to a nerve, or group of nerves. Pudendal neuralgia is typified by vulvar pain radiating from the vulva to the perineum, groin and thighs. This is a much more specific and defined pain than that described by patients suffering with dysesthetic vulvodynia (Turner & Marinoff, 1991).

Mc Kay (1992, 1993), described a series of patients with dysesthetic or 'essential' vulvodynia who did not describe any radiation of the pain from the vulva to surrounding areas, this study also indicated that there was no history of nerve damage in these patients. Mc Kay (1989), demonstrated that this subset of vulvodynia is more common in post menopausal women who may also complain of rectal and urethral discomfort. Turner & Marinoff (1991), have suggested that a majority of patients which are diagnosed as having dysesthetic or essential vulvodynia are actually suffering from pudendal neuralgia, the cause of which may be varied and the symptoms very similar to essential vulvodynia. Patients with pudendal neuralgia 'complain of an unprovoked, persistent, superficial, burning sensation that is frequently accompanied by a deep aching component' (Turner & Marinoff, 1991). Patients which are less severely affected complain of an itch-burn sensation or feelings of rawness. These symptoms may be reported over various areas of the body which are supplied by branches of the pudendal nerve. Patients studied by Turner and Marinoff (1991), reported burning on vaginal

entry and also prior to, and during sexual intercourse, similar findings to those associated with VVS and cyclic vulvodynia. Again, tricyclic anti-depressants are beneficial in the treatment of these patients, particularly in the older subgroup of patients with this disorder. Tricyclic anti-depressants affect cutaneous nerves and are not solely used for treatment of depression. However, women with vulvar pain do often show some signs of depression, and often benefit from treatment with anti-depressants, especially if pudendal neuralgia is suspected to be complicating the diagnosis (Lynch, 1986; McKay, 1989).

2.44 Cyclic Vulvodynia

A diagnosis of cyclic vulvodynia may be made on the report of a) symptom free periods and b) remission of symptoms with long term anti-*candida* therapy. Flaring of symptoms associated with cyclic vulvodynia can often be related to menses or symptom free weeks during the menstrual cycle. Infectious agents such as *Candida*, HPV, *Garderella* and *Trichomonas* should be eliminated as diagnoses, and if present, any infection should be treated. However, eradication of the infection rarely affects the symptoms associated with cyclic vulvodynia. Reports of swelling or episodic erythema of the vulva may be linked with *Candida* vulvitis which presents as itching and burning, and should always be considered a possible causative agent of vulvodynia (Carcio, 1992; Secor, 1992; Foster, 1993). Many patients with vulvodynia report being treated for *Candida* on many occasions. Although patients with cyclic vulvodynia do not often prove to be consistently *Candida* positive, treatment of these patients with long term anti-candidal therapies does often improve symptoms (Sobel, 1986). Treatment regimes have included the use of oral ketoconazole for 1-2 months, the dose being reduced after 2 months; the use of ketoconazole 5 days prior to menses; and the regular use of topical antifungals such as clotrimazole (McKay, 1985, 1989, 1991, 1993). Mc Kay (1989), treated all of her vulvodynia patients with anti-candidal therapy for six months (if they had previously responded to these treatments). A subgroup of these

patients, regardless of whether they were *Candida* positive, showed remission of symptoms whilst treatment was maintained, generally these patients had a history of cyclic vulvitis. McKay (1989), postulates that low dose anti-candidal therapy is more effective in the management of cyclic vulvodynia, as these allow the mucosal surface to recover its natural barrier to infection. In summary, although anti-candidal therapies are effective in the treatment of cyclic vulvitis, there is no evidence as to whether cyclic vulvitis is related directly to *Candida* allergy, subclinical infection, reinfection, or hypersensitivity to *Candida* antigens (Ashman and Ott, 1989; Witkin, 1987; Sobel, 1986, 1992, 1993).

2.45 Vulvar Vestibulitis Syndrome

Sensitivity and pain located within the vestibule of the vulva, have been documented for over a century. However, until the 1980s very little research had been completed to elucidate the cause and the incidence of this problem. Vulvar vestibulitis syndrome (VVS) was probably first recognised over a century ago by Skene (1889), and described a few years later by Thomas and Munde (1891) as excessive sensitiveness or hypersensitivity of the vulva. In 1928, Kelly described a reddening of the vestibule which made intercourse difficult, but it was not until 1976 that Pelisse and Hewitt associated the pain experienced during intercourse with erythemous vulvitis. The history and potential causes of the syndrome have been reviewed by many researchers who have given the syndrome a variety of names including, focal vulvitis, vestibular adenitis, and the minor vestibular gland syndrome (Friedrich, 1987; McKay, 1989; Oates, 1990; Marinoff & Turner, 1991, 1992; van der Meijen *et al.,* 1994). The term "burning vulva syndrome" was introduced at the 1975 meeting of the International Society for the Study of Vulvar Disease, and at the subsequent meeting in 1983 the terms vulvodynia and vulvar vestibulitis syndrome (VVS) were added to the existing terminology referring to vulvar disease.

Since the introduction of new terminology in 1983, VVS has been described on numerous occasions (Peckham *et al.*, 1986; Friedrich, 1987; Furlonge *et al.*, 1991; Goetsch, 1991; Marinoff & Turner, 1991, 1992 ; Secor & Fertitta, 1992; Monif & Belatti, 1993; Bazin *et al.*, 1994). Defined by Friedrich (1987), VVS is characterised by a) severe pain on vestibular touch or at attempted vaginal entry, accompanied by b) sensitivity to pressure localised within the vestibule, and c) erythema of the periductal tissue of varying degrees. This definition of the syndrome encompassing the three main diagnostic criteria is now widely accepted, and used as a means of diagnosing patients presenting with vulvar discomfort.

a) Epidemiology

The incidence of VVS in the female population is not known, however, one report has suggested an incidence of 15% of the gynaecologic population (Goetsch, 1991). It is widely agreed that reports of the syndrome have been increasing over the past 10 years, with an increasing number of women presenting at their GP's practices with vulvar pain characterised by severe entry dyspareunia. It is probable, that a significant number of women suffering with VVS do not consult their GP with problems of this nature. In contrast, other women who do approach their GP for help, frequently receive treatments which are unsuccessful, and alarmingly, these women are often not referred to a GUM clinic. A majority of patients who eventually attend a GUM clinic have seen a number of clinicians, and have tried a myriad of treatments, mainly antifungal, with no significant improvement in their condition. The etiology of this complex and distressing syndrome is yet to be conclusively proven, but it is probable that the etiology will be shown to be multifactorial.

The landmark study by Goetsch (1991) is one of the largest epidemiological studies of VVS, involving 210 women attending a vulvar clinic; however, the proposed incidence of the syndrome is higher than suggested by other studies. In contrast a study by Pinsent *et al.* (1995

personal communication) showed the incidence to be much lower, approximately 6%. The study cohort in this investigation was a group of 49 women attending their GP for well women screening. This difference in the proposed incidence of VVS, may be due to the fact that Goetschs' patients were self selected, and not a true representation of the gynaecological population, resulting in a higher reported incidence than would normally be seen in the female population.

According to Goetsch (1991), the median age for the syndrome was found to be 34 years, with a majority of women falling into the 20-40 age range. Goetsch (1991), found that the average time length of suffering was 10 years; many of the women interviewed admitted that they had reduced sexual activity as a consequence of pain, and several women attributed marriage failures to this problem. Spontaneous remission of the symptoms were found to be very rare except in cases where vestibulitis had developed post partum (Lynch, 1986; Goetsch, 1991; Schover *et al.*, 1992). A majority of patients had noticed some change over time, but the improvement was very gradual usually occurring over several months, and often over several years (Goetsch, 1991). In contrast, a study by Peckham *et al.* (1986), suggested that 50 % of VVS patients would show spontaneous remission of symptoms with time. As suggested by Goetsch (1991), if in fact this was the case, there would be a large number of women recalling past dyspareunia which had spontaneously remitted.

Many of the observations made by Goetsch (1991), are supported by Furlonge *et al.* (1991) and Secor & Fertitta (1994), and have previously been described by Marinoff & Turner (1988), and Friedrich (1987). In addition, Furlonge (1991) found that a majority of patients attending his clinic were Caucasians, mainly in social groups one and two, who were in stable monogamous relationships. Symptoms of vulvodynia had occurred for 6 months or more in 92% of patients, and for 2 years or more in 25% of patients, similar results to those recorded

by Marinoff & Turner (1992). Very few afro-Caribbeans or asians have been found to suffer from the condition, which may indicate a genetic link or a different response to disease (Friedrich, 1987; Goetsch, 1991; Marinoff & Turner, 1991, 1992; Secor & Fertitta, 1992). Friedrich (1987), who studied 87 patients, concluded that the syndrome was unlikely to be related to allergy, menstrual cycle, spinal disorders, *Candida*, HPV, *Herpes simplex*, hygiene products, sexual abuse or depilatory creams. Marinoff & Turner (1986), also demonstrated that patients with VVS seen in their clinic, did not have active infections of gonorrhoea, *Candida*, *Gardnerella*, *Trichomonas* or *Herpes simplex*.

b) Clinical findings

Patients presenting with VVS generally have a history of entry dyspareunia and post-coital pain often resulting in reduced frequency of sexual intercourse. The pain experienced by these patients is not constant but spontaneous, usually elicited by applying pressure, in any form, on the vestibule. Pain is usually described as introital or superficial; deep dyspareunia and tenderness within the vagina are characteristically absent (Monif & Belatti, 1993; Marinoff & Turner, 1991). Other findings may include an intolerance of tampons and tight clothing, and the inability to sit for long periods of time, or to ride bicycles or horses. Colposcopic examination commonly reveals physical findings limited to oedema and small foci of erythema surrounding the vestibular gland openings, accompanied by acute tenderness of the vestibule. These findings are consistent with the guidelines described by Friedrich for the diagnosis of VVS (Friedrich, 1987).

Turner and Marinoff (1988), described VVS as being either acute or chronic. If introital dyspareunia lasts more than three months and becomes the main problem the syndrome can then be considered as being chronic. Borderline vestibulitis (secondary vestibulitis) may be diagnosed if a patient has sensitivity to touch with a swab, but has not reduced sexual activity

or tampon use as a result of the pain. Patients were divided into subsets of primary and secondary VVS by Goetsch (1991). Those having the primary condition had persistent pain from the first attempt at vaginal entry, whereas patients with secondary vestibulitis had previously been able to have sexual intercourse without discomfort (Marinoff & Turner, 1991, 1992). Half of the patients interviewed by Goetsch (1991), had noted pain when first using tampons rather than during sexual activity, suggesting that the cause of the syndrome is probably not sexually transmitted or necessarily linked with sexual intercourse in all cases. This is supported by earlier observations by Peckham *et al.* (1986), who reported that 9% of their patients reported dyspareunia with first sexual intercourse. In accordance with these reports, Bazin *et al.* (1994) report that dyspareunia was evident at first intercourse in 44% of their patients, a much higher incidence than that described by Goetsch (1991) or Peckham *et al.*(1986). However, in contrast to these reports, Bazin *et al.* (1994) add that age at initial intercourse, the number of sexual partners and the use of oral contraceptives are all important factors in the development of VVS.

c) Diagnosis, assessment and counselling of VVS patients

A cotton tipped applicator or chlamydia swab has generally been used for assessing the sensitivity of the vestibule in cases of VVS. Despite the common use of this technique, this means of assessment may now be challenged by the development of a 'Vulval Algesiometer' (see Chapter 3). The cotton tipped swab is used to palpitate the openings of the vestibular glands, to determine whether the patient has vestibular discomfort. In contrast the Vulvar Algesiometer, removes the difficulty of keeping pressure constant during an examination, and also maintaining a similar pressure from one patient to another. The degree of pressure applied can be varied and the patient can indicate when the pressure is uncomfortable, at which point the reading can be recorded (see Chapter 3). A measurement of sensitivity, although subjective, is helpful for both the patient and clinician, mainly for monitoring improvement

or effectiveness of treatment regimes (Curnow *et al.*,1996). Two studies have used a measurement of the degree of dyspareunia for assessment of treatment regimes. Michelwitz *et al.* (1989), asked women to rate themselves on a scale of 1 to 10 (10 representing the most functional level) to compare their sexual activity before and after therapy. Authors agree that a means of measurement is beneficial to both the clinician and patient (Michelwitz *et al.*, 1989; Marinoff & Turner, 1991). Another means of assessment has involved the use of various dilutions of acetic acid, to gauge the sensitivity of the vulva (Sonni *et al.*, 1995).

Before a diagnosis of VVS can be made, the patient should also be tested for the presence of vaginal and vulval infections. If a patient still complains of VVS after treatment of any infection, a full explanation of the problem should be provided by the clinician or health counsellor. Suggestions that the problem is wholly psychological should be eliminated, information should be provided on current treatments available, and the proposed effectiveness and duration of these treatments outlined. Treatment of VVS is notoriously problematic, and usually a long term process, however, if symptoms persist for more than six months surgery should be considered as an option (Woodruff & Parmley, 1983).

d) Pathology

Pyka *et al.* (1988) completed the most comprehensive study on the histopathology of vulvar vestibulitis syndrome. Histologic staining failed to demonstrate fungus, Gram positive bacteria, mycobacterium species, spirochetes or Donovan bodies. This study involved 41 patients who showed a mixed chronic inflammatory infiltrate in the stroma of the vestibular epithelium, findings which have been substantiated in other accounts of VVS (Woodruff & Parmley, 1983; Peckham *et al.,* 1986; Friedrich, 1987; Marinoff & Turner, 1988; Michelwitz, 1989; Furlonge *et al.*, 1991 and Wilkinson *et al.*, 1993; Marks *et al.*, 1995). The predominant cell type was the lymphocyte, accounting for 60-70% of the inflammatory cells. Plasma cells

were found in approximately 75% of cases although they were never the predominant cell type. A small number of granulocytes were found in 25% of cases, but eosinophils were only found in one case. In no case was atypia of the inflammatory cells noted (Pyka *et al.*, 1988). Immunofluorescence showed no particular pattern of immunoglobulin deposition which may be indicative of a specific disease. The intensity of the inflammatory response was graded in each case from mild to severe. Consistent with other investigations, most patients (54%) had experienced a moderate response, and nearly all (83%) fell into the mild to moderate category, with only 17% having a severe inflammatory response (Friedrich, 1987; Michelwitz *et al.*, 1989; Furlonge *et al.*, 1991; Goetsch, 1991). The infiltrate was diffusely distributed in the superficial stroma in all cases, the deep stroma was involved only when very severe inflammation had occurred. Minor vestibular glands, when present, were always associated with a periglandular distribution of inflammatory cells; when metaplasia of the glands was evident, inflammation surrounding vestibular clefts was universally present. However, inflammatory cells were never seen to invade the gland lumens, resulting in adenitis; neither did the inflammation involve nerves or blood vessels (Pyka *et al.*, 1988).

e) Etiology of Vulvar Vestibulitis Syndrome

The etiology of this syndrome is uncertain; the initiating event may be infection or trauma, however, what causes the maintenance of the inflammatory response has not yet been ascertained. An infective etiology seems probable as the physical and pathological findings suggest an inflammatory process. A majority of the work completed on VVS includes routine tests for infectious agents, usually with negative results. It is possible that the specialised epithelia of the vulvar vestibule and the minor vestibular glands may make this area particularly susceptible to infections or morphologic changes which may influence the development of vulvar vestibulitis.

Vulvar vestibulitis may be defined as an acute or chronic condition. The separation of cases into one of these two groups has been shown to be particularly helpful when choosing a suitable treatment regime (Marinoff & Turner, 1986, 1991, 1992; Secor, 1992). Any infection which causes vulvovaginitis may result in the symptoms associated with vulvodynia and vulvar vestibulitis, for example *Trichomonas*, bacterial vaginosis, and *Candida* infections. Irritants such as soaps, douches and sprays may also have an etiological importance, especially if over cleansing with these products occurs. The use of antiseptics and suppositories are also possible causes of VVS, as are destructive therapeutic agents such as short term 5-fluorouracil therapy for the treatment of condylomata acuminata, cryotherapy, carbon dioxide lasers and treatment vehicles (base cream) (Marinoff *et al.*, 1993; Marinoff & Turner, 1992). Causes of chronic vestibulitis include a variety of infections which are the same as those described under acute etiologies (as above). However, chronic infections are characterised by recurrence and persistence of the etiological agent(s), for example recurrent bacterial vaginosis, HPV and chronic candidiasis (Table 2.5)

Table 2.5 Etiological Agents Associated with VVS.

Acute Etiologies	**Symptoms & Association with VVS**
Recurrent Candidiasis	Four documented episodes of infection in 12 months. Associated with vaginal soreness and dyspareunia. Hypersensitivity to persistent *Candida* infection may result in VVS (Marinoff & Turner, 1991)
Recurrent Bacterial Vaginosis (BV)	Alkaline vaginal discharge produced due to BV infection is irritating to the vestibular epithelia, which may be sufficient to cause VVS. Symptoms often resolve when infection is treated and discharge is removed.
Persistent HPV	Several studies have indicated an association of VVS and HPV, which may manifest in different forms of the infection. Not conclusive evidence as presence of koilocytes is not always indicative of HPV infection.

Mann *et al.* (1992), have shown 80% of their 103 VVS patients to have a history of recurrent candidiasis. Other studies have shown this incidence to be slightly lower at 50% (Lynch, 1986), 67% (Peckham *et al.,* 1986), and 63% (Friedrich, 1988). Although some studies have shown a correlation between past *Candida* infection and VVS, few patients present with acute vestibulitis associated with *Candida* (Bazin *et al.*, 1994). The correlation between past candidal infection and VVS is particularly interesting, however, the mechanism of involvement remains unknown. Ashman and Ott, suggested that some VVS patients who had suffered with candidiasis in the past may have developed an autoimmune reaction to the organism due to cross reactivity of the candidal and tissue antigens (Ashman & Ott, 1989). Others have reported hypersensitivity to *Candida* due to re-infection (Marinoff & Turner, 1986), or antichromonal (cross immunity) as an important factor in the development of VVS (Krvavac, 1992). However, bacterial, fungal and viral infections are relatively rare in VVS patients, and idiopathic causes form the largest group, with a majority of patients falling into this category, showing neither an infection nor an allergic reaction to a particular treatment.

As described previously, HPV has been well documented as an associated factor in vulvodynia and particularly VVS. The first study linking HPV with vestibulitis was carried out by Turner and Marinoff (1988). In this investigation, all of the seven women studied with VVS were shown by Southern blot and *in situ* hybridization analysis to harbour HPV (Turner & Marinoff, 1988). Since this initial investigation, the presence of this ubiquitous virus has been identified and typed in VVS samples by routine histological examination, *in situ* hybridisation, Southern Blotting, and PCR. One of the reasons behind this search for HPV includes the suggestion that, the inflammatory response in the form of a lymphohistiocytic infiltrate around the superficial capillaries, might be the hosts first attempts at eliminating the virus (Turner & Marinoff, 1988). Many of the studies investigating the incidence of HPV associated with VVS, have indicated the presence of the virus using routine histological

examination. Generally, it is considered that there is an over diagnosis of HPV based solely on histological findings (Goetsch, 1991; Bergeron *et al.*, 1992; Schover *et al.*, 1992; Dennerstein *et al.*, 1994; Prayson *et al.*, 1995). Although histological examination is essential for assessing the inflammatory infiltrate and for eliminating other diagnoses such as VIN, it is difficult to identify the presence of koilocytes, using this method. It is probable that many studies describing koilocytes in vestibular samples, are actually describing the presence of glycogenated superficial cells which have a similar appearance to koilocytes, and are commonly seen in the superficial layers of vulvar epithelium (Sargeant *et al.*, 1996). Using DNA hybridization techniques, many specimens identified as being koilocyte positive using histology, have proven to be HPV negative (Dennerstein *et al.*, 1994; Wilkinson *et al.*, 1993; Prayson *et al.*, 1995). These findings echo those from an earlier investigation by Turner & Marinoff (1988), who have recently suggested that one reason for this could be the presence of unidentified HPV types in the tissues which were not hybridizing to the DNA probes. Bergeron *et al.* (1994) showed koilocytes in 9% of their VVS specimens, a figure which was comparable to negative controls; this study did not isolate HPV DNA in any of the specimens showing koilocytes.

In addition, and contrary to the findings of Turner and Marinoff (1988), Goetsch (1991), observed that a majority of patients with VVS had no known association with HPV. However, patients who had experienced a HPV infection, were found to suffer vestibulitis as a secondary rather than primary condition. Three studies using PCR have also indicated a low incidence of HPV in patients with VVS, estimated to be in the range of approximately 5-10%. (Bergeron *et al.*, 1994; Wilkinson *et al.*, 1993; Bazin *et al.*, 1994) These studies support the findings that interferon, an anti-viral treatment, is rarely effective in the treatment of VVS. There is agreement that HPV is probably not the primary cause of VVS. However, there may be a small sub-group of VVS patients which do harbour HPV, and it is this group which

respond well to anti-viral therapies (Bergeron *et al.*, 1992, 1994; Wilkinson *et al.*, 1993; Bazin *et al.*, 1994; Prayson *et al.*, 1995).

Other conditions which may be of etiologic importance in the initiation of VVS include estrogen deficient states, severe cervicitis, IgA deficiency, reduced numbers of lactobacilli, inflammatory bowel disease e.g., Crohns disease and interstitial cystitis (McCormack, 1990; Scrimin *et al.*, 1991; Fitzpatrick *et al.*, 1993; Foster *et al.*, 1993). Bazin *et al.* (1994), have shown that hormonal factors such as the early use of oral contraceptives may be a contributing factor to VVS. However, the intricacies of these proposed relationships have yet to be determined.

f) Treatment & Management of Vestibulitis

Thomas (1880), in his Diseases of Women, described three women; one was treated with nitric acid, and two had removal of the affected mucous membrane, but all three showed no improvement. He suggested a holiday away from the patients spouse, with general tonics. Todays patient does not fare much better, as in most cases the etiology of VVS is still unknown and therefore the treatment of this condition still problematic. Treatment guidelines for VVS patients are very vague, and as yet no treatment has been firmly established, and no series of results has shown uniformly positive results in all patients treated. In an attempt to alleviate suffering the clinician often embarks on a shotgun-type of approach which often involves the use of a mixture of therapeutic regimes which may or may not be helpful in the relief of symptoms. Often, by using this type of approach the well meaning clinician may cause irritant or allergic reactions which subsequently exacerbate the patients condition. As described previously there are two categories of VVS, chronic and acute, although the duration of the symptoms does not seem immediately important, this factor has been suggested to be useful in making therapeutic decisions (Marinoff &Turner, 1986, 1991, 1992;

Secor & Fertitta, 1992).

In the case of acute etiologies, any specific infection should receive pharmacologic treatment. All therapeutic agents which may contribute to the syndrome should be discontinued. As the vestibule is endodermal in origin it is generally less responsive to sex steroid treatment than the remainder of the surrounding area consequently very little success has been achieved with the use of some steroids (Friedrich, 1988; Peckham *et al.*, 1986; Reid *et al.*, 1988; Marinoff & Turner, 1991; McKay, 1992). The rapid relief from symptoms, often seen in acute cases when the presumed cause is treated, suggests the presence of an etiological relationship. A more complicated treatment approach is required in the chronic cases of VVS. Some success has been achieved where it has been possible to eradicate the causative agent. Frequently however, treatment of the causative agent does not result in remission of the symptoms associated with vulvar vestibulitis syndrome. Generally, in the chronic form, only an association rather than a direct cause and effect relationship can be implied. Many of the causes will be identical for both groups, acute VVS becoming chronic, when the cause becomes persistent or recurrent. Perinoplasty remains a final option for women with chronic VVS of six months or more in duration, who have not responded well to more conservative measures. Generally surgery should contemplated only when all conservative therapies have failed (Woodruff & Parmley, 1983; Mann *et al.*, 1992; Bornstein *et al.*, 1995).

Conservative therapies for VVS

Symptomatic therapy usually consists of the application of topical anaesthetics such as Xylocaine (5%) ointment 15 to 30 minutes prior to intercourse. Often the use of some protective coating such as petroleum jelly or zinc oxide are useful to minimise the contact of any irritating discharges, and add lubrication during intercourse (Marinoff & Turner, 1986; 1992).

Friedrich (1988), completed an investigation of several therapeutic regimes which he used for the treatment of VVS, none of which proved particularly successful. Seven women were treated with isotretinoin, using doses of between 20 and 60mg/day depending on weight. One woman had lasting remission three months after ceasing treatment, however, only three women were able to use the drug without serous side effects. Eighteen women were treated with Dapsone, commencing at 50mg/day and increasing to 75mg/day if there was no bone marrow depression demonstrated. Three women showed subjective and objective improvement but only one was permanently cured. Fifteen women were treated with Acyclovir, commencing 600mg/day; in five patients, the dose was increased to 1600mg/day. Seven patients showed a subjective improvement and in three of these patients there was less intense vestibular tenderness, however, only two of these women have shown long term benefit. No correlation between HSV titres and the response to treatment could be shown. Eleven women were treated with progesterone cream twice daily; four women were made worse, and none of the women became better. Eight patients were treated with 0.025% Capsaicin cream 4 hourly. This caused severe burning in the initial two weeks of treatment requiring oral analgesia. After four weeks two patients reported complete relief of symptoms but had to continue to use the treatment (Friedrich, 1988).

Glazer *et al.* (1995), treated the symptoms of VVS by assessment and improvement of the pelvic floor musculature. In this study 33 women suffering with VVS were treated, the pelvic floor musculature was assessed initially and patients were shown how to assess themselves at home using a portable system. Biofeedback-assisted pelvic floor exercises were completed daily by the patients, and regular evaluations by the consultant were carried out in order to monitor progress and ensure correct use of the equipment. After 16 weeks of treatment, pelvic floor musculature had improved and reports of pain had decreased in 83% of patients, 22 of 28 of the patients treated resumed sexual relationships soon after treatment. After six months,

patients were found to have maintained therapeutic benefits and were commonly free from pain. This study is the first to assess pelvic floor muscle status in relation to VVS and the severity of vulvar pain. Unlike other studies, which have concentrated mainly on invasive treatment with interferon injections, this study has involved the use of a non-invasive treatment which has demonstrated some extremely interesting and promising results.

Reid *et al.* (1988) assessed patients with VVS by measuring the degree of acetowhitening on application of acetic acid. As described previously, the use of acetic acid for the detection of HPV is not suitable, or reliable as other epithelial conditions may result in acetowhite changes. Despite this however, Reid and co-workers treated patients with vulvar pain, who demonstrated acetowhitening of the vestibule, with 5% trichloroacetic acid, carbon dioxide laser therapy, 5% 5-flurouracil and surgery, depending on the severity of symptoms (Reid *et al.*,1987, 1988). The results of the investigation were described as 'universally disappointing'. Patients with painful vestibular erythema were described as 'substantially harder to treat', and medical and surgical regimes were generally unsuccessful. This study highlighted the problems associated with diagnosing patients by assessing the degree of acetowhitening. The use of lasers for vulvar conditions was proven to be unsuccessful. This study also demonstrated that micro-papillations of the vulva are not always due to HPV and should not be treated aggressively. However, the authors' use of 5-fluorouracil later in the investigation did successfully control the symptoms in 16 of 24 women with VVS.

The recurrence rate of vestibular pain in patients treated with lasers is high, and the recovery period long (Kaufmann & Friedrich, 1989). Reid *et al.* (1987, 1988), used carbon dioxide laser photo vaporization which they observed to cause exacerbation of the symptoms of VVS, often causing inflammation in previously normal vestibular glands. Davis (1989), demonstrated satisfactory results using carbon dioxide laser therapy, In this investigation 63%

of his patients experienced pain relief and resumption of sexual intercourse. In conclusion, some authors have suggested that laser therapy should no longer be offered as a option for treatment of VVS as the possibility of tissue damage far outweighs any benefits from this type of treatment (Mann *et al.,* 1992; Marinoff & Turner, 1992,1993).

Interferon

Until 1990, the only reliable form of treatment for long term VVS was surgery, a procedure termed perinoplasty, which was proposed by Woodruff & Parmley in 1983. Approval of the Food & Drug Administration (FDA) to use recombinant alpha interferon for the treatment of overt clinical warts (Waltz man & Wade, 1991) rapidly lead to the use of this compound for the treatment of VVS, which was proposed to be linked to HPV infection in some cases of VVS. Treatment of VVS with interferon has since received considerable attention, however, earlier reports generally showed more success than later studies (Horowitz, 1987; Hatch, 1988; Umpierre *et al.*, 1991; Mann *et al.,* 1992).

Interferons were first identified as having anti-viral properties when it was found that minute amounts of alpha interferon are produced in a variety of cells when they are infected by a virus. The way in which interferons work is unclear, but it is thought that interferon binds to the membrane receptors on the cell surface where it induces enzyme formation, in turn preventing viral replication in virus infected cells. Additionally the phagocytic nature of macrophages is thought to be enhanced, and the cytotoxicity of lymphocytes for target cells augmented by interferon. Kent and Wisniewski (1990), found that a lag period occurred between intralesional injection of alpha interferon and the cellular response. Although patients experienced a lapse period of 14-18 days before their condition began to show any improvement, the success rate of the treatment was found to be greater than 60% with only minimal side effects. The aim of the study carried out by Waltzman & Wade (1991), was to

evaluate interferon treatment in five patients suffering from VVS and subclinical HPV infection, of which none gave a history of overt genital warts, or of having sexual partners with genital warts. The degree of acetowhitening indicative of HPV infection, was estimated using a dermogram. After four weeks the size of the aceto-white area was compared to the pre-treatment estimate. The results showed that three out of the five patients had experienced an improvement of 50% or greater. The presence of aceto-whitening is not a definite association with HPV infection, but is seen in many hyperkeratotic states. Ideally, an alternative means of detecting HPV infection in such cases should be used in parallel with the acetic acid test. The use of a dermogram for the measurement of aceto white areas is also fraught with difficulties, as during examination and measurement of the affected area the skin is prone to stretching which will give a false representation of the size of the aceto-white area.

Waltzman and Wade (1991) used 5 million international units (iu) of interferon alpha 2β injected intradermally weekly for 3 weeks. Previously, Hatch (1988, 1991) and Horowitz (1989), had used a similar technique to that described by Waltzman and Wade (1991), and they also indicated that the response was favourable. Horowitz (1989), used alpha 2β interferon at a concentration of 1 million iu injected intradermally three times weekly for 4 weeks. Fifteen out of seventeen patients treated showed complete symptomatic relief, and only five out of fifteen patients complained of flu like symptoms during the treatment.

Hatch (1988), and later Marinoff & Turner (1991), also used alpha 2β for the treatment of vestibulitis patients, which they found to be cheaper and more effective than surgery. They concluded that interferon treatment seems to be more effective in patents with a short history of symptoms. Horowitz (1989), did not indicate the length of history of the symptoms in his patients, but did say that there was an absence of dyspareunia until shortly before visiting the gynaecologist. In agreement with these findings, Waltzman and Wade (1991), found that the

two patients who did not show any relief of the symptoms after treatment, were in fact long sufferers of the condition. One patient had undergone many treatments for VVS including laser removal of the surface layers of the vestibule, and the second had shown symptoms of the syndrome for 4 years (Waltzman & Wade, 1991). Kent and Wisniewski (1990), also observed aceto-whitening in 8 patients, all of whom were shown to fulfil the criteria characteristic of VVS. Using the same dose and treatment schedule as Horowitz (1989), the results of this study were similar, and were consistent with the findings of Waltzman and Wade (1991). Larsen *et al.* (1993) completed a small study of 10 women with chronic vulvodynia. Again, as in the case of Kent & Wisniewski (1990), application of acetic acid demonstrated acetowhitening in all patients. Assessment of acetowhitening and histological examination for koilocytes was completed prior to treatment, and after 1 and 3 months of treatment. This is the only investigation which has reported histological examination of vestibular biopsies subsequent to treatment of any kind. This study involved the intra-lesional injection of recombinant alpha 2β interferon, three times a week for eight weeks. All patients showed complete (70%) or partial response (30%), however, there was no reported change in acetowhitening or the histological appearance of koilocytes post treatment. Again, it seems relevant to indicate that acetowhitening is not specific for viral infection, and the presence of koilocyte like cells may be a natural variant of the vestibular epithelium. It is likely that both acetowhitening and koilocytosis are not related to VVS but that interferon is affecting the epithelium in such a way that the symptoms of the syndrome are removed.

The effectiveness of interferon therapy suggests that in some cases VVS has been caused by a subclinical infection by HPV which is eliminated by interferon in some patients. However not all VVS patients were found to benefit, again suggesting that several aetiologies may be involved (Table 2.6) The rate of detection of HPV and effectiveness of treatment in cases of VVS seems to be dependant upon the method of detection. Studies using only histology for

the identification of HPV in tissue samples generally showed lower rates of success than those using DNA probes or PCR. However, these studies are difficult to compare as there are many variables which should be considered such as; inclusion of controls, sample size, selection of patients, assessment of dyspareunia, type of interferon, method of injection, the placebo effect and the incidence of spontaneous remissions. Despite these factors, it is possible to conclude that if sensitive detection techniques are used to select VVS patients with evidence of HPV, the chances of success using interferon are greatly increased.

Table 2.6 Investigations of diagnostic criteria, followed by treatment using interferon

Author	N° of Patients	Evaluation	Result*
Hatch 1988	22	Colp, Hist, DNA	72%
Horowitz 1989	17	Colp, Hist	88%
Umpierre 1992	13	DNA, PCR	38%
Larsen *et al.*, 1993	10	Colp, Hist	70%
Marinoff *et al.*1993	55	Hist	49%
Bornstein *et al.*, 1993	7	DNA	71%
Kent & Wisniewski 1990	8	Hist	62%
Waltz man & Wade 1991	5	Hist	60%
Mann *et al.*, 1992	13	Hist	50%

* Degree of improvement obtained
DNA = In situ hybridization with DNA probes for HPV, Colp.=colposcopy, PCR = polymerase chain reaction, Hist = General histologic examination

Surgical Intervention

Surgery has been used by several authors as the last means of treatment of VVS, very rarely is the vestibule removed unless the patient has suffered from extremely intense dyspareunia for more than 6 months, and, or, would like to be sexually functional and has been treated initially using non invasive methods. Woodruff *et al.* (1981), removed painful foci by local excision, but later Woodruff & Parmley (1983), described a more stylised operation which they termed perinoplasty. In this operation, a 'Y' shaped strip of tissue is excised beginning

just below the Skenes' ducts , and including the lower 1-2cm of the labia minora, extending to just above the anal orifice. Vaginal mucosa is undermined and advanced to the perineum where it is sutured with Vicryl. The rate of success in Woodruff & Parmleys' series was major asymptomatic relief in all of the fourteen patients, although two of the patients still had some tenderness near the Skenes' ducts (Woodruff & Parmely, 1983). Since the introduction of this operative technique, a success rate of 80% was achieved in 64 patients between 1982 and 1984 (Woodruff & Parmely 1983). Peckham *et al.* (1986) described a series of eight patients who had undergone surgery. On follow up, seven were pain free and sexually active, but the authors make the point that post-operative pain disability can last for up to five months. Marinoff & Turner (1993) reviewed their series of patients treated using this operation, and reported that 71 of 73 patients experienced partial or complete relief of symptoms after an average follow up of three years. Others have achieved success in between 60% and 80% of VVS patients (Friedrich, 1987; Bornstein *et al.*, 1995). In addition Michelwitz *et al.* (1989) and Marinoff & Turner have modified the operation further to include part of the periurethral area, resulting in increased success of this procedure (Marinoff & Turner, 1992). Barbero *et al.* (1994), describe membranous hypertrophy of the posterior fourchette as a cause of dyspareunia and vulvodynia. Twenty-one patients, who had erythema and point tenderness of the vestibule characteristic of VVS, were treated surgically for this condition, using a similar technique as reported by Woodruff and Parmely (1983), but removing just the posterior part of the vestibule. Following excision of the posterior part of the vestibule and vaginal advancement, 19 patients showed elimination of symptoms, and the remaining 2 patients were much improved.

Mann *et al.* (1992), evaluated 56 patients six months after perinoplasty using a questionnaire to assess vulvar pain and dyspareunia separately. Twenty nine percent reported that they were pain free, many patients said they were much improved (37%), and that they were not

suffering any dyspareunia (36%), however some patients responded that they do sometimes experience dyspareunia (42%) (Mann *et al.*, 1992). Michelwitz *et al.*(1989), reported a group of 44 women whose symptoms related mainly to the Bartholins' ducts. They assessed themselves on an analogue scale using a scale of 1-10 to describe their symptoms. Thirty patients whose symptoms were very severe (scale 1-3) were treated using laser therapy to treat the periductal tissue to a depth of 5-10mm. Thirteen of these patients reported a return to full sexual function; eight patients failed to show any improvement and were subsequently treated using perinoplasty. In this group the cure rate was 100%. The healing phase post treatment was up to 10 weeks for laser therapy, whereas patients treated by perinoplasty generally recovered after 4-5 weeks.

Table 2.7 Vulvar vestibulitis syndrome: success with surgical excision

Author	No.Patients	Success* No.	%	Comments
Baggish *et al.*(unpublished)	15	13	87	Glands removed + vestibule
Davis (1989)	30	20	67	CO_2 laser excision + periurethral area
Friedrich (1987)	38	23	60	-
Mann *et al.* (1992)	55	37	66	-
Marinoff &Turner (1991)	73	60	82	Periurethral area
Michelwitz *et al.* (1989)	16	16	100	8 failed CO_2 laser ablation
Peckham *et al.* (1986)	8	7	88	-
Schover *et al.* (1992)	38		47	Limited excision (Barths)
	32		50	Limited excision + psych
			56	Limited excision+ psych+ sex therapy
Woodruff *et al.* (1983)	14	14	100	

* includes complete cure and much improved , adapted from Baggish *et al.* (1995)

Bornstein *et al.* (1991) have compared perinoplasty with vestibuloplasty. The difference between these procedures is that during vestibuloplasty no mucosal epithelium is removed. The submucosal glands are removed, the vestibule is undermined, and then the mucosa re-

sutured into place. Women were allocated to one of the surgical procedures using computer randomisation. After 10 vestibuloplasties had been undertaken the results were found to be so unsatisfactory, with no improvement in any patient, that the study was terminated (Bornstein *et al.*, 1991).

De Jong *et al.* (1995), comment that surgical intervention for focal vulvitis and VVS is not a suitable treatment for the condition, as many patients continued to suffer with focal tenderness of the vestibule after the operation, which lead to the authors suggesting that this radical treatment should be abandoned. In this study, the authors found all women to exhibit 'inadequate sexual behaviour', depression, lack of sexual libido, and often sexual aversion, all of which added to the problem of vulvar pain. In conclusion these authors suggested that a psychological assessment and treatment would be of more benefit to patients with idiopathic vulvar pain.

2.5 Psychological aspects of vulvodynia and VVS.

Several investigations have reported psychosexual problems associated with vulvodynia and in particular VVS. Many physicians are not willing to accept the concept of non-pathogenic vulvodynia, an unwillingness, which may stem from the terminology used to describe disorders such as dysesthetic vulvodynia and VVS in the past. Terms such as non-pathogenic vaginitis, burning vulva syndrome and psychosomatic vulvovaginitis have been used, suggesting that the etiology of the condition is mainly psychosocial (Gardner & Kaufman, 1981, as cited by Ridley, 1988).

In a study of 83 women with vulvovaginitis, women were assessed using two psychological questionaries. Women with confirmed and unconfirmed vulvovaginitis were found to score more highly on depression and emotionally distressed scores than the 32 control women.

Patients with unconfirmed vulvovaginitis were often emotionally distressed and suffered pain during sexual intercourse. Authors of this report, and others, support psychological inquiry as it is important to the clinical assessment and treatment of patients with idiopathic vulvovaginitis. Six of the ten women in this study who accepted psychological assessment and psychotherapy were found to be suffering from clinical depression, which consequently responded to treatment, as did the symptoms of vulvovaginitis (Magni *et al.*, 1984; Stewart *et al.,* 1990; de Jong *et al.*, 1995).

Table 2.8 Classification of psycho-cutaneous disease

Classification of Psycho Cutaneous Disease	
■ Strictly Psychological Origin	- Glossodynia - Dermatitis artefacta
■ Strong Psychogenic Factors Imputed	- Generalised pruritus - Pruritus of the anogenital region - Alopecia areata
■ Genetic or Environmental Factors Linked to Emotional Stress	- Psoriasis - Atopic Dermatitis - Lichen planus - Lichen simplex chronicus - Recurrent HSV

(adapted from Stewart, 1990)

Koblenzer (1983), described three main groups of psycho-cutaneous disease (Table 2.8). Although there is some overlap between the groups, a classification of this type was considered to be useful by the author in the treatment of cutaneous disorders. Koblenzer (1983), describes glossodynia, which has since been described as being analogous to vulvodynia (McKay, 1988), as rarely having a physical basis. This investigation indicated that a continued search for an etiologic agent in cases of glossodynia is often damaging to the patient, who is usually extremely anxious and often depressed by their situation. Koblenzer (1983) recommends treatment with antidepressants and short term psychotherapy, similar

treatments which have proved useful in the management of vulvodynia and related conditions.

VVS is not a psychological problem (Dodson & Friedrich, 1977), however, there are psychological factors involved in a syndrome such as VVS which need to be addressed (Reaner, 1979; Preti, 1994). One investigation has shown a degree of depression in 80-100% of VVS patients, which was demonstrated by questionnaire interviews, and often by patient admission (Lynch, 1986). Although the depression was not linked to suicidal tendencies, these patients were reported to have histories of obsessive compulsive behaviour, marital problems, and commonly showed anger about their condition and previous medical treatment (Lynch, 1986; Schover *et al.*, 1992). Patients commonly reported anger towards GP's and gynaecologists due to their inability to diagnose the problem and relieve the pain, their inability to listen, and often their quickness to indicate a psychological origin to the problem. Lynch (1986), described a spectrum of VVS patients, ranging from those women with demonstrable pathology to those with sexual dysfunction and psychologic disturbance. A majority of patients showed some degree of psychologic problem, as they were all preoccupied with the pain caused by VVS. These authors concluded that the psychologic problems which have caused this preoccupation with VVS associated pain, must be addressed regardless of whether there is a pathological cause for the syndrome (Koblenzer, 1983; Lynch, 1986; Schover *et al.*, 1992).

A particularly interesting investigation by Abramov *et al.* (1994), has indicated yet another factor which must be considered in the treatment of patients with VVS. This study describes a group of 14 women with VVS, 50% of which also have vaginismus, an involuntary vaginal spasm seen on attempted vaginal examination. Similar findings have been reported by Basson (1994). Abramov indicated the necessity of treating the vaginismus prior to treatment of VVS, as some patients with VVS and vaginismus improved significantly after treatment of the

vaginismus, which was the major cause of dyspareunia. Patients whose vaginismus was treated successfully but whom still had dyspareunia due to VVS were referred for perinoplasty (Abramov *et al.*, 1994). All patients in this investigation resumed sexual relationships 1 year after surgery (if surgery was required), and 71.5% of patients achieved this in 3 months. Surgery, although necessary in some cases, does however cause psychological stress often due to reluctance to resume sexual relationships after the operation. Therefore, sexual counselling and psychological support require careful consideration if surgery is being contemplated. Schover *et al.*(1992), showed that women who were assessed post operatively by a psychologist faired much better in terms of complete recovery than patients who refused such evaluation. In conclusion, careful selection of patients for surgery, and adequate counselling and psychological support were considered important by many researchers (Lynch, 1986; Schover *et al.*, 1992; Abramov *et al.*, 1994). These factors were indicated as essential to provide a combined surgical and psychological program to deal with the multiple factors which appear to initiate and maintain the symptoms of VVS.

The relationship of depression and immune function may play an important role in conditions such as vulvodynia and VVS. Several authors have reported that immune function is affected by bouts of clinical depression, which is commonly seen in women with these conditions. Reports by Cappel *et al.* (1978) Kronfol *et al.* (1983) and Murphy *et al.*(1986), have all shown impaired cell mediated immunity during depression, which may result in recurrent infections associated with some subsets of vulvodynia (as cited by Stewart *et al.*, 1990). It is possible that depression, often obvious in patients with VVS, may initiate altered immune function. The mucosal-like surface of the vulva may be affected by this change in immune function resulting in the symptoms associated with VVS.

2.6 Summary

In the overall context of vulvar pain, VVS does not appear to be one of the major causes. However, the symptoms of VVS are usually long term, and as described previously, notoriously difficult to treat. Researchers have reported an increase in incidence of VVS in the past 15 years, and it is proposed that many women suffering with vestibulitis feel unable to approach their GP, and are consequently suffering in isolation. Although the incidence of the syndrome proposed by Goetsch (1991) was suspected of being slightly high, it may turn out that the actual incidence of this syndrome is close to this figure. Research continues into the incidence of HPV in cases of VVS, a relationship which appears to be unfounded in a majority of studies. An alternative approach to the problem is required, perhaps one which aims to identify the cellular changes involved in this perplexing syndrome. The landmark paper by Pyka *et al.* in 1988, set the ground for immunological advances in the understanding of the syndrome. Unfortunately, as this study showed nothing conclusive, this approach seems to have been abandoned, as only one further investigation by Prayson *et al.* (1995) has been completed in this area. Many questions remain unanswered; are studies aiming to identify an etiological agent a waste of resources ? Should research be concentrating on the role of HPV in this syndrome despite its' link with only small percentage of cases ? What process is resulting in edema of the vestibular tissue ? Is a cell signalling mechanism to blame for the presence of inflammatory cells in the absence of an etiological agent ? Is cell death occurring in the epithelium resulting in the symptoms of VVS ?

It is obvious from the distribution of literature published in the last 15 years that many of the avenues of approach to vulvodynia and particularly VVS, were quickly proven unfruitful, often leading researchers in the wrong direction. Since 1990, very few papers have been published on the syndrome, the ones which have, are characteristically reviews and disputes relating to HPV. Consistent with the lack of advancement in understanding, treatments for

VVS also remain unaltered with several researchers returning to the use of surgery for the treatment of long term cases of VVS. It appears that until new approaches are developed to extend the understanding of the cellular mechanism involved in these conditions it is unlikely that novel treatments will be generated, resulting in researchers returning to the use of anti-depressants, interferon, and vestibulectomy to eliminate the symptoms rather than attacking the underlying cause of the syndrome.

CHAPTER THREE

Epidemiology and pain assessment of VVS patients

3.0 Abstract

Control patients, and women with VVS, were interviewed using a structured questionnaire to obtain epidemiological data. Several differences were obvious from the epidemiological data, however, this was a pilot investigation, and a larger study would be required in order for any statistically relevant data to be obtained. Neither group of patients had a high incidence of HPV infection, however, a large proportion of patients reported recurrent *Candida* infections. Patients with VVS reported a higher incidence of 'burning' pain, labial and vulvar irritation, and cystitis, than was reported by the control group. VVS patients agreed that their GPs could have been more helpful, and that their GPs were often unaware of VVS. Most GPs did not examine their patients either prior to treatment, or on following visits. Consequently, VVS patients were commonly misdiagnosed by their GP as having *Candida*, psychosexual or gynaecological problems with no definite cause. Assessment of vulvar sensitivity using the 'Vulvar Algesiometer' confirmed that patients with the symptoms of VVS, did have an increased sensitivity of the vulvar epithelium compared with control patients. Cumulative algesiometer readings from control and VVS patients, taken prior to treatment, were found to be significantly different ($p<0.0001$). Readings taken from the Bartholins' glands were significantly lower than those recorded on the Skenes' glands ($p<0.0034$). Over a four month period, VVS patients treated with ketoconazole showed a significant decrease in the sensitivity of the vulval epithelium. Over the first three months, readings improved significantly, however, after three months there was no significant change in the readings. In order to establish the efficacy of ketoconazole for the treatment of VVS a placebo controlled, multi-centered trial was considered essential.

3.1 Introduction

Friedrich introduced the term Vulvar Vestibulitis Syndrome at the 1987 meeting of the ISSVD, where he suggested three main diagnostic criteria associated with the syndrome, which were adopted internationally, and are still widely used for the assessment of patients with idiopathic vulvar pain (see Chapter 2). The epidemiological characteristics of VVS have been described on several occasions, and are discussed in detail in Chapter 2 (Woodruff & Parmley, 1983; Peckham *et al.*, 1986; Friedrich, 1987; Pyka *et al.*, 1988; Michelwitz *et al.*, 1989; Furlonge *et al.*, 1991; Wilkinson *et al.*, 1993; Bazin *et al.*, 1994 and Marks *et al.*, 1995). However, the most comprehensive paper is that by Goetsch, published in 1991. This study, involving 210 women attending a private gynaecological clinic in the USA, attempted to elucidate the incidence of VVS, and confirm the main characteristics of the syndrome. Many studies of VVS have involved the assessment of patients using the criteria suggested by Friedrich (1987); and many authors have subsequently interviewed patients with reference to past infections, gynaecological history, and the onset and duration of symptoms. However, several deficient areas exist, such as: self treatment, treatment by the GP or specialised clinic, the patients perception of the problem, and an assessment of the severity and location of the pain.

Assessing vulvar pain is notoriously problematic. Comparing present, and past pain is extremely hard, and may depend not only on the intensity of the pain, but on a myriad of other factors. Measuring pain is difficult, as perception of pain varies from patient to patient, and over time in an individual patient. Pain is also affected by trauma, stress, illness and psychological well being, all of which may alter the intensity and, or, how often the pain is felt. Patients presenting at their GP or GUM clinic, are currently assessed for vulvar sensitivity by applying pressure to the surface of the vestibule, using a cotton tipped applicator to palpitate the minor vestibular glands eliciting a pain response (Goetsch, 1991). However, this

is an unreliable measurement of sensitivity, as the pressure applied is totally variable from patient to patient, variable between different consultants, and is often not reproducible at subsequent consultations. Another method of assessing vulvar sensitivity has included the use of dilute acetic acid. This investigation used different concentrations of acetic acid to gauge vulvar sensitivity in patients attending a vulvar clinic with idiopathic vulvar pain (Sonni *et al.*, 1995).

A means of delivering a calibrated, measured amount of pressure for a specified time interval was, therefore, required for the continued assessment of such patients. The pressure delivered should be totally reproducible in order to provide consistent results, and comparable readings for a patient, from one appointment to the next. In order to monitor the progress of patients, assess treatments for VVS, and to compare the sensitivity of the vestibule in VVS patients, a pain meter or 'Vulvar Algesiometer' was designed as a possible means of measurement (Curnow *et al.*, 1996). The 'Vulvar Algesiometer' was designed and developed in the Medical Physics Department (Freedom Fields, Plymouth) in collaboration with researchers at the G.U.M. clinic and the University of Plymouth.

Many previous investigations of VVS have not included a control group for comparison of data, or alternatively, included a control group of women attending a vulvar clinic who have not suffered with vulvar pain in the past (Goetsch, 1991). This self selected group of women who were attending a clinic for other gynaecological problems, were not considered suitable controls for an epidemiological study, as they did not represent a true cross section of the female population. For the present investigation, a suitable control group of patients was considered important, as this was a pilot investigation, and the study group was relatively small. In order to obtain suitable control samples, two GP's surgeries were identified and asked to take part in the investigation. One surgery, was an inner city practice, with patients

from a wide range of socio-economic groups, the other, was a practice on the outskirts of the city centre. It was important that the control group of women were similar biographically to the VVS group in order for comparisons to be made. The GP's at both practices were asked to recruit patients of 18-40 years for the investigation. Only one of the practices, the rural practice, was involved in the trial of the 'Vulvar Algesiometer'. The aim of the epidemiological investigation was to address several areas: (a) Have VVS patients been diagnosed as having infections of HPV or *Candida*, and were these infections documented by the GP or the clinic ? (b) Have these patients ever treated themselves for vulvar irritation or past infection ? (c) How were women with VVS treated by their GP / G.U.M. clinic ? (d) How much do women with VVS understand about the condition and its treatment ?

The trial of the 'Vulvar Algesiometer' involved the assessment of vulvar sensitivity in both control, and VVS patients, and subsequent statistical analysis of the results obtained over four months. The trial of the 'Vulvar Algesiometer' aimed to establish: (a) whether there was any difference in the levels of control and VVS readings, (b) whether ketoconazole had any effect on the sensitivity of the vulva over a four month period, and (c) whether additional treatment with imipramine affected response to treatment with ketoconazole (Nizoral, Janssen Pharmaceutical, Belgium).

3.2 Methods

3.21 Questionnaire interview of VVS patients

The questionnaire was designed with the assistance of Ms.C. Sutton (Applied Social Science), and Dr. J. Chandler (Head of Applied Social Science). After drafting possible questions to be included in the questionnaire, assistance with the layout and coding of the questionnaire was provided by Ms. Sutton (see Appendix 2). Both the VVS and the control patients were interviewed by either Miss Sargeant or Ms. Sutton, who were trained in interview technique by Dr. Chandler. Ethical committee approval was obtained for the investigation, allowing both control and VVS patients to be interviewed. Confidentiality was of paramount importance, and to ensure this, completed questionnaires were only given an identification number. Twenty five VVS patients were chosen at random from the Genito-Urinary Medicine (G.U.M.) clinic records. These women were invited by letter, to attend a confidential interview at the G.U.M. clinic. Not all patients responded to the letter, so in order to maintain a suitable sample number for the pilot study, several additional VVS patients were randomly selected from the clinic records. In total, nineteen VVS patients completed the questionnaire. The control group consisted of twenty nine women, who were of a comparable age to the VVS group. Control patients were women attending their GP for Well Woman screening; a majority of these patients were recruited from the rural practice, and also participated in the trial of the 'Vulvar Algesiometer'. The questionnaire was delivered in the form of a confidential, informal interview, a format which was preferred to a self administered questionnaire, as the study group was relatively small, and a 100% completion rate was required. Interviews lasted for approximately 20-60 minutes; patients were informed that the questionnaire was confidential, that they did not have to answer all questions, and that they could terminate the interview at any time. Written consent was obtained from each patient before the interview commenced in accordance with ethical committee guidelines. Essentially, the questionnaire consisted of five sections (Table 3.0), however, not all of the sections were

completed by each patient. The number of sections which were completed, was dependent on past infections, and on whether the patient was in the control or VVS group (see Appendix 1 & 2). Each of the responses was marked as a variable, and the responses for each patient were entered into a statistical package, SPSS for the Apple Macintosh, for analysis. The data was entered by Ms. Sutton, the analysis of the data for the present study was completed by Miss Sargeant. The raw data is available, but was too unwieldy to be included in an appendix.

3.22 Development of the 'Vulvar Algesiometer'

The 'Vulvar Algesiometer' was designed and developed in the Medical Physics Department at Freedom Fields, Plymouth. The design of the equipment was completed by Mr. J. Curnow, Mr. L Barron in consultation with Dr. G Morrison and Miss P.Sargeant. The system is made up of 3 parts, a hand held probe, a control unit incorporating the power supply, and a foot switch to increase and decrease the force setting during clinical use (Fig. 3.0). The thrust probe is made of stainless steel, and is surrounded by a carbon fibre end sleeve, both of which are removable for sterilization purposes. The diameter of the probe tip is 1.5 mm, which is connected by a thrust rod to the core of the solenoid which produces the impact force. The outer sleeve is supported by a sleeve guide which comprises part of the outer casing. There is a boss at the inner end of the outer sleeve which acts as a forward stop and is also used to sense when the probe is correctly in contact with the vulval surface. The control unit provides the controlled pulse of current which drives the solenoid. There are eight calibrated levels for the drive force ranging from 178 mN (0) - 2046 mN (7). The force level can be selected using the foot pedal or the button on the front of the control unit. The force setting, which is displayed on the front panel, steps up from one level to the next from 0-7, and then returns to 0 when it has reached the maximum. The length of the tap pulse was made variable from 0.005 seconds 0.5 seconds in the pilot model, but was set at 0.5 seconds in all later models. (Fig. 3.0) (Curnow *et al.*, 1996)

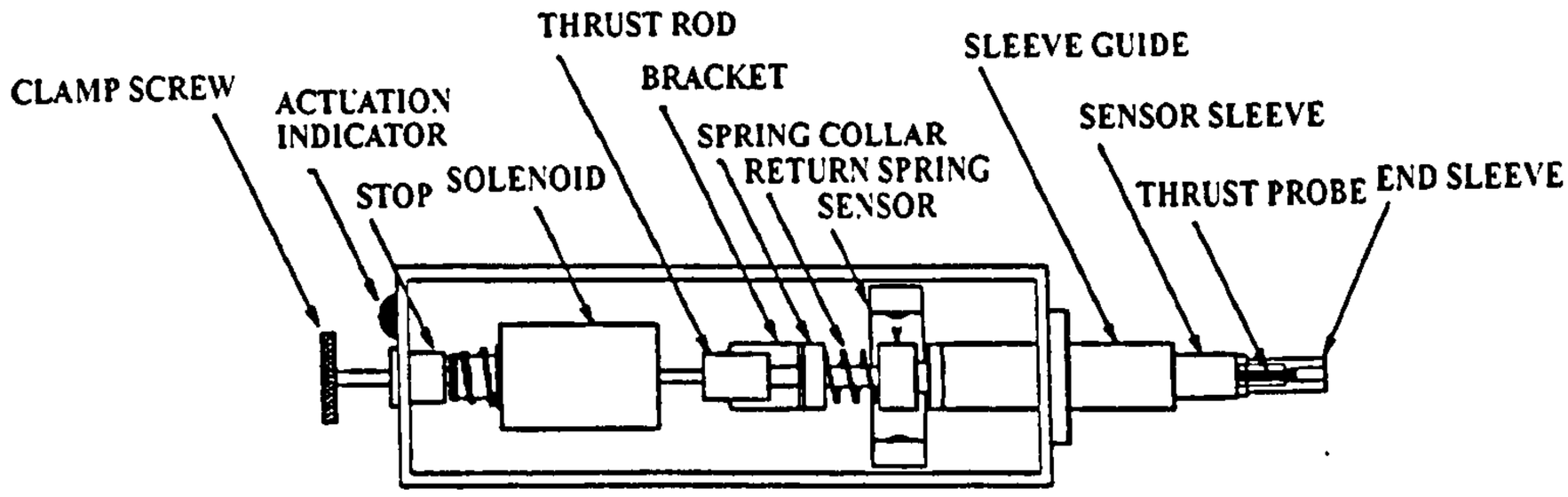
Table 3.0 Format of the Questionnaire

Section One	• *Biographical details*: age, marital status, children, ethnic origin, and occupation. • *Obstetric History*: details of pregnancies, problems after childbirth, use of tampons. • *Sexual History*: current relationship, contraception, age at first sexual intercourse. • *Menstruation*: age at start of menses, use of sanitary products, problems with the use of these products. • *Hygiene Regimes*: type and usage of toiletries, washing powders, and other detergents. Problems associated with their usage. • *Irritations:* location, description, diagnosis, and treatment of infections / irritations.
Section Two	Only to be completed by women who have suffered with *Candida* (thrush). • Symptoms experienced, self management and / or treatment of symptoms, medical diagnosis and length of treatment if provided. Recurrence of symptoms.
Section Three	Only to be completed by patients who have been diagnosed as having HPV. • Self assessment, medical diagnosis, treatment and management. Recurrence of warts.
Section Four	Only to be completed by women diagnosed as having VVS. • Symptoms experienced, self treatment, medical diagnosis and treatment prescribed, length of treatment, recurrence of symptoms. • *GP consultation:* examination, diagnosis and treatment provided, referral to G.U.M., follow up visit to GP.
Section Five	Only to be completed by women attending the G.U.M. clinic for treatment of VVS. • Number of visits to the clinic, description of symptoms, what factors affect symptoms, duration of symptoms, self treatment. • Relationship of current symptoms to initial symptoms, effectiveness of treatment, problems with daily routines and sexual relationships.

3.23 Use of the 'Vulvar Algesiometer'

The use of the 'Vulvar Algesiometer' is very similar to that of the cotton tipped applicator, used frequently in gynaecological practice to test sensitivity of the vulva (Goetsch, 1991). The probe is placed over the minor vestibular glands using a colposcope to position it accurately. When lightly placed onto the surface of the vulva the outer sheath moves back into the main body of the instrument until the slot in its boss end aligns with the light sensor. This ensures that the skin surface is sufficiently flattened, and that the thrust probe is the correct distance from the skin. When the control unit receives the sensor signal, it provides a drive pulse to the solenoid, that causes the thrust probe to tap the skin at a pre-calibrated level of force. If the patient finds this comfortable the force is increased using the foot pedal until slight discomfort is felt. This is carried out at all pressure settings and if no discomfort is felt at level 7 a reading of 7+ is recorded. On obtaining a result for one duct the clinician moves onto the next duct and repeats the procedure. In total, four readings are taken, from the two Skenes' (S1, S2) and the two Bartholins' glands (B1, B2).

Figure 3.0 Line drawing illustrating the internal components of the Vulvar Algesiometer



(Reproduced from Curnow *et al.*, 1996)

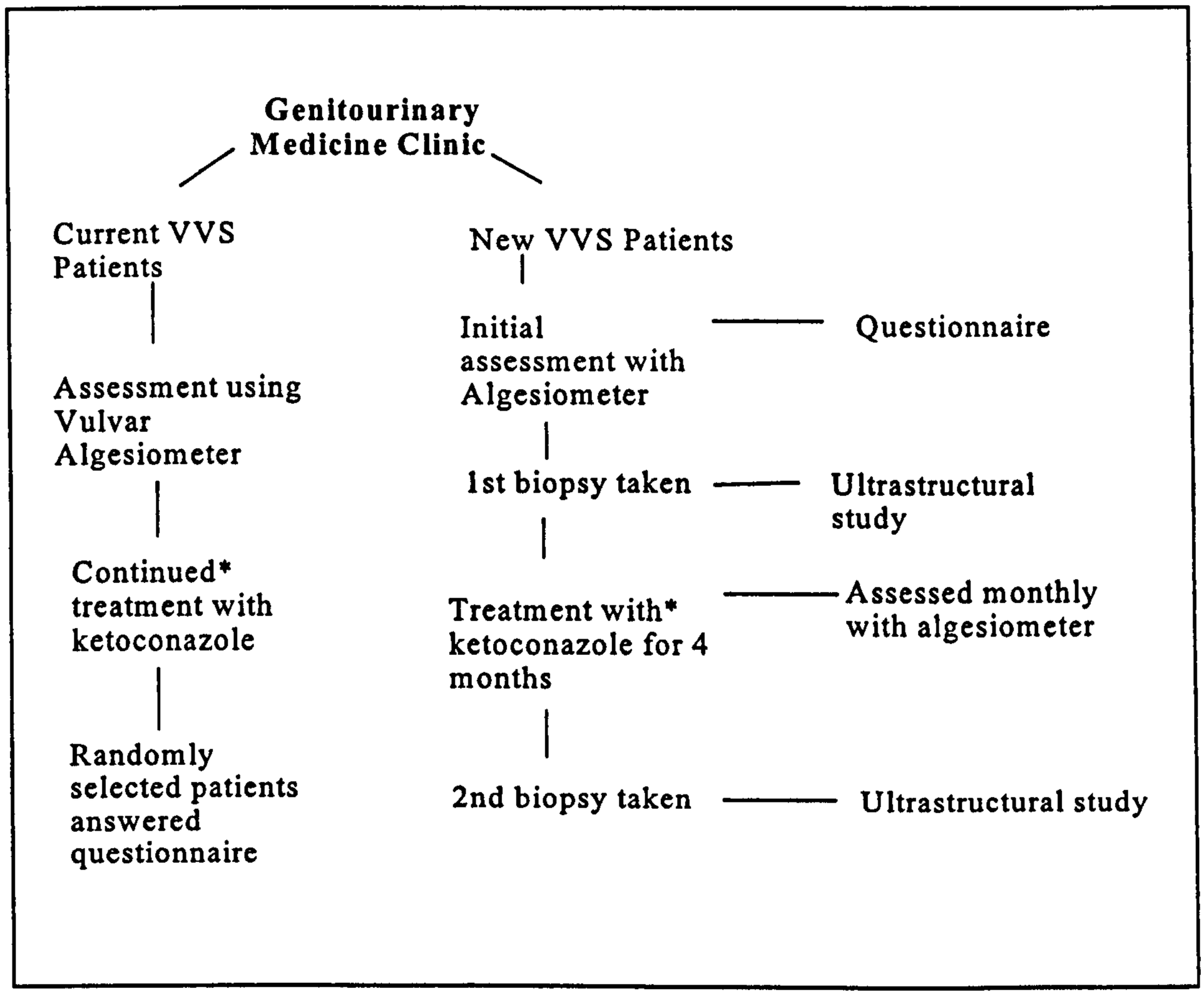
3.24 Assessment of vulvar sensitivity using the 'Vulvar Algesiometer'

A pilot study of the 'Vulvar Algesiometer' was carried out in Plymouth and Exeter to test the reliability and sensitivity of the device and the reproducibility of results using this means of measurement. All women with VVS were asked to participate in the study, so that assessment of vulvar sensitivity and effectiveness of treatment regimes could be established. All women who were asked, were pleased to co-operate in the investigation. As a comparison, and in order to assess the level of sensitivity in VVS patients, it was important to determine a base line reading from control patients with no vulvar discomfort. Twenty nine women, between the ages of 20-40 years, from a rural GPs surgery were approached by their GP and asked to participate in the pilot study. All women were asked several questions about their medical histories to determine whether patients had suffered any past vulvar discomfort. After having the assessment fully explained, each woman had a brief interview, followed by a short examination by their GP, and an assessment of vulvar sensitivity using the 'Vulvar Algesiometer'. The assessment of vulvar sensitivity in control patients is an ongoing investigation, and to date 47 women have been included, the results of which are included in this study.

All patients registered at Plymouth G.U.M. clinic, who were being treated for VVS were assessed using the 'Vulvar Algesiometer'. All patients consented to the use of the 'Vulvar Algesiometer' prior to being assessed. New VVS patients, who had not received any treatment, were assessed prior to treatment, and were asked to participate in the ultrastructural investigation (Fig.3.1). These patients were assessed prior to a biopsy being taken, and then at monthly intervals for four months. After four months of treatment with ketoconazole a second biopsy was removed (see Chapter 6). Some patients required treatment with imipramine, a tricyclic anti-depressant, prescribed at a low dose as a pain killer. Additional treatment with tricyclic anti-depressants is common in the management of vulvodynia (see

Chapter 2). This group of VVS patients were treated separately in the analysis of the data, as it was anticipated that the perception of pain, and therefore the algesiometer readings, may be affected by the use of imipramine. All statisitical analysis was completed using Excel 5.0 and Statsgraphics for Windows.

Figure 3.1 Treatment and assessment of patients involved in the investigation of VVS



* Some VVS patients received additional treatment with imipramine, a low dose anti-depressant, prescribed for its pain killing properties

3.3 Results

3.31 Epidemiological characteristics of patients with VVS

The age distribution of VVS and control patients (Fig. 3.2) illustrates that the majority of control patients (88%) were in the age range 25-35 years, whereas VVS patients were mainly between the ages of >20-29 years (74%). There were no patients in the 5th or 6th decade in the control sample, in comparison, there was a distribution of VVS patients across the age range, one of the VVS patients was 54 years old. The average age of control patients was 30.6±5.0 years, compared with 27.7±7.9 years for the VVS group. The biographical details of each group indicated that the control group began menstruating later than the VVS group, but became sexually active slightly earlier (Figs. 3.3 & 3.4). The number of children from control and VVS patients were very different, 68.2% of VVS patients had not had children, in comparison, only 17.2% of the control group were nulliparous (Fig. 3.5). Patients were assigned to a social class by their occupation, using the HMSO guidelines (1980). Generally VVS patients were class III non-manual, and III manual. Class VI included patients who described themselves as housewives, or unemployed (Table 3.1). All of the patients in the VVS and control groups were white. In the control group 75.9% were married or cohabiting, in the VVS group this figure was slightly lower at 63.2%. A majority of patients in both groups were involved in a sexual relationship with a man; the duration of the relationship ranged from 6 months to 12 years for VVS patients, and 2 -22 years for the control group. Out of the control group, 89.7% reported having 'regular' sexual intercourse, compared with 63.2% of the VVS study group (Table 3.2).

Table 3.1 Social class of control and VVS patients

Social Class	VVS (%)	Controls (%)
I	0	0
II	10	11
III NM	40	52
III M	20	0
IV	5	0
V	0	3
VI	25	34
	n=19	n=29

Table 3.2 Relationships and sexual activity of control and VVS patients

Group	*Married /Cohabit*	*Regular sexual intercourse*	*Duration of Relationship*
VVS	63.2%	63.2%	6 months - 12 yrs
Controls	75.9%	89.7%	2 yrs - 22 yrs

The use of contraception did vary between the two groups, 68% of controls, and 80% of VVS patients reported using some form of contraception. The use of condoms was similar in both groups, approximately 20%. However, the use of oral contraceptives was much higher in the VVS group than in the control, 60% compared with 38% (Table 3.3).

Vestibulitis patients showed a higher usage of sanitary towels than tampons when compared with the control group. Control patients used tampons only (48.3%), or both towels and tampons (82.8%), whereas VVS patients used mainly towels (60%) (Table 3.4). VVS patients who did not use tampons, or had used tampons in the past (26.3%), reported that they stopped using them due to pain on insertion.

Table 3.3 Methods of contraception in VVS and control patients

Method of Contraception	*Percentage of Patients*	
	VVS	***Controls***
Condoms	20	20
Pill	60	38
Cap	0	3
Coil	0	7
None	20	32
	n=19	n=29

Table 3.4 Types of sanitary products used by both VVS and control patients

Sanitary Product	*VVS*	*Control*
Tampons only & both tampons and towels	8 (40%)	24 (82.8%)
Sanitary towels	11 (60%)	5 (17.2%)
	n=19	n=29

Despite being advised to use non-biological washing powders and hygiene products, only 40% of VVS patients use non-biological powders. Both groups reported washing their vaginal area in addition to bathing or showering (50% VVS, 58% controls). Both groups used soap (75% of controls, 52.6% of VVS), however, a smaller percentage of patients in both groups used soap to wash their vaginal area (15% VVS, 34.5% control). Despite using soap, very few of these women had experienced any irritation from cleansing (27.6% of controls, 31.6% of VVS) (Table 3.5). Only one patient in each of the two groups had used any special cleansing products for the vaginal area.

Table 3.5 Hygiene regimes and cosmetic products used by control and VVS patients

Action	*VVS (% Patients)*	*Control (% Patients)*
Biological washing powders	60	62.1
Fabric softeners	65	69
Bath products	40	51.7
Soap	55	75.9
Wash vaginal area separately	50	58.6
Use soap for vaginal area	15	34.5
Irritation after washing	5	13.8
	n=19	n=29

All of the VVS patients had suffered irritation of some kind in the past due to infection or allergic reaction (Fig. 3.6). Irritation of the pubic area, clitoris, urethra, and rectal areas were similar in both groups. However, there was an obvious difference in past irritation reported for the vulva, vagina and labial areas. Amongst VVS patients, 68.4% reported irritation of the vulva, compared with 41.4% of control patients. Irritation of the labia was reported in both groups, with 31% of controls and 63.2% of VVS patients reporting past irritation. Perineal discomfort was noted by 31.6% of VVS patients and only 17.2% of controls (Fig. 3.6). When asked to describe their symptoms in their own words, 62% of controls and 36.8% of VVS patients described an irritating, itching sensation. Burning was described by 36.8% of VVS and 10.3% of control patients who had suffered past irritation (Table 3.6). Only the patients who were able to describe their symptoms were included. General unspecified irritation was described by 14 of 19 VVS patients (73%), a description which was not common in the control group (6.8%).

Table 3.6 Description of symptoms associated with infections

Symptoms	*VVS*	*Controls*
Burning	7	3
Itching	7	18
Discharge	0	4
Pain on urination	3	3
Pain during sexual intercourse	2	1
Swelling	4	1
General unspecified	14	2

Amongst VVS patients, 57.9% attempted to treat irritations themselves, whereas 48.3% of control patients who had suffered past irritations had tried to treated themselves (Fig. 3.7). On average more VVS patients reported their symptoms to their GP, local G.U.M. clinic or pharmacist, than control patients. Patients who consulted their GP, had swabs taken in 37.9% of control and 68.4% of VVS cases (Fig. 3.7). Documented infections were reported by both groups of patients (Fig. 3.8). Thrush was reported by 89.5% of VVS and 79.3% of control patients. Cystitis was more common in the VVS groups as was herpes, HPV, vaginitis and VVS. Of patients who had suffered with *Candida* infections, 36.8% of controls and 41.4% of VVS patients had treated themselves.

Only patients who had symptoms associated with VVS completed the last two sections of the questionnaire. These sections dealt with: self treatment of the symptoms of VVS; treatment by GPs or clinics; effectiveness of the treatment received; and the attitude of the consultant. Less than half of the VVS patients had attempted to treat themselves (42.1%) for the symptoms of VVS. In addition to self treatment, 78.9% of VVS patients consulted their GP and 21.1% attended a G.U.M. clinic. Of those who reported their symptoms to a GP, only 52.6% were examined, and only 10.5% were diagnosed as having VVS (Fig. 3.9). Other diagnoses by GPs' included: *Candida* (57.9%); cystitis (5.3%); psychological problems

(5.3%); and undetermined cause (31.6%). Only 63% of GPs' offered any treatment, 37% of which was reported to be ineffective. A majority of VVS patients returned to their GP (63.2%), and received more treatment which was unsuccessful (47.4%). A small percentage of patients reported that their GP was unhelpful (5.3%), or that they changed GP (36.8%), or that they were referred to a G.U.M. clinic (15.8%) (Fig. 3.10)

Following treatment with ketoconazole, 13 of 19 patients (68.4%) described their symptoms as less intense, and 7 of 19 patients (36.8%) described less frequent discomfort. Only 21% had seen no improvement in their symptoms (Table 3.7). Patients were also asked to gauge the severity of their symptoms and say whether the pain interfered with their daily routine; in order to determine this, patients were asked to respond to one of the five statements provided (Tables 3.7 & 3.8). Moderate discomfort was reported by 68.4%, and 31.5% found that the pain did not interfere with their daily routine (Table 3.9). Sexual relationships were commonly affected by the symptoms of VVS (52.6%), and 36.8% of patients reported that they were unable to have sexual intercourse.

When asked what treatment they had received at the G.U.M. clinic, 10.5% did not know what they were using, 63.2% said that they were using Nizoral (Janssen Pharmaceuticals, Belgium), and 5.3% referred to the cream as ketoconazole (chemical name). On several occasions patients were asked what colour the tube of cream was, in order to determine the treatment.

Table 3.7 Initial and current symptoms as described by VVS patients

Description of symptoms	*Number of Responses**
More frequent	2
Less frequent	7
More intense	1
Less intense	13
Same	4
	n=27 responses

* Patients were allowed to answer 'yes' to one or more statements

Table 3.8 The severity of discomfort experienced by VVS patients

Severity of Discomfort	*No. of Patients*
Severe - I find it difficult to do anything	1
Severe - I can only perform some tasks which require little concentration	1
Moderate - I always know the pain is there but can perform most tasks	13
Slight - I can ignore the pain by not thinking about it	5
Slight - I only notice the pain when I think about it	4
	n=19

Table 3.9 Degree of interference with the daily routine

Degree of interference	*No. of Patients*
Interferes with daily routine	0
Some interference with daily routine	6
No interference with daily routine	13
Medical attention required	0
Confinement to bed	0
	n=19

When asked about how they were treated by their GP, only 26.3% of VVS patients agreed that they were completely satisfied with their initial visit to their GP, 26% did not agree with this statement, and 10% were neutral. When asked if aspects of their consultation could have been better, 42% of VVS patients agreed with this statement, 10% disagreed, and 27% were neutral (Fig. 3.11). Patients were also asked if the GP was, in their opinion, interested how the symptoms were affecting them. More than half (57.9%) of the VVS patients reported that they disagreed, or strongly disagreed that the GP was interested, only 10.6% said that their GP appeared interested, and 10% were neutral. After numerous visits to their GPs, 57.9% of VVS patients were referred by their GP to the G.U.M. clinic at Freedom Fields, Plymouth, the remaining patients referred themselves. The average number of visits by each patient was approximately seven, however the number of visits ranged from 1 to 12. Only 5.3% of VVS patients suspected that they were suffering from VVS when they attended the G.U.M. clinic. Frequently, these patients did not know what was wrong with them (36.8%); 21.1% believed thrush was the cause, 10.5% were convinced that they had a psychosexual problem, and 5.3% reported that they thought their symptoms were related to old scar tissue, usually episiotomy scars. On average, the duration of the symptoms was reported to be between 1 and 4 years (42.1%), however the duration of symptoms in this group of VVS patients ranged from 6 months to 10+ years (Fig. 3.12). Only 26.3% of patients in this study had ever been free of symptoms.

3.32 Assessment of patients using the 'Vulvar Algesiometer'

'Vulvar Algesiometer' readings were taken from the Skenes' and Bartholins' ducts. In total there were four readings for each patient. Occasionally, one or more ducts were unable to be detected. In order to compare readings from different patients, and individual improvement over time, cumulative readings (S1+S2+B1+B2) were recorded. Control patients generally gave readings of 7 on all ducts (cumulative reading of 28) (Table 3.10), however 11 of the 47

control patients assessed, produced cumulative readings lower than 28, and several of these patients had readings of 1- 4 on one or more ducts (Table 3.11). Using analysis of variance, it was found that the initial readings from control and VVS groups, were significantly different ($p<0.0001$), and that the variation within the groups was less than the variation observed between the two groups. The average cumulative reading for the control group was 26.1± 4.81 compared with 6.9 ± 5.8 for the VVS group (Fig. 3.13). Cumulative readings for both groups, ranged from 0-28, and in control group 39 of 47 patients (82.9%) scored 25-28, only 8 patients scored under 25 (17%). In comparison, VVS patients showed a wide range of cumulative readings. The initial assessment of VVS patients showed no patients with cumulative readings of 25-28, patients commonly scored in the range of 0-16 (91.6%), and more than half of the cumulative readings were in the 0-8 range (68.3%) (Table 3.10).

After treatment with ketoconazole cumulative readings from VVS patients appeared significantly different when compared with the initial reading. Some patients responded quickly to treatment with ketoconazole, and several became much improved after 2 months, frequently maintaining readings of 7 on all four ducts. However a significant number of VVS patients took much longer to respond to treatment, a majority began to see some improvement in their condition after four months. In total 29 patients were treated for four months or more at the G.U.M. clinic, and had not received any other medication apart from ketoconazole, the cumulative readings from these patients were plotted over four months (Figs. 3.13 - 3.17).

Analysis of variance of the readings from all VVS patients (n=60) over five months showed that there was a significant difference between the readings from the first, to the third assessment. However, when readings from month 3 and month 4 were compared there was no significant improvement in the readings (Table 3.12). Cumulative readings from assessments 1-5 exhibited a large intra-group variation when compared to the variation

between the different assessments. Cumulative readings were seen to change considerably, particularly within a 3 month period. When assessed initially, none of the VVS patients had cumulative readings of 25-28, and only 3 patients had cumulative readings of 17+ (10%).

Table 3.10 Initial cumulative 'Algesiometer' readings from control and VVS patients

Cumulative Algesiometer Reading	No of Patients *Controls*	No. of Patients *VVS*
0 - 4*	1	29
5 - 8	0	12
9 - 12	2	7
13 - 16	0	7
17 - 20	2	4
21 - 24	3	1
25 - 28	39	0
Average	*26.1*	*6.9*
	n=47	n=60

* 0 representing the most intense vulvar sensitivity, and 28 the least intense.

Table 3.11 Algesiometer readings from borderline control patients

Algesiometer Reading	Number of Readings
0	0
1	5
2	5
3	3
4	6
5	3
6	2
7	20
	n=44 readings

In comparison, after three months of treatment, 17 of the 29 patients had readings of 17+ (58.6%), however, the number of patients with cumulative readings of 25-28 remained the same as before treatment. After four months treatment the number of patients with readings of 25-28 decreased to 15 of 29 patients (51%), however there was an increase in the frequency of readings in the middle of the range, i.e., from 5-24.

Table 3.12 Comparison of cumulative readings from VVS patients from 1st to 3rd assessment

Assessment	Number of Patients	Standard Deviation	Mean
1	60	6.04	6.84
2	60	7.77	12.06
3	59	9.33	15.29
4	42	8.70	15.72
5	27	8.47	17.56

(n=60)

Additional treatment with imipramine was quite common amongst the VVS patients treated at the G.U.M. clinic, 17 of the 60 patients had received imipramine at some time during their treatment. Of 17 patients, 5 became much improved within three months and did not require further treatment, the remaining 12 patients also responded well to treatment with ketoconazole, but improvement was slower. On the second assessment, cumulative readings from patients treated with imipramine were quite evenly distributed across the range of readings, whereas in VVS patients who did not receive imipramine a majority of readings were in the range of 0-12. Cumulative readings of 25-28 were seen to increase steadily over the four months from 0-17.6%, in addition readings of 0-4 fell dramatically from 41.1% to 17.6% after just one month of treatment, and to 5.8% after 4 months of treatment. In comparison VVS patients not treated with imipramine showed a slower response with only 17.2% of patients showing readings of 25-28 after four months of treatment. Readings of 0-4

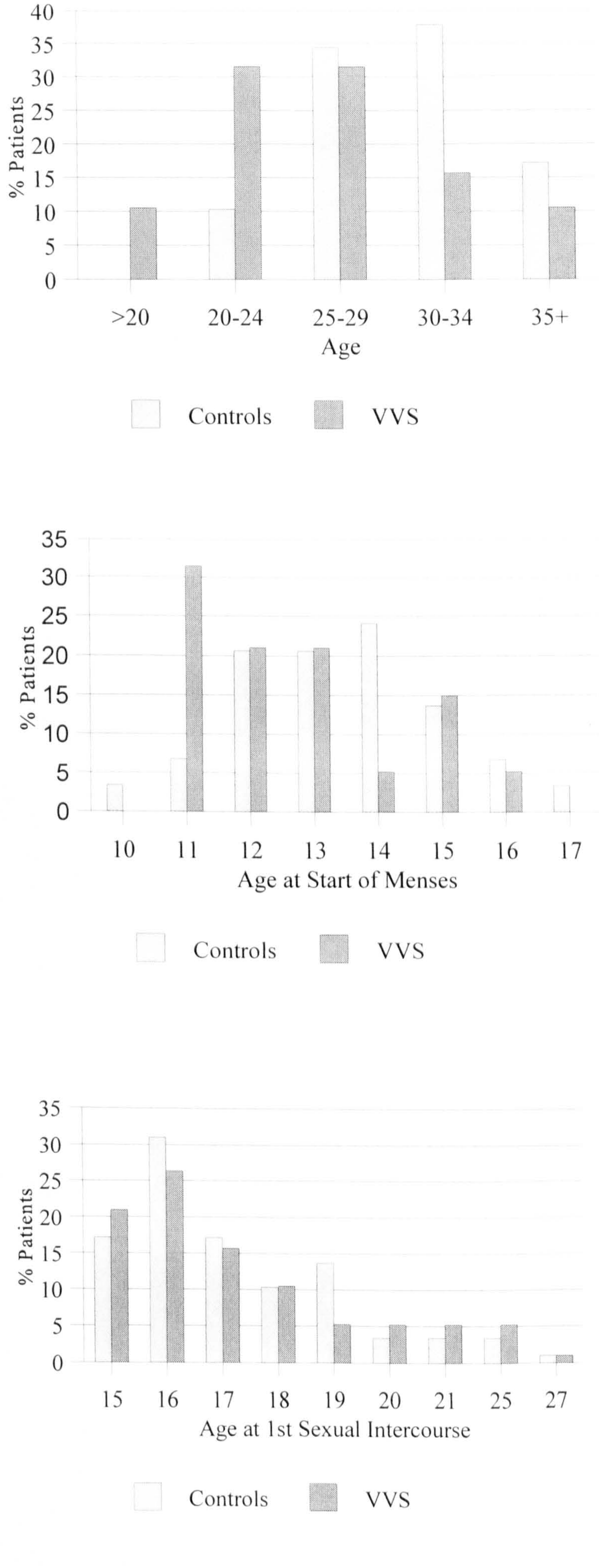
were not as common on the initial assessment of patients not treated with imipramine (34.4%) compared with 41.1% in imipramine patients. Statistical analysis of the data revealed that the two groups of VVS patients, one of which received additional treatment with imipramine, did in fact behave as a homogenous group. No statistical differences were observed between the two groups in response to treatment over four months (Figs. 3.13 - 3.17).

Algesiometer readings also appeared different between the Skenes' and the Bartholins' glands. A high percentage of Bartholins' glands gave readings of 0 and 1, when compared with the Skenes' glands. Of readings taken from the Bartholins' glands 73% were 0 or 1, compared with 52% of readings taken from the Skenes' glands in the same patients. Readings from the Skenes' glands were generally more evenly distributed over the range of readings when compared with the Bartholins' glands (Fig 3.18 & 3.19). Statistical analysis indicated that the mean reading from the Bartholins' glands was significantly different from the mean reading from the Skenes' glands ($p=0.0034$); and that the readings from the Skenes' glands were significantly higher than those from the Bartholins' glands ($p=0.0017$).

Figure 3.2 Age distribution of VVS and control patients (n=29, controls, n=19 VVS).

Figure 3.3 Age at first menstruation (n=29 controls, n=19 VVS)

Figure 3.4 Age at fist sexual intercourse (n=29 controls, n=19 VVS).


% Patients
40
35
30
25
20
15
10
5
0
>20
20-24
25-29
30-34
35+
Age
Controls
VVS
% Patients
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25
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16
17
Age at Start of Menses
Controls
VVS
% Patients
35
30
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15
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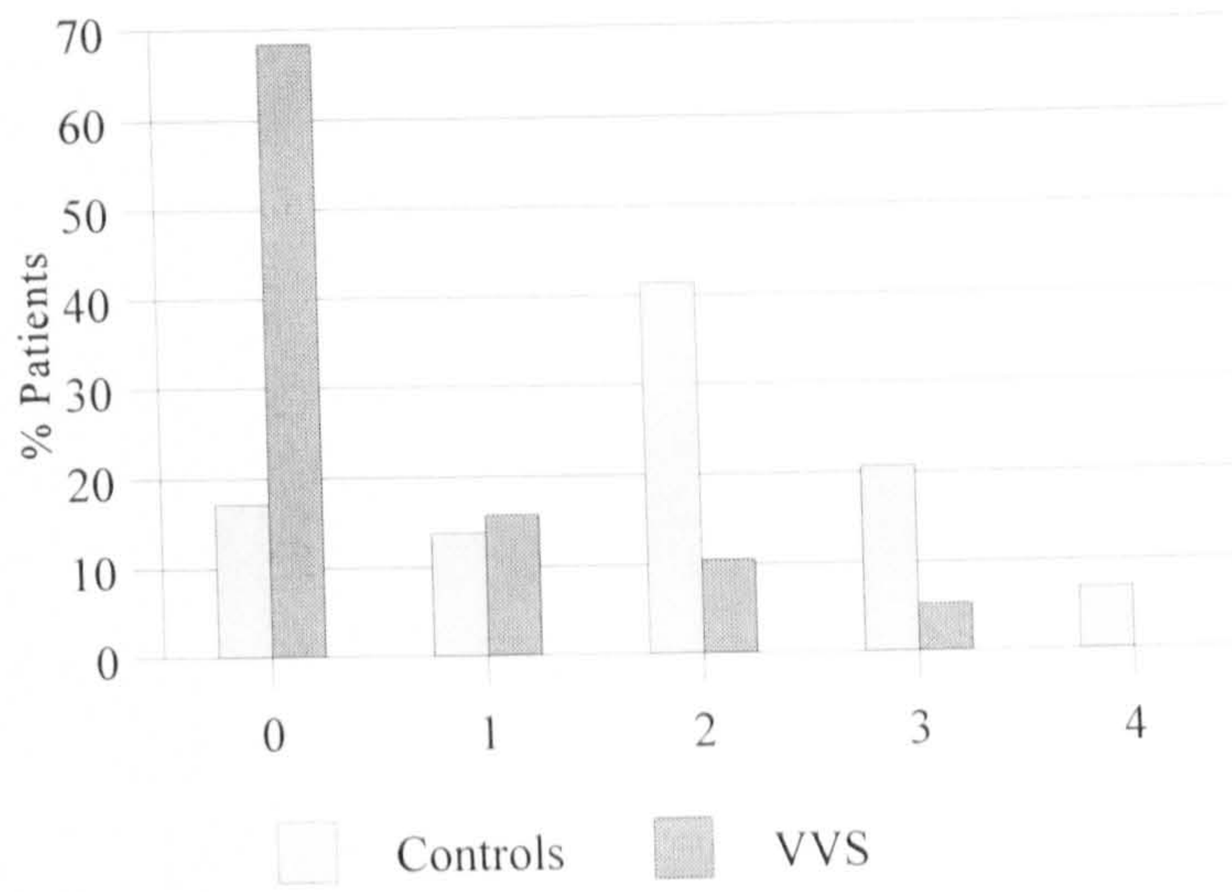
5
0
15
16
17
18
19
20
21
25
27
Age at 1st Sexual Intercourse
Controls
VVS

Figure 3.5 Number of children born to VVS and control patients.

Figure 3.6 Past irritations. Patients were asked whether they had experienced any irritation in the past, and if so what was the location of the irritation.


70
60
50
40
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10
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% Patients
0
1
2
3
4
Controls
VVS

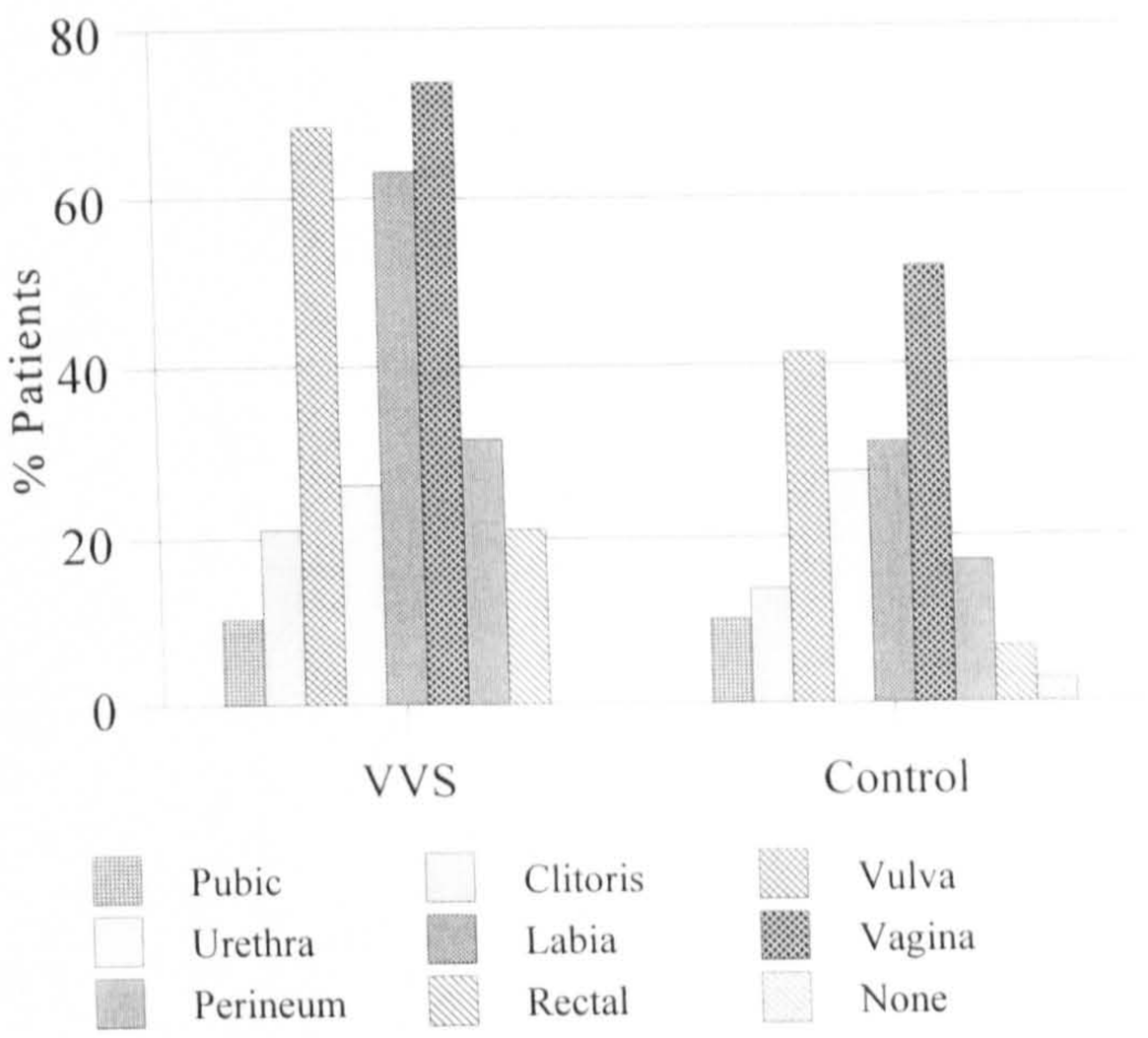

80
60
40
20
0
% Patients
VVS
Control
Pubic
Urethra
Perineum
Clitoris
Labia
Rectal
Vulva
Vagina
None

Figure 3.7 Action of control and VVS patients. Patients were asked whether they treated themselves for genital irritation. If they did not treat themselves, who did they consult? The responses of both groups of patients were very similar. However, more VVS patients were reported to have suffered irritation than control patients. If patients consulted their GP did he take a swab for microbiological identification?

1 = Self treatment of irritation

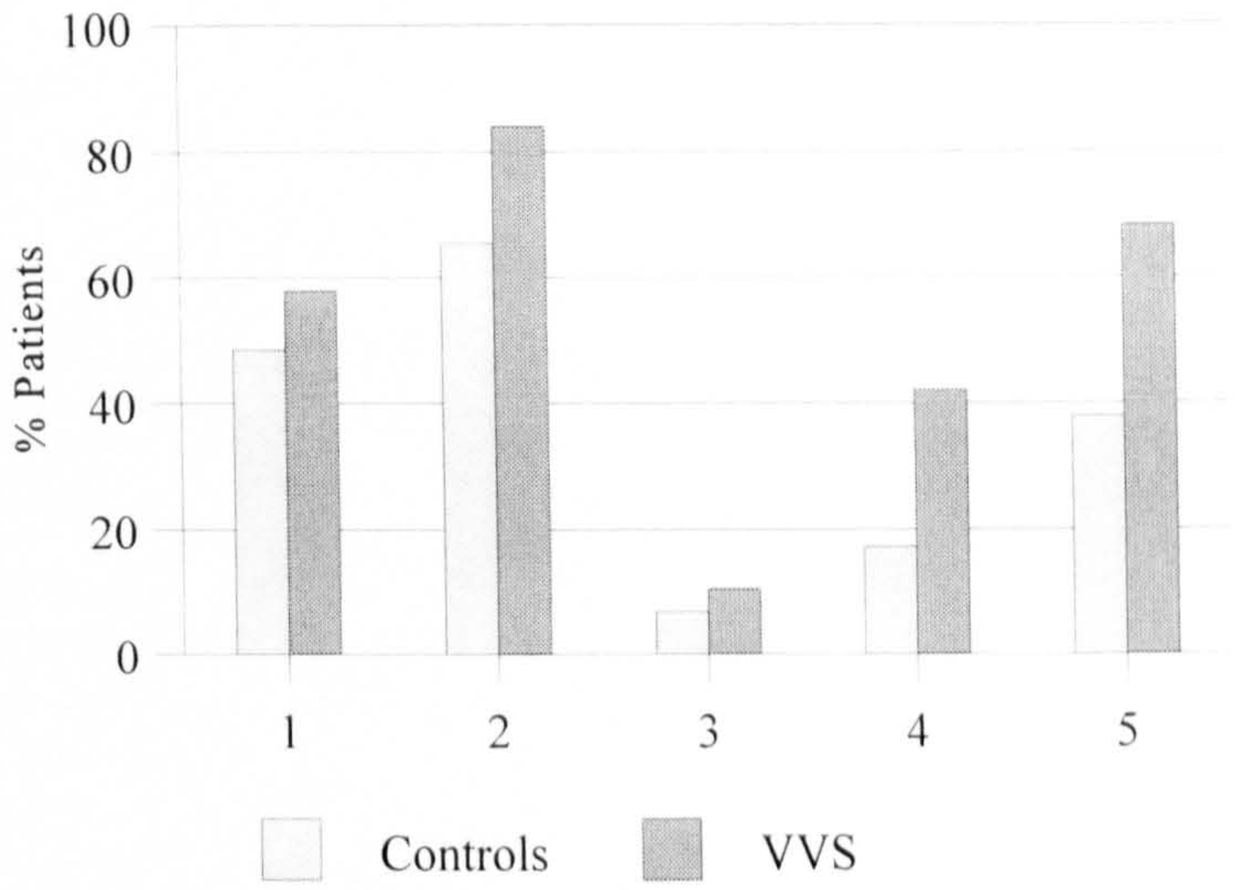
2 = Consulted GP

3 = Consulted a chemist

4 = Attended a G.U.M. clinic

5 = Did the GP take a swab?

Figure 3.8 Documented past infections. Patients were asked to report past infections which were documented by their GP or G.U.M. clinic.


100
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20
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% Patients
1
2
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4
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Controls
VVS

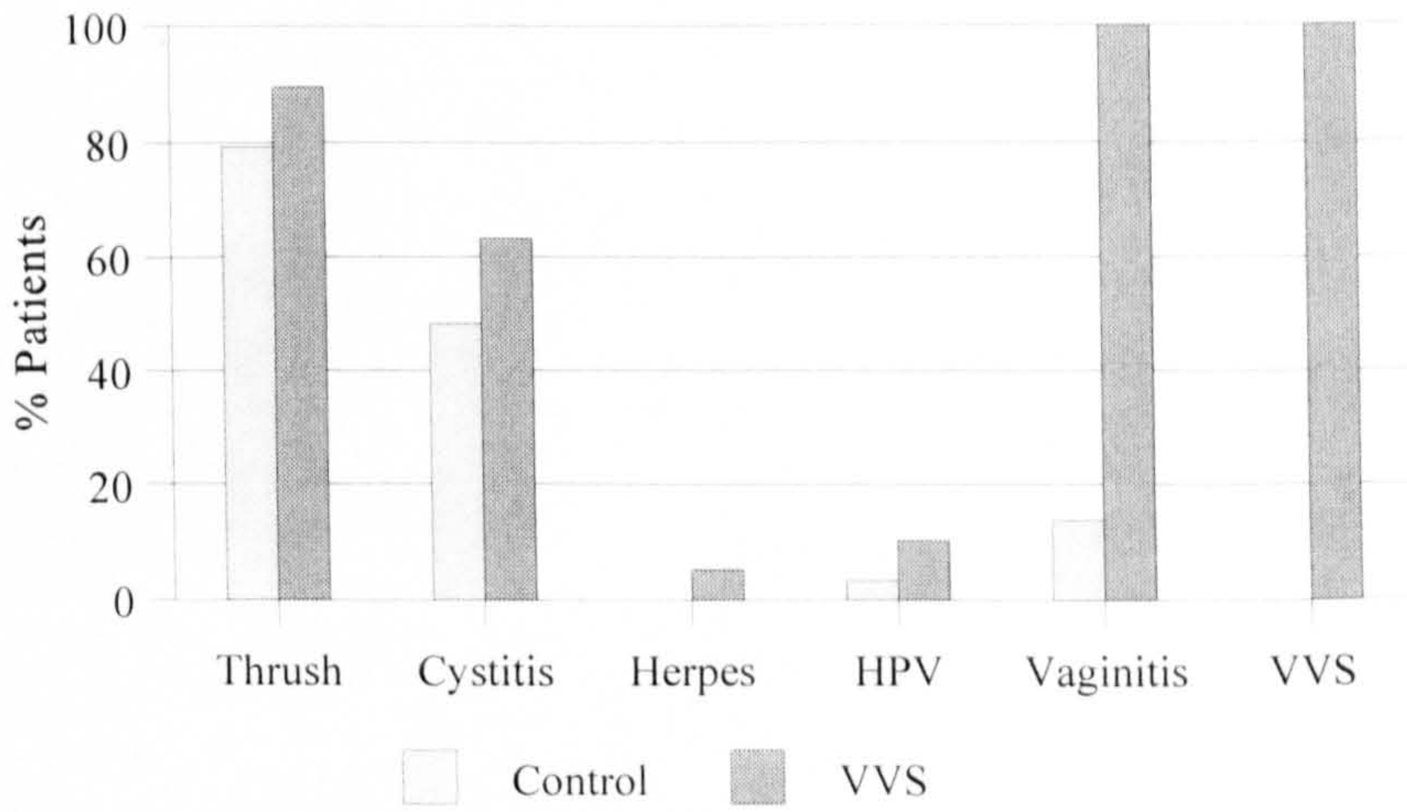

100
80
60
40
20
0
% Patients
Thrush
Cystitis
Herpes
HPV
Vaginitis
VVS
Control
VVS

Figure 3.9 Action of VVS patients. VVS patients were asked about their course of action when they developed the symptoms of VVS. Patients were asked several questions regarding their diagnosis:

Did you attended a G.U.M. clinic?

Did you consulted your GP?

Did tour GP examine you?

Did your GP diagnose VVS?

Figure 3.10 Treatment of VVS patients by their GP. VVS patients were asked to comment on how they were treated by their GP on their initial, and subsequent visits, and on the effectiveness of the treatment prescribed.

1 = GP treated symptoms

2 = Treatment ineffective

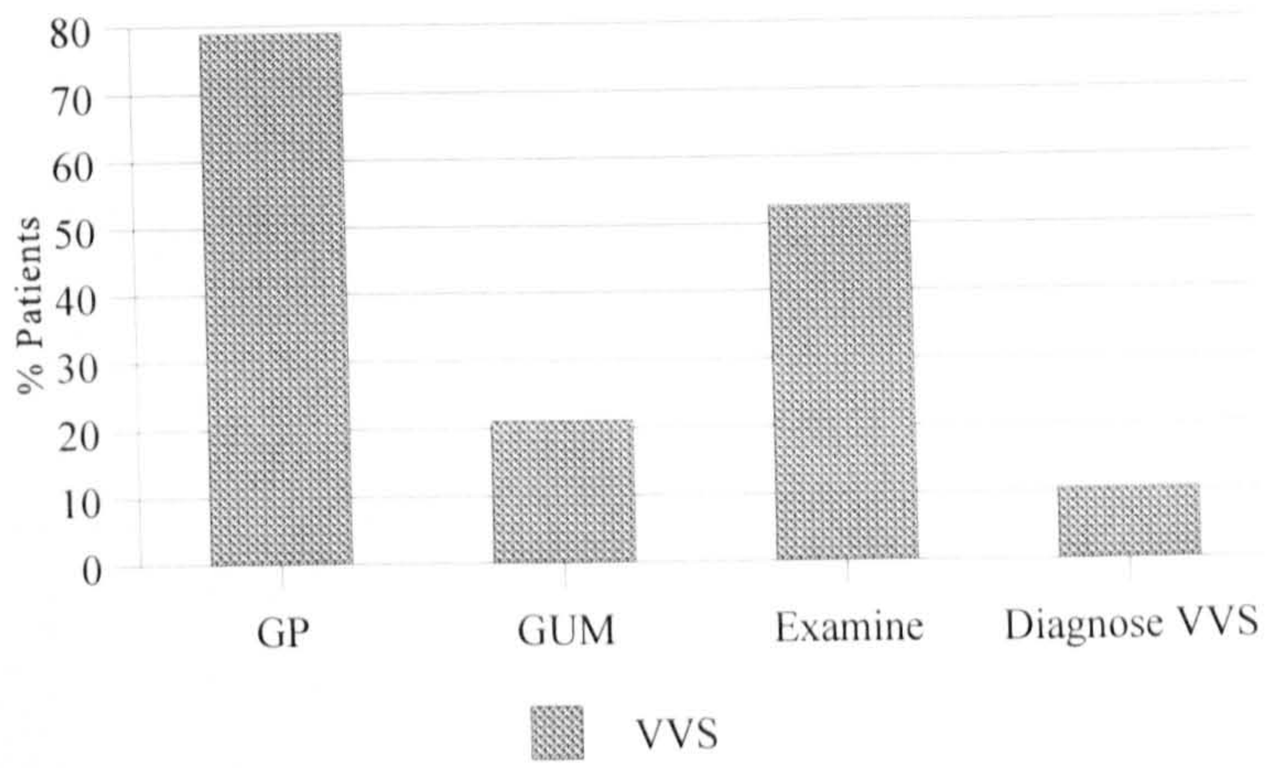
3 = Returned to GP

4 = More ineffective treatment

5 = Referral to gynaecologist / G.U.M.

6 = GP was not helpful

7 = Consulted other GP's


80
70
60
50
40
30
20
10
0
% Patients
GP
GUM
Examine
Diagnose VVS
VVS

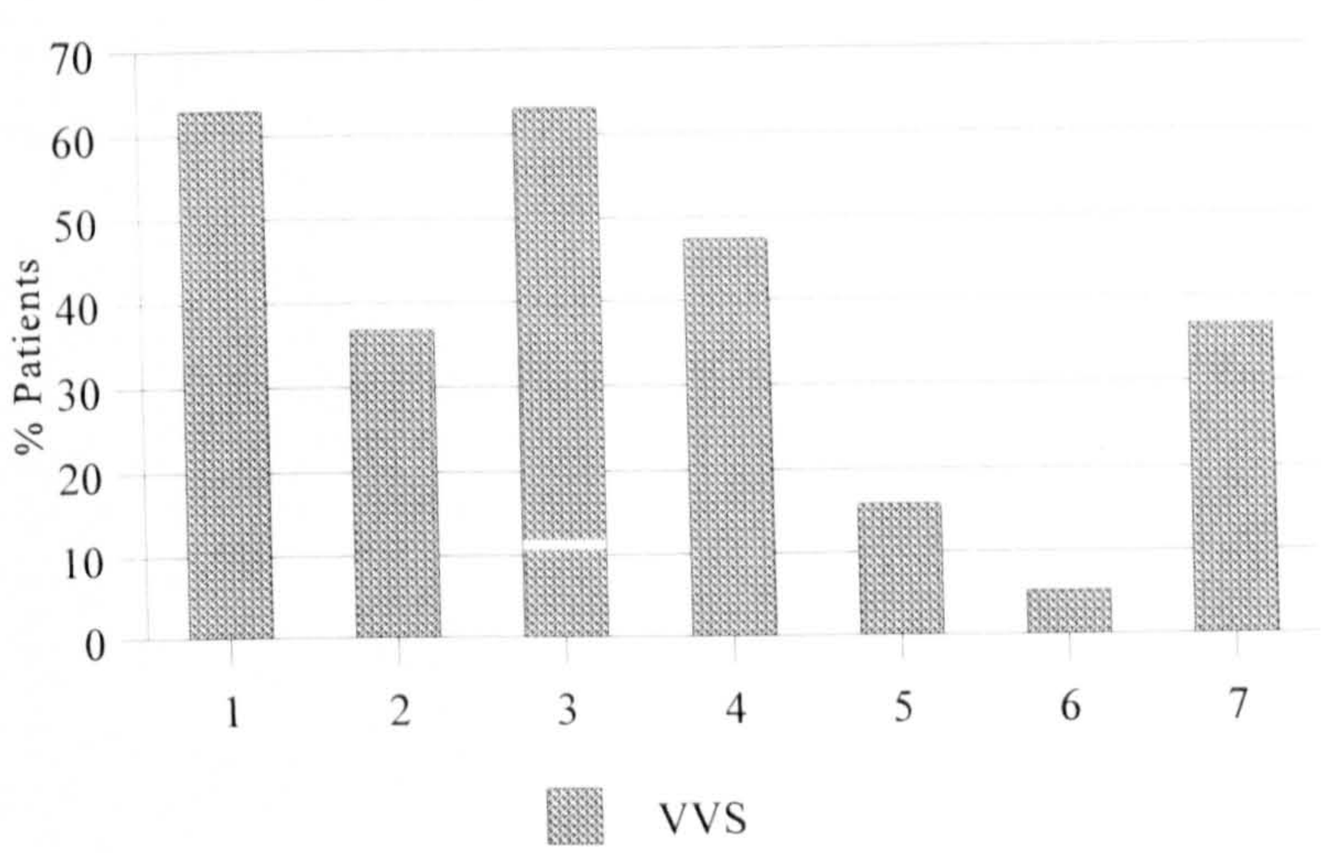

70
60
50
40
30
20
10
0
% Patients
1
2
3
4
5
6
7
VVS

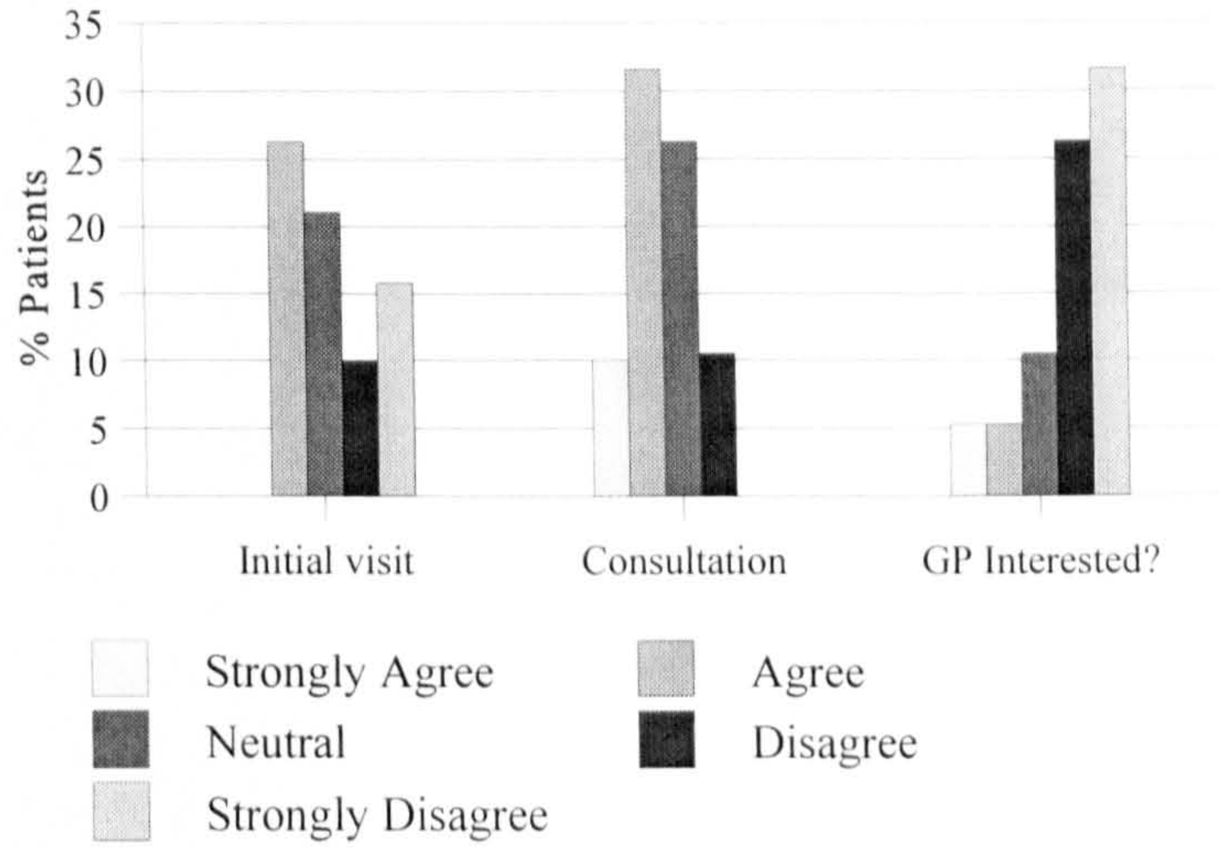
Figure 3.11 Assessment of treatment by the GP. Patients were asked to respond to 3 statements:

1. I was completely satisfied with my initial visit to my GP.

2. Aspects of my consultation could have been better.

3. The GP was interested in how the symptoms were affecting me.

Figure 3.12 The duration of the symptoms associated with VVS.


35
30
25
20
15
10
5
0
% Patients
Initial visit
Consultation
GP Interested?
Strongly Agree
Agree
Neutral
Disagree
Strongly Disagree

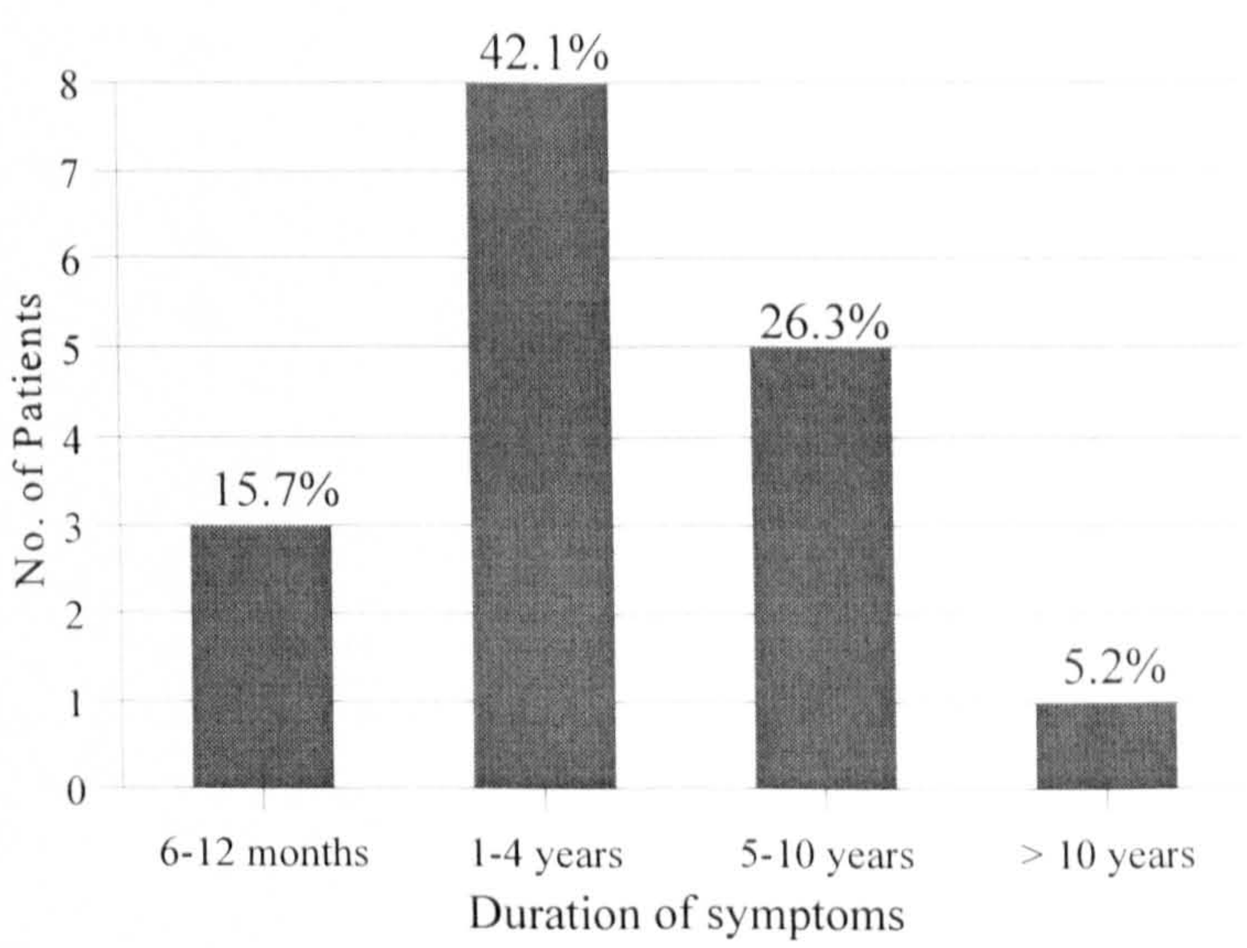
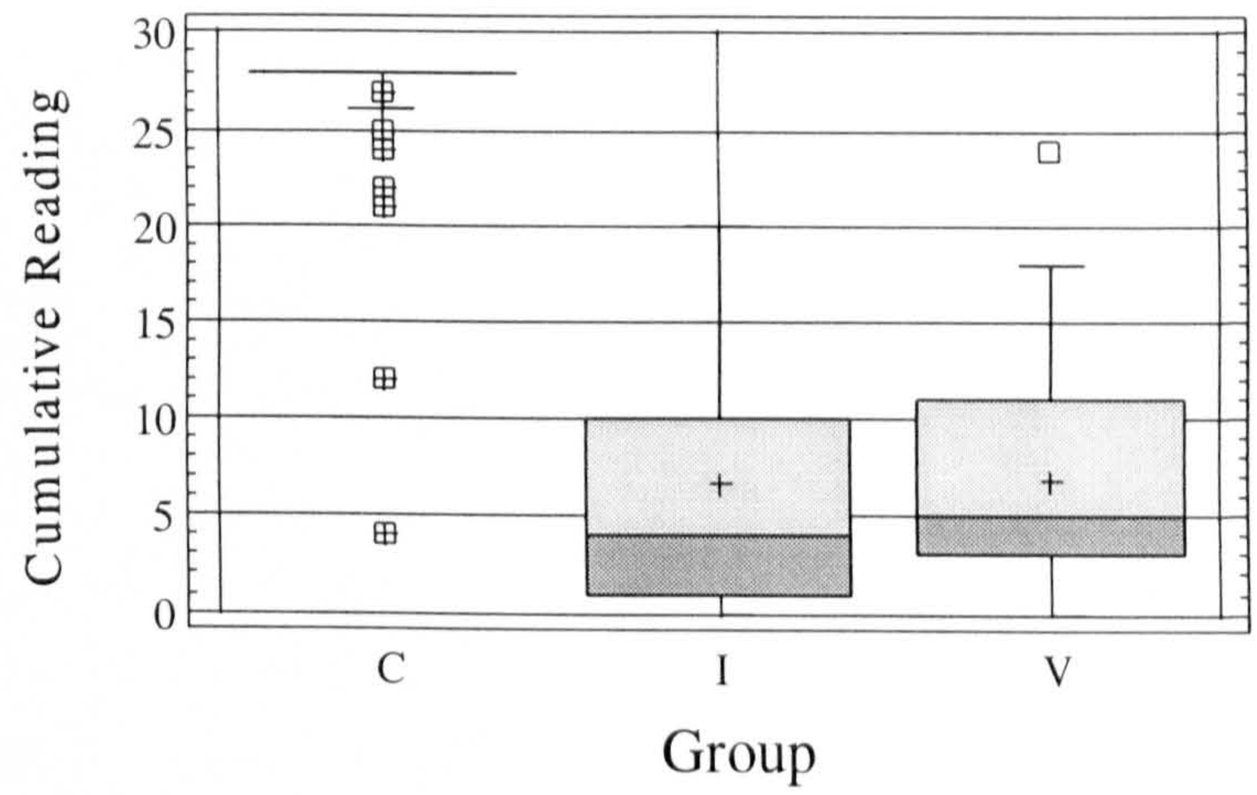

8
7
6
5
4
3
2
1
0
No. of Patients
15.7%
42.1%
26.3%
5.2%
6-12 months
1-4 years
5-10 years
> 10 years
Duration of symptoms

Figure 3.13 'Box and whisker' plot of initial cumulative readings from control and VVS patients (month =1, initial assessment). Two groups of VVS patients are included, patients who received additional treatment with imipramine (I), and those who received only ketoconazole (V). There is a highly significant difference between the readings of the control and VVS patients in both groups (Control n=47, VVS (I+V) n=60). Refer to Appendix 6 for an explanation of the Box & Whisker Plot.

Mean Value	V = 6.7	I = 6.9	C = 26.1
Standard Deviation	V = 5.89	I = 6.55	C = 4.81

Figure 3.14 Cumulative readings taken at month two (2nd assessment). There is no difference in the readings in groups I and V. Control patients were not assessed.Refer to Appendix 6 for an explanation of the Box & Whisker Plot.

Mean value	V = 11.6	I = 13.0
Standard Deviation	V = 7.78	I = 7.82


Cumulative Reading
30
25
20
15
10
5
0
C
I
V
Group

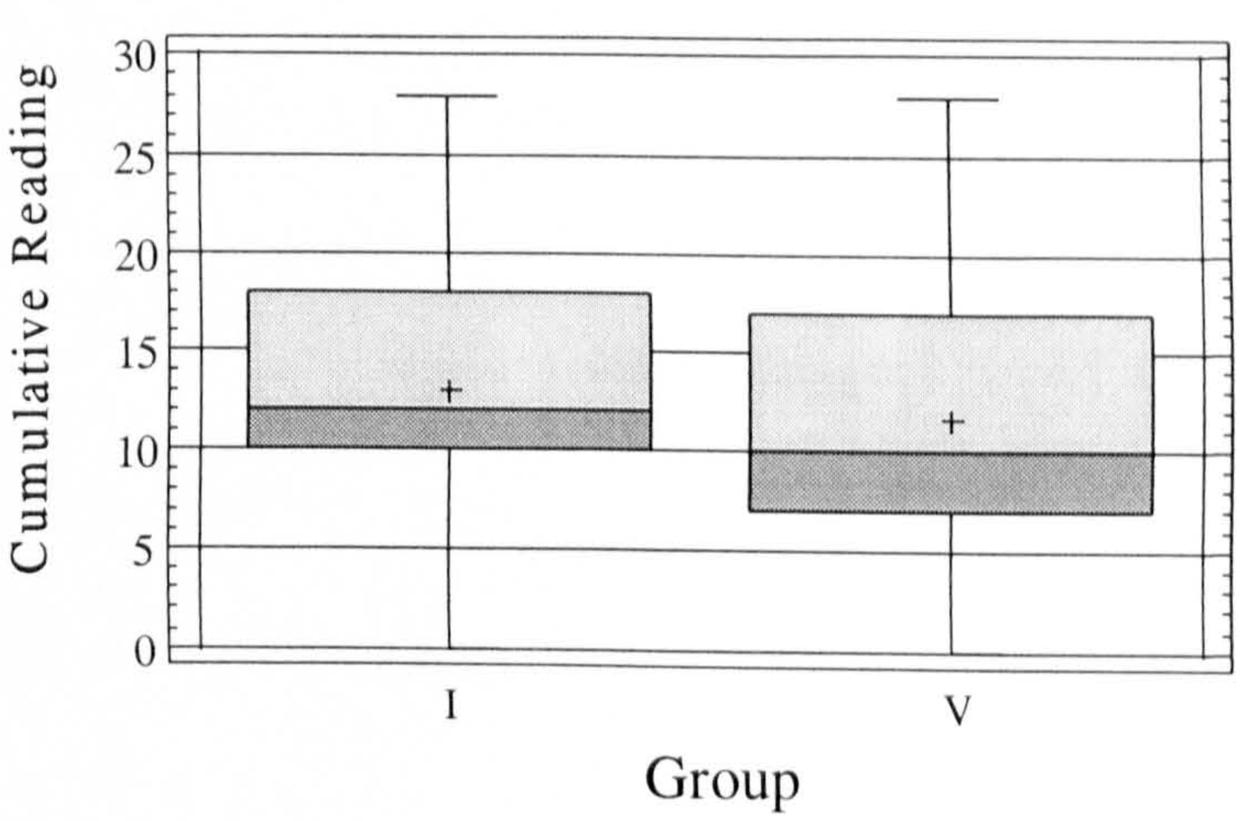

Cumulative Reading
30
25
20
15
10
5
0
I
V
Group

Figure 3.15 Cumulative readings from VVS patients treated with ketoconazole (V), and patients receiving imipramine in addition to ketoconazole (I). There is no significant difference in the cumulative readings between the two groups at month=3 (3rd assessment). Refer to Appendix 6 for an explanation of the Box & Whisker Plot.

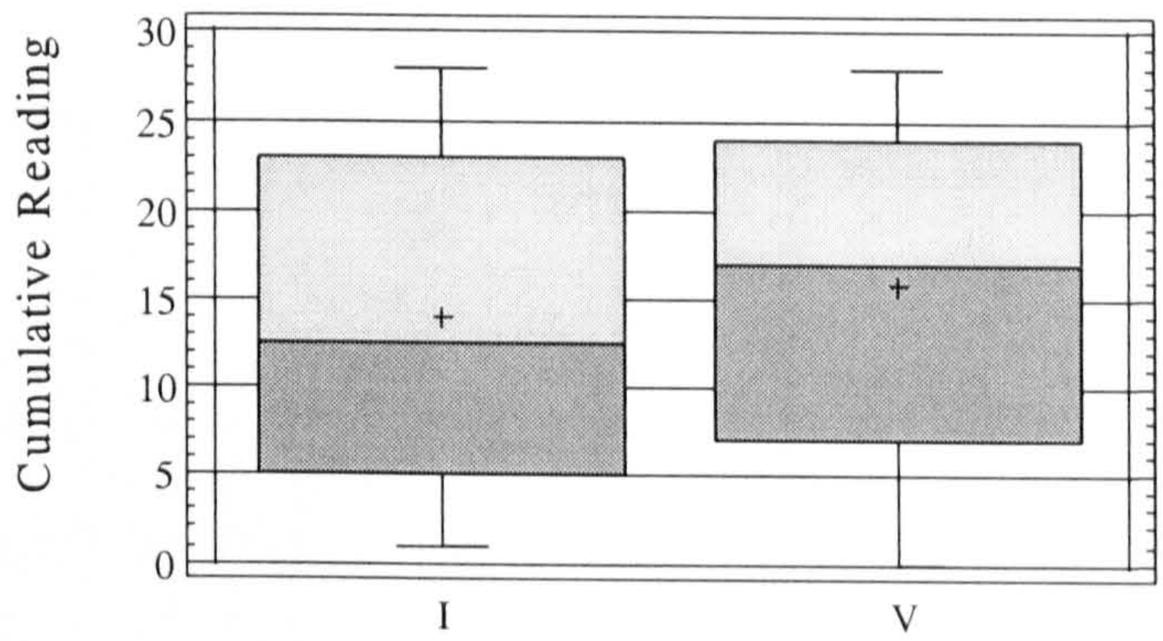
Mean	I = 14.06	V = 15.70
Standard Deviation	I = 10.01	V = 9.1

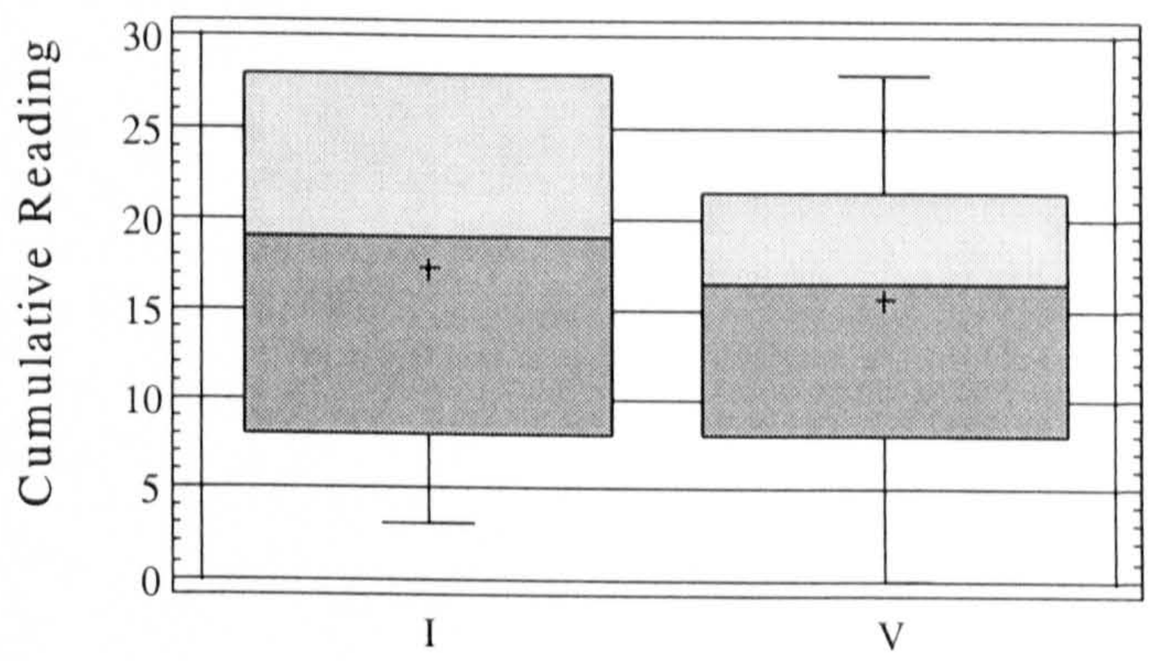
Figure 3.16 Cumulative readings from VVS patients at month four (4th assessment). Refer to Appendix 6 for an explanation of the Box & Whisker Plot.

Mean	I = 17.36	V = 15.36
Standard Deviation	I = 9.23	V = 8.28

Figure 3.17 Cumulative readings after four months of treatment (month=5). There is no significant difference in the two groups response to treatment. Refer to Appendix 6 for an explanation of the Box & Whisker Plot.

Mean	I = 18.2	V = 17.5
Standard Deviation	V = 8.5	V = 8.3


Cumulative Reading
30
25
20
15
10
5
0
I
V
Group


Cumulative Reading
30
25
20
15
10
5
0
I
V
Group

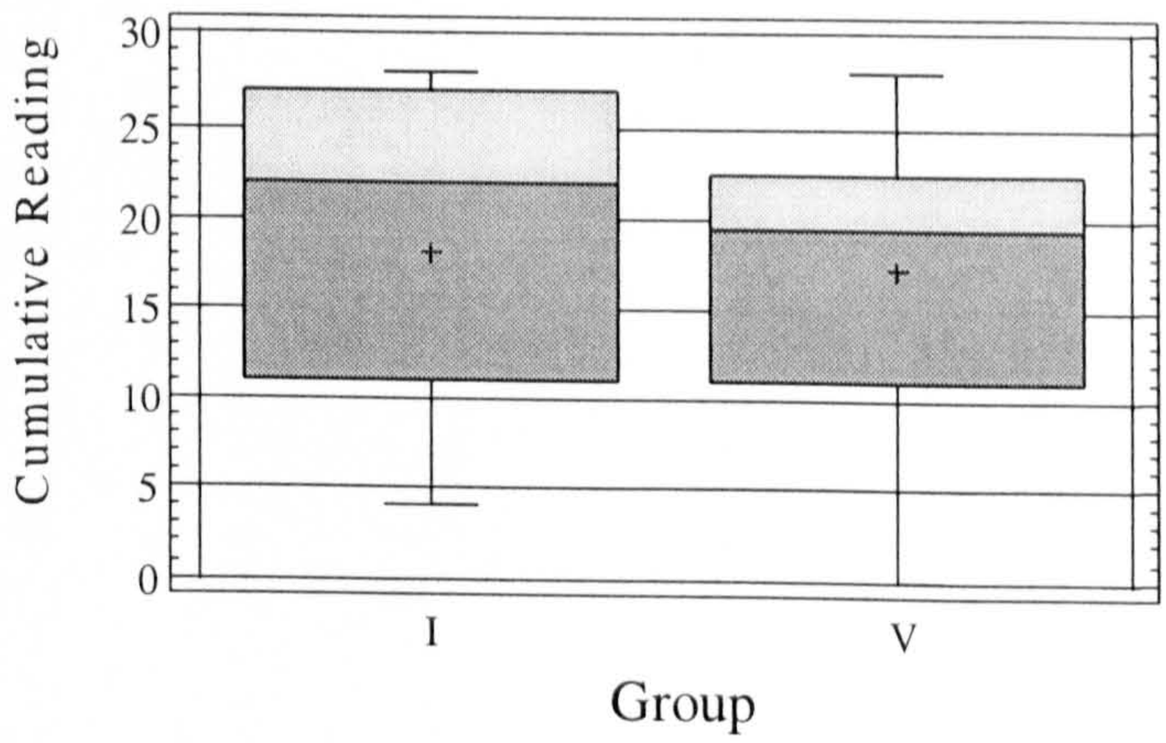
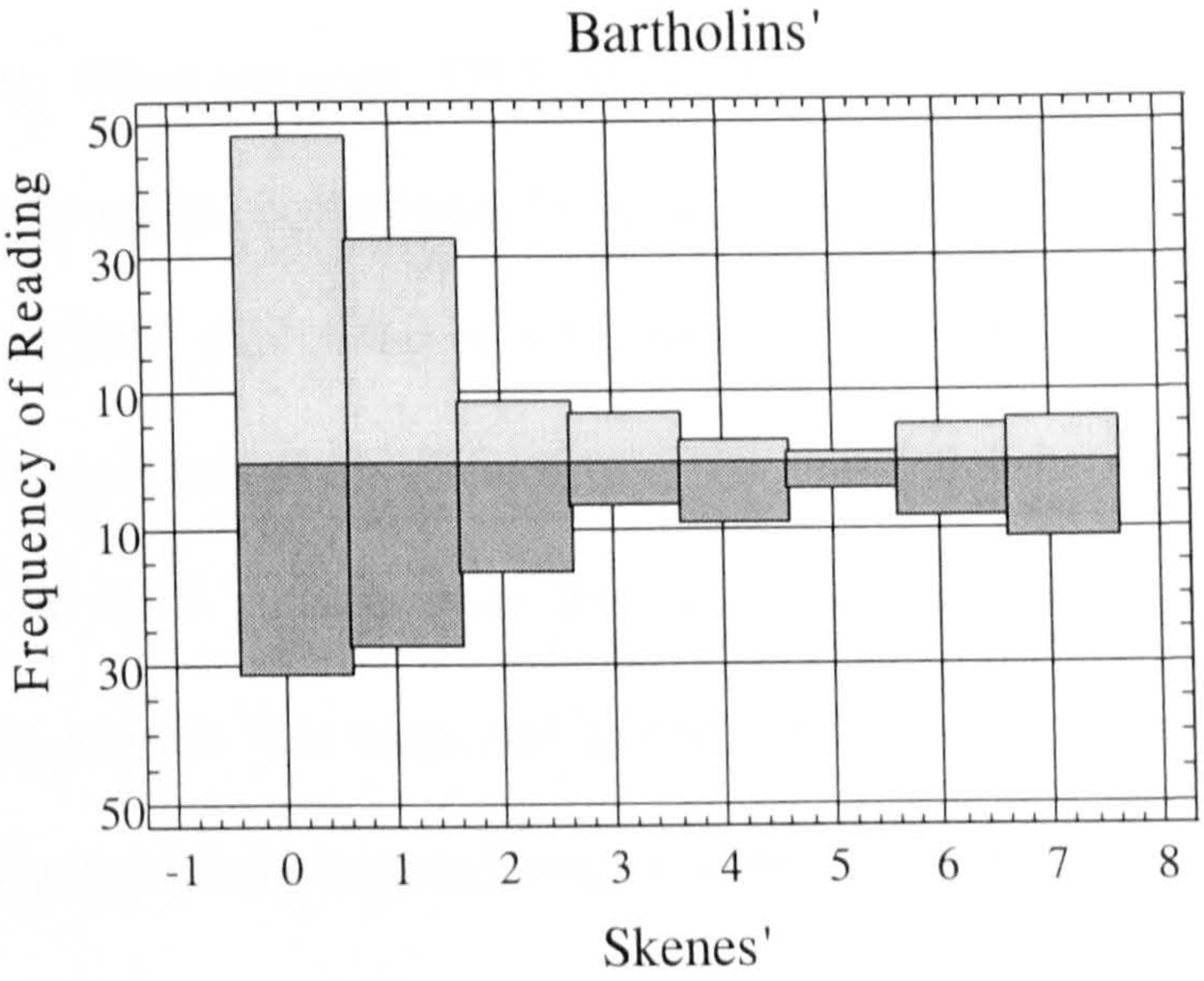
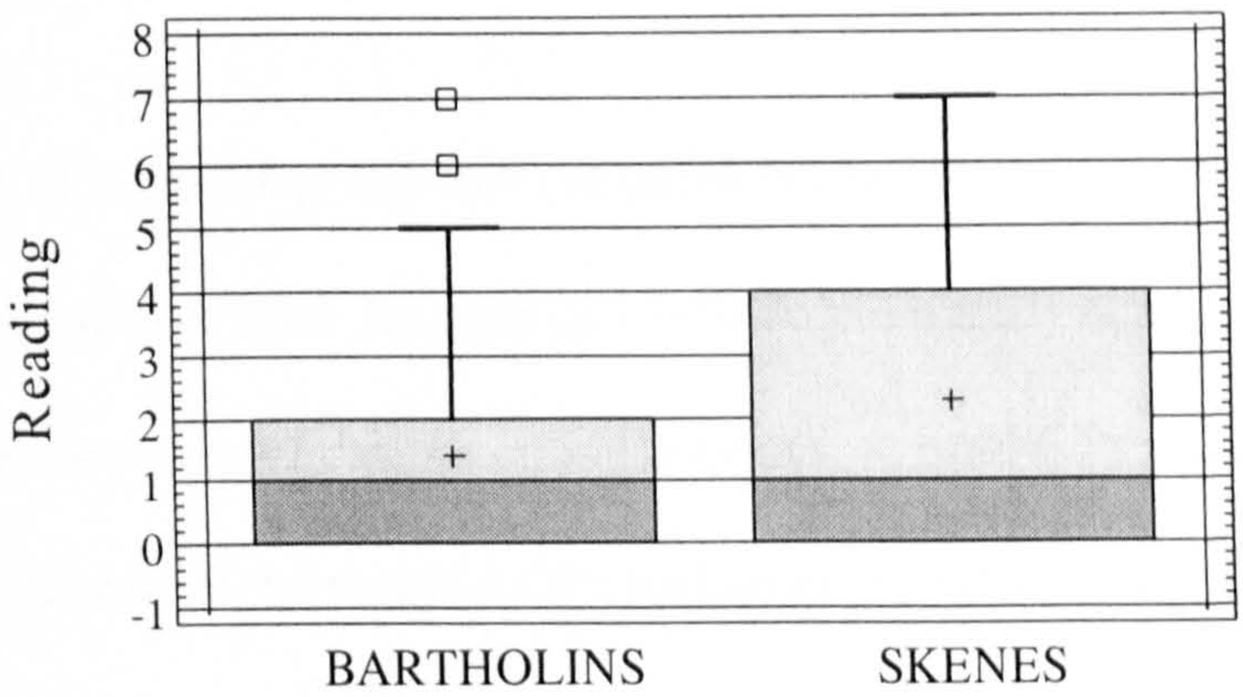

Cumulative Reading
30
25
20
15
10
5
0
I
V
Group

Figure 3.18 Algesiometer readings taken from the Skenes' and Bartholins' glands on the initial assessment. There was a significant difference between the two groups, generally the readings from the Bartholins' glands were lower than those obtained on the Skenes' glands.

Figure 3.19 'Box and Whisker' plot of the initial readings from the Skenes' and Bartholins' glands.


Bartholins'
50
30
10
10
30
50
Frequency of Reading
-1
0
1
2
3
4
5
6
7
8
Skenes'


Reading
8
7
6
5
4
3
2
1
0
-1
BARTHOLINS
SKENES

3.4 Discussion

3.41 Epidemiology of VVS and control patients

Many of the studies completed on VVS have included assessment of pain, and characterisation of the symptoms associated with the syndrome (Woodruff & Parmley, 1983; Peckham *et al.*, 1986; Friedrich, 1987; Pyka *et al.*, 1988; Turner & Marinoff, 1988; Furlonge *et al.*, 1991; Goetsch, 1991; Wilkinson *et al.*, 1993; Marks *et al.*, 1995). However, very few investigations have included a suitable control population as a comparison for epidemiological characteristics (Bazin *et al.*, 1994). Our group of VVS patients were of a similar age to those reported in previous investigations, with a majority of patients being between the ages of <20-25. The distribution of VVS patients over five or six decades was commonly reported in past investigations, and this was supported by our results which indicated one women of 54 years. Some investigations have reported women with VVS in the 7th decade, however the median age of the syndrome is usually quoted to be approximately 36 years which is similar to the results obtained in this investigation, where the mean age was 27.7 years (Friedrich, 1987; Wilkinson *et al.*, 1993; Marks *et al.*, 1995; Bergeron, *et al.*, 1994, Baggish *et al.*, 1995).

The inclusion of a control group of women for comparison of the epidemiological data proved difficult, as women between the ages of 17-50 do not usually attend their GP on a regular basis, apart from the necessity of contraceptive services. A rural GPs practice was suggested for the investigation, as one of the resident GPs had an excellent working relationship with the female patients. Patients between the ages of 18-40 were contacted by the GP personally and asked whether they would participate in the study, all patients who were approached were pleased to co-operate. In comparison, the inner city practice chosen for its location, and range of socio-economic groups did not prove to be as useful. The GPs at the inner city practice consented to patients being approached by their receptionist when they phoned to book an appointment. Although this arrangement worked adequately in the initial stages of the study, the receptionist

was obstructive and unreliable, and did not remember to recruit patients for the investigation. Consequently, as a suitable number of patients were recruited from the rural practice, this became the sole centre for control patients. The age range of the control patients was very similar to that of the VVS study group, however, when examining the age distribution, it became obvious that the VVS group were on average much younger. Of the control patients, 88% were between the ages 25-35+, over half were 30+, and there were no patients in their 5th or 6th decade (Fig. 3.2). The difference in age between the control and VVS groups was notable and it was anticipated that this difference would become apparent in some of the other sections of the questionnaire, particularly in the sections regarding pregnancy and contraception.

Several past investigations have suggested that the incidence of VVS may be linked to the age of the woman at first sexual intercourse (Bazin *et al.*, 1994). Our study showed findings confirming that the age of first sexual intercourse is probably not related to the onset, or the initiation of the symptoms of VVS. Results indicated that the control group became sexually active slightly earlier than the VVS group, however, this was only a pilot investigation and would need to be repeated with a larger sample number in order for a statistical analysis to be completed (Fig. 3.4). Bazin *et al.* (1994) showed that the relative risk of developing VVS was increased in women who became sexually active before 15 years. This study showed a 3.3 fold increase in relative risk, if women became sexually active at 15 years, compared with 16 years or later. Bazin *et al.* (1994) also indicated that women with early menarche also tended to have a higher risk of developing VVS. Control patients in the present investigation were found to start menstruating later than the VVS patients, 76% of VVS patients compared with 51% of control patients began menstruating before the age of 13 years.

The number of children from both VVS and control patients were as expected. The control group of women being slightly older were considered more likely to have had children than the

VVS group. In fact 63.2% of VVS patients did not have children, compared with 17.2% in the control group. Past studies have indicated that a percentage of VVS cases develop post partum (Goetsch, 1991), in the present investigation only 36.8% of patients had given birth, with only 21.1% of patients had experienced any lasting discomfort due to childbirth. Problems using tampons (5.3%) or having sexual intercourse (10.5%) were not common post partum in the present investigation. Goetsch (1991), refers to a group of patients who developed the symptoms of VVS post partum, this group of women were described as having secondary, rather than primary VVS. Goetsch (1991), separated patients into 1^0 and 2^0 cases, as there were obviously two main groups of patients in her study group. Those with primary VVS were often nulliparous, with a history of particularly intense pain and a strong familial history. In comparison, secondary or 'acquired' VVS was described as having a post partum onset, and is associated with infections or irritant reactions, and does not appear to have a genetic link (Goetsch, 1991). Women with secondary VVS, constituted 21% of the VVS population in Goetschs' study, and were therefore a significant sub-group of patients. This group of patients also reported that the worst period of pain was immediately after giving birth, after which the pain had lessened but did not resolve (Goetsch, 1991).

A majority of the women in this investigation, and in past studies, have not had periods of vulvar pain followed by spontaneous remission, findings which are contrary to the report by Peckham *et al.* (1985), who reported that there was a remission rate of 50% in their population. If this was the case, it is probable that there would be a notable number of women in either the control and VVS groups, reporting periods of vulvar pain in the past which spontaneously resolved. The large number of women with primary VVS both in our investigation and that of other authors (Goetsch, 1991; Bazin *et al.*, 1994), suggests that there may be an inherent variation in the sensitivity of the vestibule in women; and that many women may suffer with varying degrees of VVS, without having had an irritant reaction to a chemical or infection.

Consistent with most other studies, all of the patients in this investigation were white. Studies in the USA have indicated a prevalence of the syndrome in whites, the syndrome is rarely reported in ethnic minorities and Afro-Caribbeans. Goetsch (1991), studied a group of 210 women attending a vulvar clinic, 40 patients were diagnosed as having VVS or borderline VVS; of these patients one was Japanese, and another was Chinese. Furlonge *et al.* (1991), reported similar findings in their study group of 24 VVS patients, 22 were Caucasian, one was Mediterranean and another was Afro-Caribbean. Similar findings have been reported previously by Peckham *et al.* (1986), and Friedrich (1988). It has been suggested, that the predominance of the syndrome in Caucasians, may be due to a predisposition to the cause of the syndrome, or alternatively due to socio-cultural differences in response to disease. Alternatively, there may be a difference in presentation of the condition linked to social class. Friedrich (1987), comments that the "predilection for a single race in a majority of cases, makes an infectious agent unlikely as the only cause of the syndrome".

Other authors have reported that the syndrome is more common in social classes I and II (Furlonge *et al.*, 1991; Goetsch, 1991). These studies do not say how they classed their patients, so it is difficult to compare our data with information from these investigations. Social class may be assessed by: the patients occupation; the partners occupation; the area in which you live; or your income. The group of women in the present study were classed according to their occupation using the 'Classification of Occupations' (HMSO, 1980). Problems were encountered as women were classed by job title. For a more accurate assessment the full job description would be required. In contrast with previous studies, the present investigation showed a prevalence of the syndrome in classes III (manual and non- manual) and VI, rather than in I & II as reported in other studies. The group of patients classified as group VI included women who described themselves as housewives or not in employment. This discrepancy in social class, may be related to the class system in the USA where the majority of work on VVS

is undertaken. However, as differences in class ascription vary between the USA and the UK, this data is extremely difficult to interpret.

Consistent with the findings of Bazin *et al.* (1994), a significant number of the VVS patients in the present study were using oral contraceptives (OCs). Bazin *et al.* (1994), associated the use of OCs at an early age, with a greater risk of developing VVS, when compared with patients who have never used OCs. The relative risk of developing VVS was not reported to alter with the duration of use, or the strength of the pill. In the present study 60% of the VVS study group were using OCs compared with 38%of the control group. Despite this significant difference, the variation in contraceptive use may be linked to the average age of the patients, which was higher in the controls than in the VVS group. Many of the patients in the control group (82.8%) had already had children, compared with only 36.8% of the VVS group. Frequently, after having a family, control patients reported a change in their contraception from OCs to another method. Commonly, control patients used the cap or the coil, or alternatively, they or their partners were sterilised, all of which may account for the difference in contraception in the two groups. A direct comparison of these two groups of patients is difficult, as the patient numbers are limited, and the majority of patients were using OCs.

All patients attending the G.U.M. clinic in Plymouth were advised to use non-perfumed soaps and detergents, and to use non-biological washing powders in order to minimise the chance of allergic or irritant reactions. Despite this advice, less than half of the VVS patients (40%) used non-biological powders. However, only 5% of VVS and 13.8% of controls had ever experienced any discomfort or irritation from detergents, soaps, or cosmetic products. In comparison, a larger number of patients in both groups had suffered with irritations due to an infection. Irritation of the pubic, clitoral, urethra and rectal areas were similar in both groups. Differences were however apparent in the number of patients who had suffered with vaginal,

vulval and labial irritation. Vestibulitis patients were expected to report intense vulvar irritation, however, only 68.4% described irritation or discomfort in the vulvar area. More commonly (73.7%), VVS patients described vaginal irritation, which suggests that either, they are unable to pinpoint the source of the problem, or that they have actually had vaginal discomfort due to an infection such as *Candida*. It became apparent whilst interviewing both VVS and control patients, that both groups had difficulty describing discomfort, and reporting the location of past irritations. Diagrams were provided for this purpose but did not appear to be of much assistance in a majority of cases. Notoriously, pain perception is unclear in these patients, which is also complicated by the fact that a degree of pain referral may also be occurring. Irritation of the labia minora was described in 63.2% of VVS patients, which is probably a more accurate description of the location of the pain than those patients who reported vulvar discomfort. The labia minora blend with the vestibule of the vulva at Harts' line on the inner aspect of the labia, this is the junction of keratinised skin and mucosal-like epithelium. In comparison, control patients did not commonly describe irritation in these areas; vaginal irritation was commonly reported (51.7%), whereas vulvar and labial irritation were less frequently described (41.4% and 31.0%).

As reported, the patients' description of their symptoms related to irritation also proved problematic, many control and VVS patients were unable to describe past symptoms clearly. Consistent with the findings of other authors, pain was commonly described as 'burning', and was reported by 36.8% of VVS patients, but only 10.3% of control patients. In comparison 'itching' was reported in 36.8% of VVS cases and 62% of controls. Itching is a different physiological process when compared to burning (see Chapter 2).McKay (1988) reported that the differentiation between patients who describe itching and those who describe burning is important in the treatment of vulvodynia and VVS. Despite this, patients found it difficult to describe their symptoms and to differentiate between itching and burning.

It was interesting that only 21.0% of VVS patients described post-coital pain, as this is often an additional criteria used for the diagnosis of VVS. This figure was expected to be greater as a majority of VVS patients in the present study had described pain during and after sexual intercourse when interviewed by their consultant. Frequently, VVS patients appeared to find describing their symptoms more difficult than control patients. Many VVS patients described their pain as general rather than being located to a particular area (73%). This may be due to the structure of the question, or the diagrams used. However, the pain associated with VVS does not appear to be as well defined, for example, as the itching reported with *Candida* infection.

An investigation into the frequency of self treatment for irritation was included for several reasons. The availability of 'over the counter'preparations (OTC) for *Candida* was expected to affect whether patients treat themselves or consult a GP / G.U.M. clinic; alternatively, the use of OTC preparations may affect the onset of VVS by initiating an allergic or irritant reaction. The true incidence of VVS may be masked by patients repeatedly treating themselves for what they believe to be 'thrush'. Goetsch (1991), reported an incidence of VVS of 15% in her gynaecological practice population, which is much greater than anticipated by other authors (Friedrich, 1987). This is probably due to many patients not consulting their GP regarding vulvar discomfort, especially if the condition is not severe, and sexual relationships are not adversely affected. The aim of this investigation was to determine: what patients were using to treat themselves; whether the self treatment was successful; and if the treatment was unsuccessful did they go to a GP or G.U.M. clinic? It was interesting that approximately 50% of both the control and VVS groups had attempted to treat themselves in the past before going to their GP. When having symptoms associated with *Candida,* 36.8% of controls and 41.4% of VVS patients attempted self treatment. Recurrence of the symptoms occurred in 6.9% of controls and 21% of VVS patients. These results suggest that the control group were probably treating *Candida,* which is generally able to be treated using OTC preparations. In comparison,

a majority of the VVS group may have been trying to treat the symptoms of VVS thinking they had *Candida*. It is probably true to say, that a small percentage of VVS patients do have a yeast infection when they present at their GP, and therefore, these patients respond well to OTC anti-fungal preparations.

VVS patients found it extremely difficult to distinguish between the symptoms they previously described as thrush, and their current symptoms, which they now know to be related to VVS. Many VVS patients commented that in hindsight, past diagnoses of thrush were probably not in fact thrush but VVS, as on most occasions, infections were not confirmed by microbiological testing. Less than half of the VVS patients said that they had attempted to treat themselves for vulvar burning associated with VVS, however, those patients who did try to treat themselves did not have any success in the long term. From this investigation it was evident that nearly half of all GPs did not examine patients when they presented with vulvar pain. This may be due to time, they way in which patients described the symptoms, or the routine procedure of the practice. However, when patients present at their GP with undiagnosed vulvar discomfort, an internal examination should be completed if physically possible, and a swab should be taken in order to exclude infections. Although a colposcope is deemed necessary for the visualisation of the gland openings, several authors have commented that it is possible to see erythema of the Skenes' and Bartholins' glands with the naked eye. A routine examination will also determine whether any abnormal discharge is present which is not indicative of chronic VVS; or whether any fissuring of the vulvar skin has occurred, resulting in vulvar discomfort.

Only 10.5% of the VVS patients in this investigation were diagnosed as having VVS by their GP. Generally GPs gave a diagnosis of thrush (57.9%), however, it was interesting that a majority of GPs attempted to treat the symptoms of VVS without knowing what etiological agent(s) they were treating. Consequently, only 26.3% of treatment had a long term effect on

the symptoms of VVS. This may indicate that there is a subset of acute VVS patients who have *Candida* infections. This suggests the importance of an examination prior to treatment, as long term treatment with topical antifungal preparations is both unfounded, and also potentially aggravating to the patients condition. Despite unsuccessful treatment by a majority of GPs, many VVS patients returned to their GP for further treatment on one, or more occasion. The GPs attitude on subsequent visits was described as unhelpful by many patients, very few of which showed any improvement in their condition with additional treatment. This is a typical situation described by VVS patients in other investigations who have often consulted many GPs, dermatologists and gynaecologists for an explanation of their condition. Many potential VVS patients may be deterred in this way by their GP, and consequently, never receive any treatment for their symptoms. These patients, together with borderline cases of VVS who do not consider their problem sufficient to consult a gynaecologist, probably make up a significant percentage of women who are never recorded as having VVS. If GPs routinely asked questions regarding sexual relationships, and openly discussed problems related to vulvo-vaginal pain during consultations, the true incidence of VVS may be discovered, a point which has been demonstrated by Goetsch (1991). The incidence of VVS is reported to be increasing (Friedrich, 1987), however, this apparent increase in the number of women reporting vulvar pain, is probably not in fact an increase in the incidence of the condition, but more likely, an increase in the awareness of women, who now feel more comfortable discussing such issues with their GP.

Despite being a pilot investigation, several conclusions were able to be drawn from the questionnaire data. As described by other authors, VVS patients often report a burning sensation rather than an itch. Patients with VVS also have a higher usage of OCs and a higher incidence of cystitis. The group of patients in this investigation had no evidence of HPV infection on colposcopic examination, and did not report any past infections of HPV. One of

the most obvious, and disturbing observations from this pilot study, was the number of patients receiving unsatisfactory treatment from their GP, whom in a majority of cases, did not perform an examination and consequently did not identify VVS.

3.42 Assessment of control and VVS patients using the 'Vulvar Algesiometer'

One of the main concerns of vestibulitis patients, which was demonstrated in the epidemiological investigation, is that they can not describe their symptoms, or say how much pain they are feeling, or whether the pain they have is more or less intense or more or less frequent than at their last consultation.

Assessment of 47 patients attending their GP for Well Woman screening, using the 'Vulvar Algesiometer', resulted in 11 of these patients having slightly low, possibly abnormal readings. Two of these patients had readings which were similar to those commonly recorded in cases of VVS. Control patients with initial duct readings of 5 and 6 did not report vulvar pain, and did not have problems during sexual intercourse or when using tampons. These patients were asked if they had ever had any vulvar pain or related problems; one patient recorded an incident whilst riding a bike, which she believed was linked to the development of vulvar pain, another patient was considered by her GP to be psychologically unstable, and consequently was unable to discuss past events. All of the patients declined a consultation at the G.U.M. clinic at that time, and unfortunately, these patients have not been assessed again. It is possible that the remaining nine patients with no reported vulvar discomfort but abnormal vulvar sensitivity may be borderline VVS cases as described by Goetsch (1991).

Vestibulitis patients who were assessed using the 'Algesiometer' had varied responses to the device, but generally found that an assessment of their condition was a helpful addition to the management and treatment of the syndrome. On several occasions, patients described severe

burning, characteristic of VVS, after being assessed using the 'Algesiometer', which was attributed to the direct pressure applied to the duct openings during the assessment. This discomfort was often parallelled to that experienced during sexual intercourse, and was reported to subside over several hours. In comparison with readings from control patients, VVS patients demonstrated statistically different readings on initial assessment ($p<0.0001$) (Fig. 3.13)

On several occasions, imipramine was prescribed for VVS patients, who were in considerable pain and complaining of insomnia due to vulvar discomfort. Imipramine is primarily, a tricyclic anti-depressant, however, in this study, the drug was used solely for its pain killing ability. All patients were informed about the medication they were prescribed; patients taking imipramine were told that in higher doses the drug is an anti-depressant, but in low doses it acts only as a pain killer. In this study imipramine was prescribed at extremely low levels of 10-50 mg, and was not considered sufficient to act as an anti-depressant. However, whether the use of tricyclic anti-depressants has any effect on the psychological well being of the patient has not been determined. The use of tricyclics is common in the treatment of VVS and vulvodynia, and they are also used for the treatment of pudendal neuralgia (McKay, 1895; Turner & Marinoff, 1991).

It was interesting to find that there were no differences in response to treatment in patients who received imipramine in addition to ketoconazole. Patients who were prescribed imipramine appeared to respond more rapidly to treatment than those patients who did not take imipramine (Fig 3.13 - 3.16). However, statistical analysis of the data indicated that these two groups of VVS patients responded to treatment as a homogenous group. A larger study group is required to investigate the effect of low doses of imipramine on the perception of pain in VVS patients. It may be the case that patients treated with imipramine did actually respond better to treatment, or alternatively, the knowledge that imipramine is an antidepressant, may have subconsciously altered the patients perception of pain.

This pilot study primarily aimed to characterise the VVS patients included in the investigation, and to compare the gynaecological histories of these patients with a group of control women. The use of this pilot questionnaire has been particularly useful for this purpose, and has indicated some key areas which would be investigated in more depth in a larger epidemiological study of VVS patients. The histories of the VVS patients were particularly useful in the design of the electron microscopy investigation, where initially, the incidence of HPV was to be investigated. The trial of the algesiometer, also provided some significant results, allowing some tentative conclusions to be drawn.

- It is possible to detect women with VVS from a control population.
- The 'Algesiometer' is useful for monitoring patient progress and for assessment of different treatment regimes.
- It is possible to differentiate between women with vulval pain and control patients.
- Borderline or developing cases of VVS may be detectable.
- There is a difference between the readings of VVS patients pre- and post-treatment

Although the 'Vulvar Algesiometer' has proved to be an important part of the assessment of VVS patients, it was essential to remember that the readings obtained were subjective, and that other factors may also affect the sensitivity of the vestibule when patients are assessed. In conclusion, a measuring device is not only useful for the clinician and researcher when assessing the benefits, or shortfalls of a treatment, but also an excellent source of progress for the patient who often reports that she receives very little information from the consultant. A measurement of progress also helps boost the patients morale, or provides a reason for why the pain has intensified; all information is helpful as recovery may take many months.

CHAPTER FOUR

Ultrastructure of the epithelium of the normal human vulva: a comparison with related epithelial surfaces

4.0 Abstract

An ultrastructural study of the pathology of Vulvar Vestibulitis Syndrome (Friedrich,1987) necessitated a detailed knowledge of the epithelial surface of the normal vulva. A comparison of vulvar, vaginal and keratinised perineal epithelia, confirmed the resemblance of the vulvar epithelium to partially thickened mucosal-like epithelia. No conclusive evidence of keratinisation was observed and the characteristic granular and spiny cell layers found in skin were absent. The prominent cell type was the glycogenated intermediate cell, which formed a structurally homogeneous layer of cells. Other cell types present were: superficial flattened cells, many having nuclei, traces of organelles and large glycogen deposits; supra basal cells, and basal cells, some of which appeared mitotically active and rich in organelles. All cell layers in the vestibular epithelium were characterised by the presence of numerous interdigitating cytoplasmic processes, infrequent desmosomal junctions and relatively pale staining cytokeratin filaments. Granules similar to membrane coating granules, were present in close contact with the membranes of superficial cells. The granules were commonly electron lucent and found in small groups; occasionally granules were found to have electron dense cores. Using SEM, surface projections were identified as microridges; raised, rounded projections of the plasma membrane forming interlacing ridges covering the surface of the vestibule. When compared with the ultrastructural characteristics of perineal and vaginal epithelium, the surface projections evident on vestibular epithelium were similar to those covering the vaginal surface, but different from the flattened microridges of perineal epithelium. Leukocytes were seen in all cell layers, but were particularly common immediately below the basement membrane.

4.1 Introduction

As stated in the general introduction, a study of the some what neglected vulvar epithelium was considered paramount to the investigation of VVS. When examined using light microscopy, vulvar epithelium is found to be composed of four layers as in true skin (Wilkinson, 1992). However, the prominence of each of the cell layers is variable depending on the location of the epithelium within the vulva. The labia majora are covered by keratinised epithelium, characterised by the presence of both a prominent granular and a horny cell layer. In comparison the inner aspect of the labia minora which merges with the vestibule, is devoid of these characteristic cells, which has resulted in the epithelium being described as mucosal or sub-mucosal (Ridley 1988). The labia majora, labia minora and the frenulum and prepuce of the clitoris are ectodermal in embryological origin. The inner aspect of the labia major and the whole of the labia minora are covered with non hairy epithelium, however, sebaceous glands and mucous secreting glands are commonly seen in this epithelium (Ridley, 1988). The surface epithelium of the vestibule is particularly interesting as it is the only part of the external female genitalia that is endodermal in origin, originating from the same sinus as the urethra and the trigone of the bladder (Friedrich, 1983; Woodruff and Friedrich, 1985). The vestibule is reported to have similarities to both skin and mucosal surfaces (Lawrence, 1993), and is described as a collision zone at the junction of two germ layers (Woodruff and Friedrich 1985).

While studying the pathology of Vulvar Vestibulitis Syndrome using electron microscopy, it became apparent that it was essential to characterise the normal structure of the vestibular epithelium affected by this perplexing condition, before any attempt could be made to describe the ultrastructure of tissue from vestibulitis patients. Limited research on the vulva in the last few decades has led to the formation of active working groups within the International Society for the Study of Vulvar Disease (ISSVD), whose principal aim has been to increase research

into vulvar conditions. By looking at the terminology, pathology, dermatology and epidemiology of vulvar diseases, the society aims to make advances in the study of the vulva. This is an area which has been merged with vaginal and cervical diseases for many years and as a result has consequently received scant attention. Several researchers have described the embryology and anatomy of the vulva whilst investigating various conditions affecting it (Skene, 1889; Friedrich, 1983, 1987; Woodruff and Friedrich, 1985; Marinoff and Turner, 1991, 1992), and minimal light microscopy work has outlined the main structural characteristics of the tissue (Marks *et al.*, 1986; Pyka *et al.*, 1988; Reid *et al*, 1988; Michelwitz *et al.*, 1989; Furlonge *et al*, 1991; Sternberg, 1992; Lawrence, 1993; Prayson *et al.*, 1995; Marks *et al.*, 1995). Pyka *et al.* (1988), made the most comprehensive investigation of vulvar vestibulitis at the light microscopy level, examining the structure of the minor vestibular glands, the presence of infectious agents, and the composition of the inflammatory cell infiltrate using monoclonal antibodies. A comparable study was recently completed by Prayson *et al.*, (1995), which produced similar results to the study completed by Pyka *et al.*(1988). It was therefore surprising that the histology of the normal vestibule has been investigated only as part of pathological studies by light microscopy, and that an ultrastructural survey of the vestibule has not been undertaken. Ferenczy & Richart (1974), have commented on the ultrastructure of keratinised vulvar epithelium, but have failed to include details of non-keratinised, mucosal like-epithelium, characteristic of the inner aspect of the labia minora and the vestibule. Limited work on the normal vulva may be attributed to a persistent bias towards the study of neoplasias being created by the nature of the specimens available for study; most being radical vulvectomies as a consequence of tumours or suspected tumours of the vulva. In comparison the structure of the vagina and cervix is better documented using light and electron microscopy as these tissues are commonly removed during repair operations, and for cervical dysplasia.

Scanning electron microscopy of epithelial surfaces has been well documented. Ferenczy & Richart (1974), completed a comprehensive text on scanning and transmission electron microscopy of the female genital tract, which described epithelial surfaces ranging from the endometrium to the cervix, however, the epithelium of the vestibule has been overlooked. Areas of keratinised vulvar epithelium and mucosal vaginal epithelium have been described without reference to the vestibular part of the vulva. Apart from investigations of vaginal and cervical epithelium, reported descriptions of oral / buccal surfaces appear to be the most suitable comparison for vestibular epithelium. Oral epithelium is bounded by keratinised and mucosal epithelia, a similar situation to that of vestibular epithelium; consequently oral surfaces have much in common with the surface of the vestibule (Squier *et al.*, 1976; Landay & Schroeder, 1977, 1979; Schroeder, 1981).

The influence of ovarian hormones on the epithelium of the vestibule was considered to be a possible complicating factor in this investigation. It was expected that hormonal status of the control patients would determine the number of patients suitable for inclusion in the investigation, as well as affecting the ultrastructural appearance of the vestibular epithelium, and ultimately its comparison with biopsies from women with VVS. The epithelia of the vulva, vagina and urethra are all reported to be influenced by ovarian hormones. Tozzini *et al.* (1971 as cited by Ridley, 1988) report corresponding changes in the epithelia of the vagina, and mucosal surfaces of the vulva in response to progesterone and estrogen. The influence of these hormones is demonstrated during the first few weeks of life when the female infant is under the influence of maternal sex steroids received transplacentally. For approximately four weeks after birth the vagina of the infant is lined with glycogenated squamous stratified epithelium usually only seen in the adult. As estrogen levels fall, the endometrium breaks down and the vaginal epithelium begins to thin and loses its stratification. The vaginal epithelium remains thin and unstratified until puberty when ovarian

hormones are produced associated with the development of secondary sexual characteristics. Increased levels of estrogen has been shown to result in thickening of the epithelium of the vagina and vulva, and ultimately, differentiation of these epithelia into well defined cell layers (Dickenson, 1949; Eddy, 1969, Burgos & Roig de Vargas-Linares, 1978; Ridley, 1988). The percentage of superficial cells present in the epithelium is positively correlated with estrogenic activity. In comparison, the presence of progesterone causes a reduction in the numbers of superficial cells, and an increase in the intermediate cell population (Friedrich, 1983a). Under the influence of estrogens the vaginal epithelium is highly glycogenated. Glycogen is utilised by lactobacilli and corynebacterium which produce lactic acid and consequently a low vaginal pH to prevent the overgrowth of opportunistic organisms such as *Candida*. The use of oral contraceptives, particularly combined hormone pills, has resulted in an increase in progesterone and estrogen levels and consequently a reduction in the superficial cell population. Subsequently, a reduced level of glycogen is available to lactobacilli, and opportunist infections are more likely to occur (Ridley, 1988).

Due to the possible influence of ovarian hormones on the epithelium of the vulva, it was important that women donating control biopsies were pre-menopausal, consistent with the study cohort. Only pre-menopausal women were included in the study in order to exclude any physiologic changes in the vestibular epithelia linked with hormonal change. In post-menopausal women, the decrease in progesterone and estrogen results in a much thinner epithelium than is evident during the reproductive years. The period of time involved in the thinning of the epithelium, and also the degree of thinning are variable. Similar changes in structure have also been reported in oral mucosa, which, in response to alterations in hormone levels, becomes thin and atrophic in post-menopausal women (Squier *et al.*, 1976). Unfortunately variations in cyclic hormones due to the stage of the menstrual cycle were unavoidable in this investigation. Possible differences in ultrastructural appearance, such as

thickness of cell layers and glycogen content, due to varying hormone levels, were taken into consideration during the TEM investigation.

The unique nature, and lack of documentation on the ultrastructure of vestibular epithelium, form the basis for this study, which principally aimed to describe the ultrastructure of the epithelial surface of the vulva using transmission electron microscopy (TEM). Knowledge of the normal ultrastructure of the vulva was considered critical to the ultrastructural study of the pathological tissue from vestibulitis patients. Particular emphasis was directed towards characterising the various cell types present, their inter-relationships, and comparability with similar cells found in related epithelia. Perineal and vaginal tissue, obtained during gynaecological repair surgery, was processed using the same method as used for vulval epithelia. These tissues were included as controls for the investigation and for comparison of the fine structure.

Routine chemical processing, and low temperature processing techniques were used to identify possible artefactual changes in tissues processed by traditional methods, and to maintain antigenicity of tissues for immuno-labelling. Routine processing for TEM generally involves the use of aldehydes, osmium tetroxide, alcohol dehydration, and heat polymerisation of resin; all of which have the ability to alter protein structure and render antigens unrecognisable to specific antibodies. Several processing methods have been developed to overcome these problems whilst maintaining acceptable ultrastructural detail (Table 4.0) As vestibular epithelium had not previously been described using electron microscopy, both routine and low temperature processing protocols were considered important to establish an accurate description of vestibular epithelium. Low temperature freeze substitution (LTFS) for TEM was selected for this study, as antigenicity and ultrastructural preservation are reported to be much improved compared with other low temperature processing techniques (Atherton

et al., 1992; Robertson *et al.,* 1992). LTFS also compares well with other low temperature techniques (Table 4.0); freezing is extremely rapid, aldehyde fixation is minimised and heat polymerisation of the resin is avoided. If, as anticipated, the study of tissue from VVS patients culminated in an immunological study, tissue processed using low temperature methods would be essential for immuno-gold marking of specific antigens linked to the condition. Low temperature processing was not expected to be completed on all biopsies.

Table 4.0. Comparison of Low Temperature Processing Techniques for TEM

Method	Advantages & Disadvantages
Progressive Lowering of Temperature	- Low concentration aldehyde fixation - Use low temperature resin. - UV polymerisation of resin. - Low temperature dehydration reduces extraction of cellular components. - Good morphology.
Thawed Cryosections	- Aldehyde fixation - Cryoprotected with glycerol - Frozen in LN_2 and sectioned at -80°C - No resin, no heat polymerization - Morphology difficult to interpret
Freeze Substitution	- No aldehyde fixation - Slam frozen onto copper mirror at -80°C - Ice removed by methanol at -80°C - Ice crystals often form during freezing - Good preservation of 10-15um
High Pressure Freezing	- Same advantages as freeze substitution - High pressure - no ice crystals form - Very expensive

4.2 Materials and Methods

The structure of perineal, vaginal and vestibular epithelia was investigated using transmission electron microscopy (TEM), light microscopy (LM), and scanning electron microscopy (SEM). Tissue biopsies were obtained from pre- / peri-menopausal women attending a local hospital for gynaecological repair surgery. Approval from Plymouth Local Ethical Committee was obtained (February, 1994 (see Appendix 1)), and women between the ages of 20 and 45 years were approached by their consultant and asked whether they would participate in the study. Informed consent was obtained from each patient. Only pre- / peri-menopausal women, who had not suffered with vulval pain in the past were included in the investigation. Biopsies of vulvar epithelium were taken under general anaesthetic prior to repair surgery. Biopsies of vulvar (Sample A), perineal (Sample B) and vaginal epithelium were processed for TEM and SEM (Fig. 4.0). Generally, samples were used for one of the four possible routes of processing as the biopsies were very small. Occasionally routine TEM and SEM could be completed on a single biopsy. Only three vestibular samples were available to be processed using both LTFS and cryo SEM (Fig. 4.1).

Figure 4.0 The anatomy of the vulva, indicating the extent of the vestibule and the position of samples

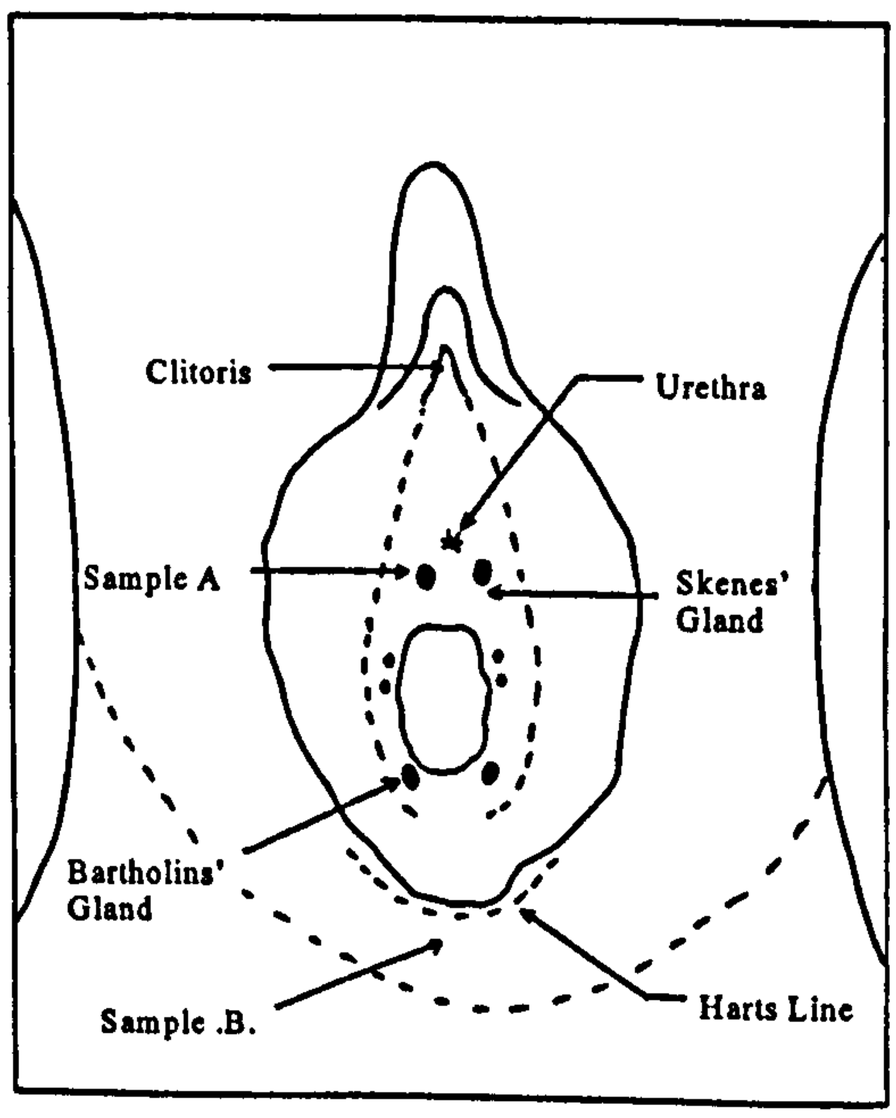
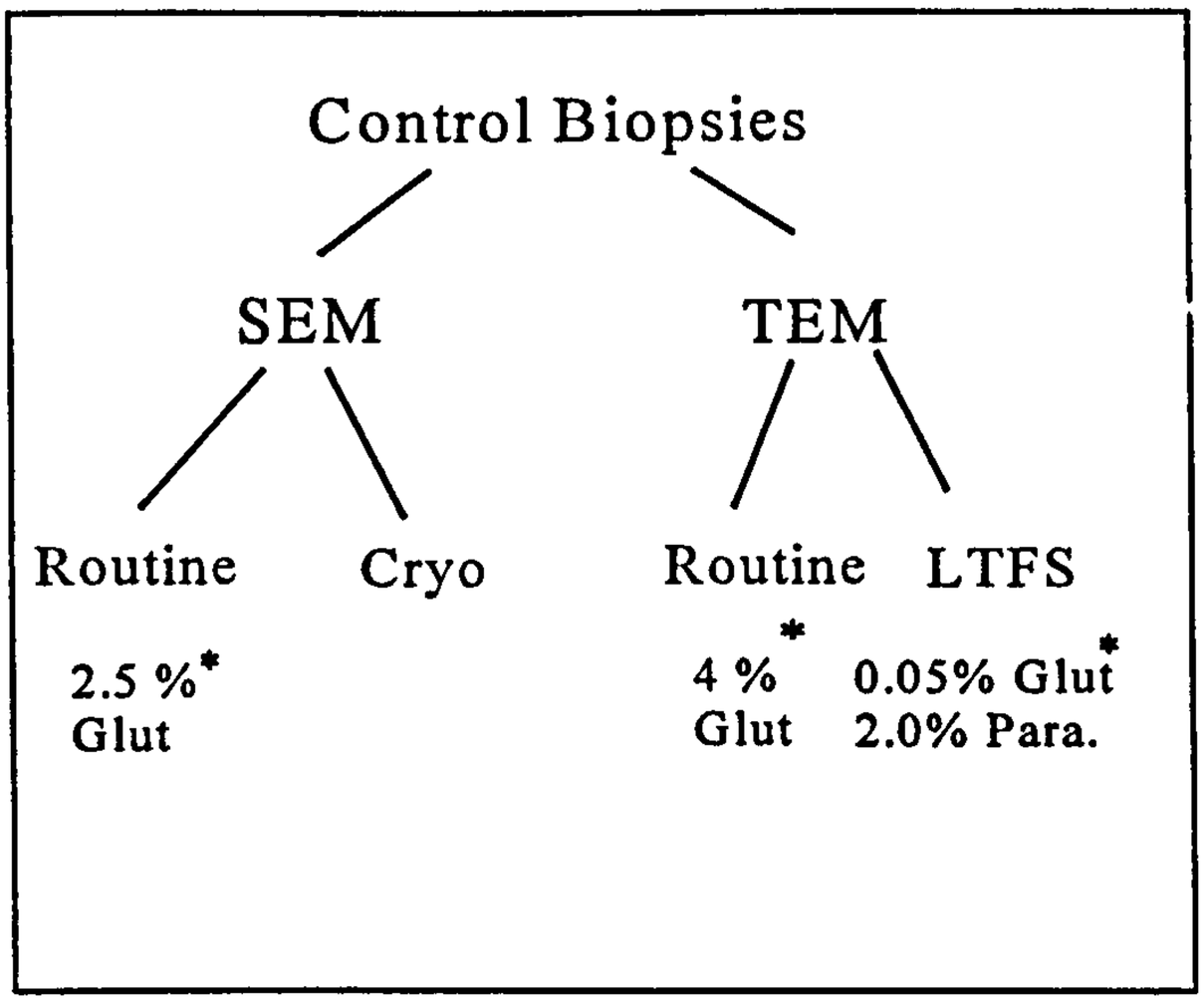


Figure 4.1 Route of Processing for Control Biopsies

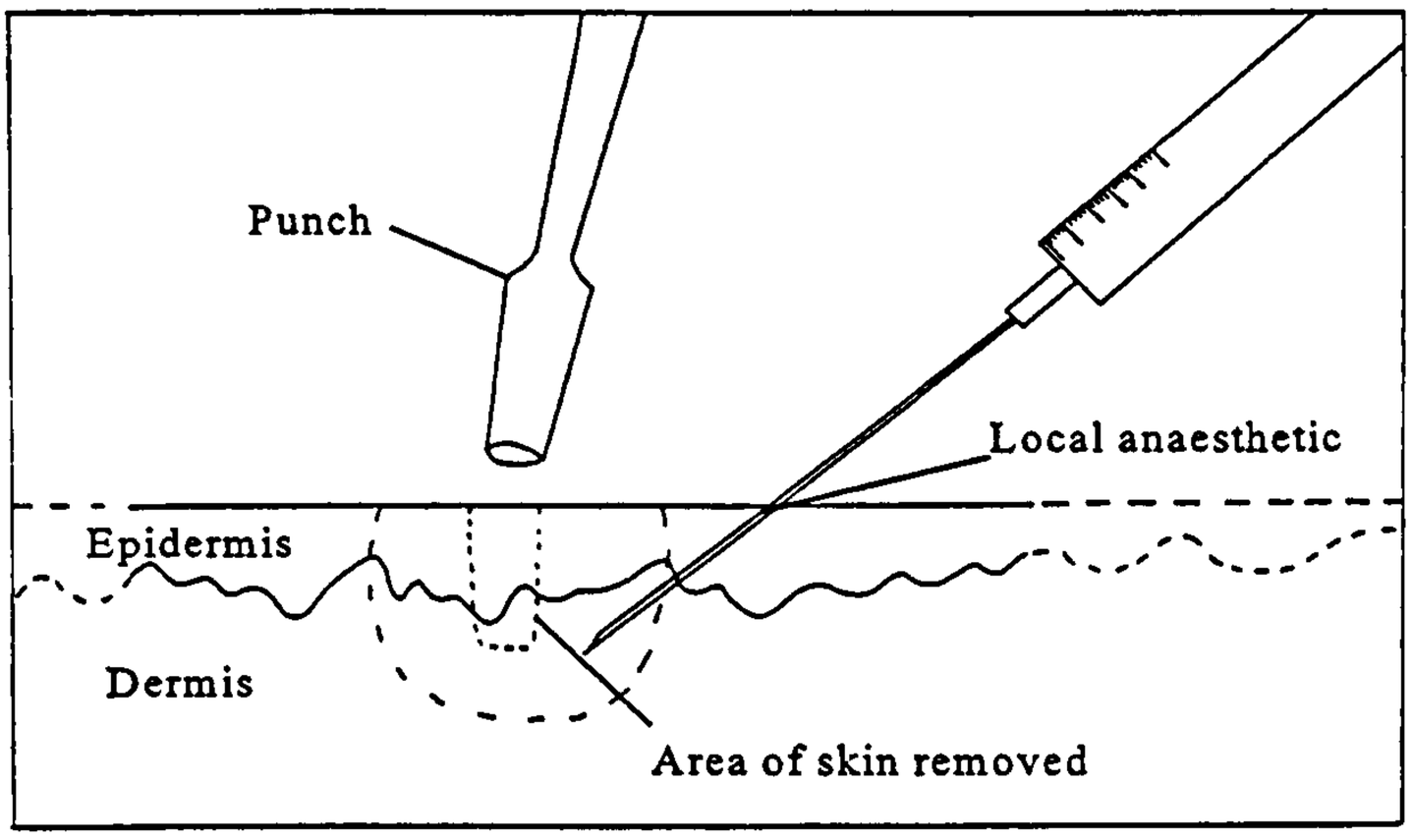


* Glut = Glutaraldehyde Para = Paraformaldehyde

4.21 Obtaining a Vulvar Punch Biopsy

Unlike the cervix and endometrium, removing a biopsy from the vulva required some form of anaesthetic. Anaesthesia may take the form of a local injection or a pudendal nerve block, generally, however, a local injection was considered sufficient. A Keyes cutaneous punch was used to remove a small circular plug of skin, working on the same principal as a cork borer. Keyes punches range from 2-12mm, however, a 3- 4mm punch was considered suitable for biopsying the vulvar area. It was important that the dermis was penetrated as a superficial biopsy would not be suitable for pathological diagnosis. Once the incision was made the specimen was grasped beneath the epidermis with forceps and the dermal tissue cut with a scalpel (Figure 4.2) (Pinkus, 1977).

Figure 4.2 Obtaining a punch biopsy



4.22 Scanning electron microscopy

Both routine and cryo processing techniques were used to investigate the surface structure of the vestibular epithelium. Samples of vaginal and perineal epithelium were also processed for routine SEM to compare their ultrastructure. Tissue samples were briefly rinsed in s-carboxymethyl-L-cysteine prior to fixation by cryo or routine processing, to remove surface mucus.

a) Chemical Fixation and Critical Point Drying

Blocks retained for SEM were fixed in the primary fixative for 2 hours, subsequently washed in sodium cacodylate buffer (0.1M, pH 7.2), and post fixed in 1% osmium tetroxide for 1 hour. After washing in buffer, tissue blocks were dehydrated in ethanol (30%, 50%, 70%, 90%, and absolute ethanol) for 10 minutes in each mixture. Samples were subsequently critical point dried for SEM. The intermediate fluid was amyl acetate and the transitional fluid liquid carbon dioxide. Dry samples were mounted on brass stubs and gold coated in a Polaron E1500 sputter coating unit to a nominal thickness of 5-10nm, and examined in a Jeol JSM 5300 scanning electron microscope operated at 15kV.

b) Cryo SEM

Only control vestibular samples were investigated using cryo SEM. Tissue samples were subdivided into blocks of approximately 3mm x 3mm and rinsed in s-carboxymethyl-L-cysteine to remove surface mucus. The tissues were transported in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.2). Prior to cryofixation the tissue blocks were rinsed in distilled water, and blotted dry on filter paper to remove excess water detrimental to visualisation of the fine surface structure. The cryostage was pre-cooled to -170^{0}C. The tissue was secured to the specimen arm with silver paste and subsequently rapidly frozen by plunging into LN_2 slush. The specimen was withdrawn into the vacuum transfer device, and under vacuum the specimen arm was inserted into the cryostage. Ice crystals on the surface of the epithelium were removed by sublimation and gentle surface etching carried out by raising the temperature to -80^{0}C. The specimen was then withdrawn from the cryo chamber, sputter coated with gold and viewed under a Jeol 6100 SEM operated at 10kV and at a temperature of -170^{0}C.

4.23 Transmission Electron Microscopy

a) Chemical fixation

A 3mm Keyes punch biopsy sample was removed from the lateral border of the right Skene's gland (Fig.4.2) and immediately transferred to a vial containing the primary fixative, 4% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.2) at 4°C, for two hours. Biopsies of perineal and vaginal tissue were obtained from women having posterior repair surgery using a 3mm Keyes punch and processed in the same way as the vulvar samples (Fig.4.2). After 30 minutes the biopsies were subdivided into tissue blocks of approximately 1mm x 1mm whilst immersed in fixative (4^{0}C). Several blocks of tissue were retained in the primary fixative for routine SEM. The tissue blocks for TEM were returned to fresh primary fixative for a further 90 minutes. Blocks were subsequently washed twice in 0.1M sodium cacodylate buffer (pH

7.2), post fixed in 1% osmium tetroxide for 60 minutes (4°C) and finally washed twice in 0.1 M sodium cacodylate buffer (pH 7.2). Tissue blocks were block stained in 2% uranyl acetate for 20 minutes in the dark, washed in distilled water and dehydrated in a series of graded ethanol, 30%, 50%, 70%, 90% and absolute alcohol, for 10 minutes in each mixture. Dehydration was completed by two 10 minute washes in propylene oxide. Infiltration of resin was carried out over several days to ensure full penetration of the resin into the tissue. A series of graded mixtures of propylene oxide and Spurr resin (Spurr, 1969) were used (3:1, 1:1, 1:3, Pure x 2). Blocks were polymerised at 70°C for 8 hours. Ultrathin sections, cut on a Reichert Ultracut ultramicrotome, were collected on copper grids, stained for 10 minutes in uranyl acetate and 20 minutes in lead citrate, and subsequently viewed using a Jeol 1200 transmission electron microscope. Semi-thin sections, of 0.5µm were cut and stained with 1% methylene blue for routine light microscopy.

<u>b) Low temperature freeze substitution (LTFS)</u>

To avoid chemical fixation, samples were rapidly frozen and the ice removed at -80^{0}C by substitution with methanol i.e., freeze substitution (Robertson *et al.*, 1992). All processing was completed using Reichert Automated Freeze Substitution (AFS) equipment and a Leica Impact Freezer. Several samples of control vestibular tissue were processed using this technique, other epithelial samples were not available for processing using this method. Tissue blocks (no larger than 1mm x 1mm) for low temperature processing were fixed in 2% paraformaldehyde and 0.05% glutaraldehyde for 60 minutes (Atherton *et al.*, 1992). The specimens were cryoprotected by placing in 30% glycerol for 30 minutes. Rapid freezing of the tissue was completed using an impact freezer, which brings the tissue in contact with a pre-cooled copper mirror at -80^{0}C (Fig.4.3). The pressure, speed and force of impact are variable, and were altered depending on the thickness or hardness of the tissue. For vestibular epithelium, a moderate force setting suitable for mammalian skin was selected. Tissue blocks

were placed on transparent office tape which adhered to the foam disk on the specimen carrier. Tape was used on the surface of the foam disks to prevent the tissue blocks adhering to the foam during freezing. Prior to rapid freezing the specimen carrier was stored in a humidity chamber to prevent dehydration of the specimen. The specimen carrier was held in place on the plunger rod by a magnet with the specimen facing downwards (Fig. 4.3). The polished metal mirrors were cooled in liquid nitrogen for 10 minutes prior to slamming to ensure a surface temperature of -190^{0}C . As the plunger rod holding the specimen is released, the pre-cooled mirrored surface rapidly freezes the tissue block as they make contact. The foam disc prevents the tissue from being compressed during the slamming process. After slamming the magnet was released leaving the specimen carrier and tissue sample in contact with the copper mirror. After 30 seconds the specimen carrier was lifted from the mirrored surface using forceps cooled with liquid nitrogen, and the sample gently removed from the surface of the tape. The frozen samples were placed in dry plastic capsules and stored in a pre-cooled cryotransfer container. This container was used to transfer the samples from the impact freezer to the AFS, preventing any increase in temperature and subsequent thawing of the sample. Ultra-pure methanol was pre-cooled in the AFS during the slamming process. Samples in the cryotransfer container were transferred to a freeze substitution chamber in the AFS. Ice crystals were then removed by replacement of ice with an organic solvent (methanol) at -80^{0}C. After rinsing the samples with pre-cooled methanol, used methanol was withdrawn via the central compartment and replaced with fresh solvent using a dry disposable pipette. Substitution with methanol was carried out at -80^{0}C for 36 hours, T1, (Table 4.1), the methanol was changed twice during this time. After 36 hours the temperature was increased by 4^{0}C/hour up to -50^{0}C, approximately 7.5 hours (S1). Infiltration of Lowicryl resin (HM20) took place over 8 hours at -50^{0}C (T2). Resin / methanol mixtures, 1:1, 1:2, 1:3, Pure x 2, were used, two hours in each mixture. The AFS was programmed to 'pause' after the first pure resin change, at which point the tissue blocks were left in pure resin overnight. Polymerization

was achieved with UV light for 2 days at -50^{0}C (Robertson *et al.*, 1992). After polymerisation of the tissue blocks, the AFS was warmed to +25^{0}C by stepping up the temperature by 10 ^{0}C/hr. All equipment was cleaned in acetone and dried at 50^{0}C.

Figure 4.3 Diagrammatic representation of rapid freezing using the Reichert impact freezer

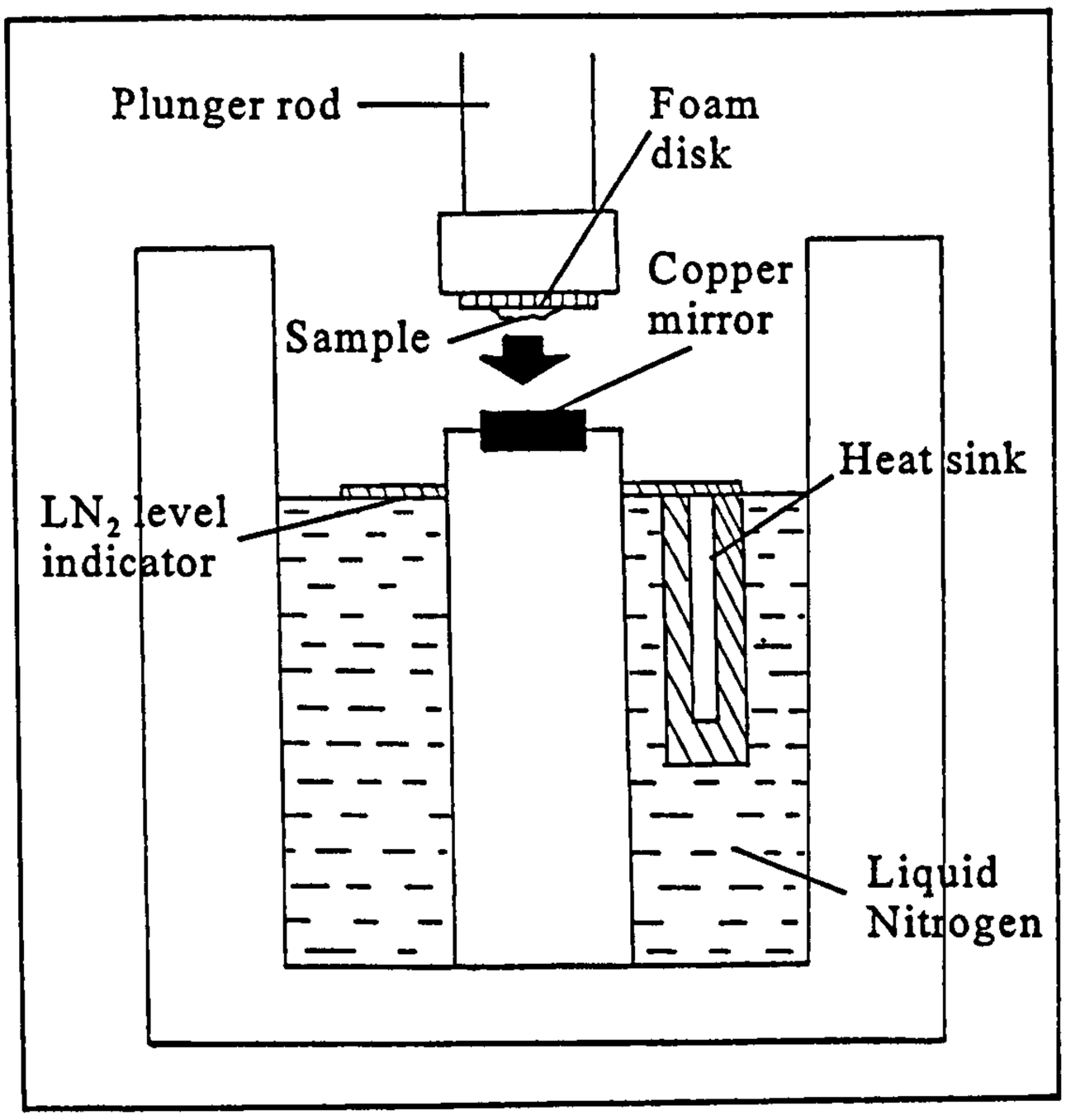


Table 4.1. Processing Protocol for LTFS

Stage of Processing	Time (hrs)	Temp. (°C)
T1 (Substitution)	36	-80
S1 (Substitution)	7.5	4°C/hr
T1 (Infiltration) 1:1	2	-50
S1 (Infiltration)	0	-50
T2 (Infiltration) 1:2	2	-50
S2 (Infiltration)	0	-50
T3 (Infiltration) 1:3	2	-50
Pure	2	-50*
T1 (Polymerisation)	48	-50
S1 (Polymerisation)	7.5	10°C/hr

*Pause & maintained at -50°C overnight in pure resin

3.3 Results

4.31 Light microscopy

The overall appearance of vulvar, vaginal and perineal epithelia is illustrated diagrammatically in Figure 4.4.

a) General structure of vaginal epithelium

The differentiation of the epithelium into specific cell layers was not clearly defined. Keratinised squames and granular cells were absent, replaced by large, slightly flattened polygonal shaped cells (Plate 1a). Superficial cells were often seen packed with glycogen (Plate 1b). Superficial cells often retained their nuclei, however the intercellular space appeared swollen and the cells were often devoid of cytoplasm and organelles. Intermediate cells were hexagonal in shape with large nuclei, these cells did not have the characteristic spiny appearance associated with epidermal cells at this stage of maturation. Leukocytes were present below the basement membrane and occasionally interposed between epidermal cells. Melanocytes were not visible using light microscopy (Plate 1a).

b) General structure of vulvar epithelium

Semi-thin sections (0.3-0.5μm) stained with methylene blue showed an epithelium about 20-25 cells thick. Differentiation into specific layers was difficult to observe using light microscopy, but it was possible to identify some differences in cell type, moving from the basement membrane upwards, to the superficial cell layers (Plate 1c). Notable differences included: a significant increase in the size of cells, a decrease in staining intensity, and often the loss of nuclei linked with maturation. Cells of the intermediate cell layer are slightly spiny in appearance due to the presence of cell junctions which are just visible by light microscopy. The surface cells did not stain heavily with methylene blue; in comparison the cells along the dermal junction were generally more heavily stained (Plate 1c). The epithelium was

infiltrated by white blood cells, which were seen infrequently in all cell layers. The basement membrane is gently undulant in appearance forming wide, shallow projections of the dermis into the epidermis. Melanocytes were not visible interposed between basal cells, however, the occasional clear cell profile suggestive of a Langerhans cell was present (Plate 1c).

c) General structure of perineal epidermis

Semi-thin sections (0.3-0.5μm) stained with methylene blue showed an epidermis of about 20 cells thick. Differentiation of the epidermis into four specific cell layers was possible due to distinct changes in cell morphology. Staining with methylene blue illustrated a distinct granular cell layer, 2-3 cells in thickness, and cornified layer, 5-15 cells in thickness. Nuclei were absent in the intensely staining, flattened, cornified cells. Using light microscopy, it was possible to identify spiny cells, characterised by distinct, intensely staining cell junctions. Basal cells were intensely staining, between which pale staining melanocytes and Langerhans cells were visible. Very few leukocytes were visible beneath the basement membrane, or penetrating the epidermis. Capillaries were not common close to the basement membrane (Plate 1d).

4.32 Scanning Electron Microscopy

a) Surface characteristics of perineal epidermis

The surface cells of perineal epidermis were flattened in appearance with prominent, raised, and thickened cell boundaries. The cells were polygonal in shape, variable in dimension and ranging from 5-30 μm in diameter (Plate 2 a-c). Some surface cells appear to have a more flattened surface topography than neighbouring cells (Plate 2c). Cytoplasmic projections are ridge-like, with a flattened, worn appearance. Neighbouring cells with more prominent surface structures appear to have slightly raised microridges, not flattened as seen on surrounding

cells. Microridges formed a dense network on surface cells, the orientation of ridges varied between individual cells, some areas being completely flattened. Pore-like structures were visible infrequently between surface cells of perineal epidermis (Plate 2a).

b) Surface characteristics of vaginal epithelium

Surface cells of vaginal epithelium were relatively large, ranging from 10 - 40μm in diameter, and varied considerably in shape and size. Cell junctions were prominent and raised, but did not appear thickened. Microridges characterised the surface of this epithelia. Mucus was present covering large areas of the epithelial surface, making it virtually impossible to visualise the surface structure.

c) Surface characteristics of non-keratinised vulvar epithelium

The surface of vestibular epithelium was undulant in appearance (Plate 3a). Surface cells were polygonal, approximately 10-30μm in diameter. Cells of this epithelial surface had particularly clear microridges, with a raised, rounded appearance. At high magnification some microridges appeared nodular (Plate 3d). Microridges were not straight, but generally curved and interlocking. These structures appeared to occur at the same density over individual and neighbouring cells, but were variable in length (Plate 3a-e). Orientation of ridges varied from cell to cell. Cell boundaries were prominent and raised (Plate 3c). Mucus was common on this epithelial surface, however, no bacteria were seen. Occasionally pore-like structures were visible interposed between surface epithelial cells, these structures varied in size, and were commonly isolated.

LTSEM was not successful for the study of vaginal and vestibular epithelium. Although tissue samples were rinsed in s-carboxymethyl-L-cysteine prior to fixation, visualisation of the surface revealed mucus which was evidently masking the ultrastructural detail. Areas which

were able to be visualised showed very little morphology, and many of the structures seen using traditional processing were not visible. The surface appeared flat and structureless, possibly due to the presence of ice. Due to poor visualisation of the epithelial surfaces, micrographs are not included.

4.33 Transmission electron microscopy

a) Ultrastructure of perineal epidermis

A schematic diagram of the characteristic cell layers is shown in Fig 4.5. The stratum corneum was approximately 5-15 cells thick, these cells appeared particularly electron dense, however the intensity of staining appeared to decrease as the squames reached the epidermal surface (Plate 4a). Surface cells of perineal epidermis showed thickening of the plasma membrane, loss of organelles and the nucleus (Plate 4a & b). The surface of the cornified cells was characterised by short, blunt ridges, giving the surface an undulant appearance (Plate 4a). The cells of this layer contain closely interwoven, intensely staining, cytokeratin filaments (Plate 4b & d). The cell layer immediately below this, the stratum granulosum, was only 2-3 cells thick (Plate 4c-f). The cells of the granular cell layer are characterised by intracellular, irregularly shaped, intensely staining, keratohyalin granules (Plate 4d). Cells in this layer sometimes retain their nuclei, but generally the nucleus is absent or flattened, and the cytoplasm has few organelles (Plate 4e & f). Melanin granules were often visible in these cells (Plate 4f). Cells of the spinous cell layer are polyhedral in shape with numerous dark staining cytokeratin filaments forming bundles surrounding the nucleus. The plasma membrane of these cells is moderately folded between numerous desmosomal junctions which are studded around the periphery of the cell (Plate 5a). It is the numerous desomosomal junctions and associated cytokeratin filaments which result in the spiny appearance of these cells, which are characteristic of keratinised epithelial surfaces. At the transition from spinous cells to basal cells it is evident that basal cells of perineal epithelium have a more diffuse network of

individual cytokeratin filaments than seen in the spinous cells. Basal cells were oval in shape, and the cytoplasm was rich in mitochondria with occasionally some rough endoplasmic reticulum and Golgi present (Plate 5b). Few desmosomal junctions were evident in the basal layer, the majority were observed in the stratum spinosum. When present in the basal layer, cytoplasmic junctions were more common adjoining the spiny cells rather than between individual basal cells. Folding of the cytoplasmic membrane was more obvious between basal cells than in other cell layers. Leukocytes were not common in perineal epithelium. Occasionally the profile of a lymphocyte was visible, characterised by few organelles and sparse cytoplasm (Plate 6). Other non-epidermal cells included melanocytes and Langerhans cells. Other leukocytes were not able to be identified, such as the cell shown in Plate 6, in which dense granules, vesicles and numerous organelles are evident.

b) Ultrastructural characteristics of vestibular epithelia

Superficial cell layer

A schematic representation of the ultrastructural characteristics of vestibular epithelium is shown in Fig.4.6. Using transmission electron microscopy, the surface of the vulvar epithelium was seen to be covered with microridges at intervals of approximately 0.2 - 0.7μm (Plate 7a & d). Irregular in shape and length, and ranging from 200-400nm in height. The remains of desmosomal junctions were evident on the surface of superficial cells (Plate 7d). Desmosomal disintegration was visible between living surface cells and exfoliating cells. Filaments attached to desmosomal junctions in the superficial cell layer were diffuse, and few in number. The majority of the surface cells were moderately flattened, containing some cytoplasmic filaments but generally few organelles (Plate 7a & d). These cells were particularly characterised by the presence of large deposits of glycogen, visible as aggregates or rosettes (Plate 7a & b). Surface cells generally had an electron lucent matrix, but occasionally an intensely staining cell was present, slightly shrunken; these appeared to be

apoptotic (Plate 7a). Microvillous processes were seen to interdigitate between surface cells; desmosomal junctions were short and less frequently seen here than in the deeper cell layers (Plate 7a & c). Nuclei were not uncommon in the surface layers of vulvar epithelia. Nuclei, when present, appeared flattened with a spherical nucleolus, frequently the nuclear matrix was partially degenerated (Plate 7a). Displacement of the nucleus, possibly due to the presence of large glycogen deposits, was common (Plate 7a & Fig.4.5). The cytoplasmic membrane appeared to be slightly thickened, but no prekeratin or keratohyalin granules were visible (Plate 7c). Occasionally, vesicles, similar in appearance to membrane coating granules, were visible in superficial cells (Plate 7b). Vesicles were not common in the normal tissue; however, when they were present, they were seen in close contact with the plasma membrane; and were generally electron lucent in appearance, occasionally with an electron dense core (Plate 7b&d).

Upper intermediate cell layer

Immediately below the flattened surface cell layer is the upper intermediate cell layer which is analogous with the spinous cell layer in keratinised epidermis (Plate 8a). These cells are polyhedral in shape, becoming increasingly flattened towards the surface layers. Upper intermediate cells have slightly flattened nuclei and contain large deposits of glycogen commonly situated in a halo around the nucleus (Plate 8a). The cytoplasm is granular in appearance characterised by numerous polyribosomes, however, few organelles were visible in these cells. Generally, leukocytes were visible in the basal or lower intermediate cell layers. However, occasionally the pale staining profile of a leukocyte was visible interposed between upper intermediate cells (Plate 8a). These were usually small lymphocytes with sparse cytoplasm and few organelles. Infrequently the lobed nucleus and organelle-rich cytoplasm suggestive of a macrophage or neutrophil was evident (Plate 8b). A definitive identification was impossible due to the absence of characteristic granules.

Lower intermediate cells layer

Deeper or lower intermediate cells were rich in mitochondria in the perinuclear region; profiles of mitochondria were commonly small and round (Plate 9 & 10a). Some rough endoplasmic reticulum and occasionally Golgi regions were seen. The cytoplasm of these lower intermediate cells was characterised by the presence of free and poly-ribosomes. As in other cell layers, glycogen granules were common, seen here mainly in the form of small clusters. Deeper intermediate cells generally have more abundant cytoplasmic organelles than cells of the upper intermediate layer, with less glycogen stored in the cytoplasm (Plate 9). Glycogen is in the form of rosettes rather than large aggregates. Cytokeratin filaments, which are lightly staining in this epithelium, form tonofibril bundles which converge at electron dense desmosomal plaques (Plates 9, 10a & c). Single cytokeratin filaments of low electron-density form a diffuse network in the cytoplasm leaving a clear zone immediately round the nucleus (Plate 10a). The plasma membrane is highly convoluted; microvillous projections extend into the intercellular spaces between neighbouring intermediate cells, between the desmosomal junctions (Plates 9 & 10a). Cells which appeared electron-dense using transmission electron microscopy also stained intensely with methylene blue for light microscopy. These electron dense cells were often slightly shrunken in appearance with few organelles and dense cytoplasm (Plate 9).

Basal cell layer

Cells of the basal layer were characteristically round or oval in shape, often slightly elongated at the proximal end of the cell where the epithelium meets the dermis (Plate 10b). The nuclei were large and oval in the perpendicular direction having one or more nucleoli present (Plate 10b), some mitotic figures were evident. Mitochondria, free ribosomes and endoplasmic reticulum are numerous in the proximal perinuclear region; mitochondrial profiles are round or oval in shape and usually situated in small clusters close to the basement membrane (Plate

10b). Cytoplasmic projections, as seen in the spinous and supra basal cells, are present around the periphery of basal cells. In this cell layer the cytoplasmic processes are visible forming close connections with neighbouring basal cells (Plate 10b). A tonofilament network, as seen in the supra basal cells, is also present in basal cells, again leaving a fibre free halo around the nucleus. Tonofilament bundles are generally more darkly staining in the proximal region of the basal cells than in cells of other layers. Desmosomal junctions are infrequent along the lateral borders of the basal cells. Cytoplasmic projections of basal cells form pseudopodia-like feet which extend into the dermis following the undulating contours of the basement membrane. The projections are smooth in appearance and larger than the villous processes on the other faces of the basal cells (Plate 10b). Where the foot-like projections of the basal cells meet the basement membrane, hemidesmosomes are formed, characterised by dense patches where tonofilaments converge on anchoring plaques (Plate 10b). Some lucent cell profiles, characterised by the absence of desmosomal junctions and cytokeratin filaments are also apparent (Plate 9). These cells, infiltrating the epidermis from the underlying dermis were mainly lymphocytes, with little cytoplasm and few organelles. Occasionally a wall of a capillary was visible in close contact with the basement membrane.

c) Ultrastructural characteristics of vaginal epithelium

Vaginal epithelium was found to be very similar ultrastructurally to vestibular epithelium. The surface cells often appeared flattened, however, in several biopsies surface cells were large and swollen. The plasma membrane of surface cells was slightly thickened but non-keratinised, neither membrane coating granules, or keratohyalin granules were present in the superficial cells. The surface structure of vaginal epithelium was commonly masked by the presence of electron dense mucus (Plate 11a) The cytoplasmic matrix of large superficial and upper intermediate cells was frequently degraded, organelles were characteristically absent, and many cells only had the remains of a nucleus present. A small number of cytokeratin

filaments were still present in these cells, mainly associated with desmosomal junctions. As in vestibular tissue, desmosomes were short in length in the superficial cell layer. The surface and intermediate cells were frequently seen to contain large deposits of glycogen, which, in the form of aggregates, formed a halo around the nucleus (Plate 11a & b). Glycogen was seen to occupy most of the cell space in intermediate, and particularly in superficial cells (Plate 11b & c). Basal cells were characterised by dark-staining cytokeratin filaments and numerous organelles. Mitochondria were seen in clusters in the proximal cytoplasm. The cytoplasmic membrane of these cells was folded to form cytoplasmic processes, particularly numerous at the junction of basal and intermediate cells. Cytoplasmic processes were finger-like in appearance, and of varying length and dimensions. Leukocytes were visible in small numbers in the epidermis, but were more commonly seen in the dermis, immediately below the basement membrane.

d) Ultrastructure of vulvar tissue processed by LTFS

Vestibular tissue processed by LTFS appeared similar in structure when compared with tissue processed by routine chemical fixation for TEM. Semi-thin sections, stained with methylene blue, demonstrated an epithelium of similar dimensions, and characteristics as seen in chemically fixed tissue. However, when examined using electron microscopy, several differences were apparent. Ultrastructural preservation of the surface and upper intermediate cells was satisfactory, and all membranes appeared intact and stained well, however, organelles when present appeared damaged (Plate 12 a-c). Surface cells were characterised by the presence of microvillous projections, nuclei were flattened and often displaced. Glycogen was not evident in a halo around the nucleus of these cells, instead a structure less area was apparent (Plate 12a). Vesicles were identified in close contact with the plasma membrane of superficial cells in all biopsies processed by LTFS (Plate 12b & c). Often, vesicles were electron dense with an internal lamella-like structure (Plate 12b & c). Lower

intermediate and basal cells appeared extremely shrunken and electron dense, with minimal ultrastructural detail (Plate 13a-c). Organelles were indistinct and it was impossible to visualise the cytoplasmic membranes of these cells; the nuclear membrane appeared intact in a majority of cases. Collagen in the dermis was well preserved, and banding was clearly visible (Plate 13d). The basement membrane was characterised by hemidesmosomes, and did not appear damaged. Vesicles and anchoring fibrils were evident along the basement membrane. Cytokeratin filaments were clear, and arranged in bundles in the basal cells, however, no mitochondria, endoplasmic reticulum or Golgi were visible.

4.4 Discussion

The epithelium covering the vestibule of the vulva is unlike extra-genital skin because it is non-keratinised, with a non-pigmented surface, and consequently has more similarities to mucosal surfaces than to skin, at least at the ultrastructural level. Although mucus is not secreted by the vulval epithelial cells, the vestibule is bathed in mucus produced by the cervix, and to a lesser extent by the vestibular glands of the vulva (Skene, 1889; Huffman, 1948; Dickenson, 1949; Friedrich, 1983b; Woodruff and Friedrich, 1985, Ghadially, 1988; Ekert, 1989). Like skin, the vestibular surface has an epithelium comprising several layers of closely packed cells, which mature and differentiate as they approach the surface (Breathnach, 1971; Menton and Eisen, 1971; Toner and Carr, 1982; Sternberg, 1992). However, unlike keratinised skin, appendages such as hair follicles and sweat glands, and the characteristic granular and cornified cells, are absent from vestibular epithelium. It is in this respect that the epithelium of the vestibule resembles vaginal epithelia and oral mucosal surfaces (Squier *et al*, 1976; Landay and Schroeder, 1977; Schroeder, 1981).

As evident in vestibular epithelium, the individual cell layers of vaginal epithelium are not readily distinguishable, one cell type merging to the next (Plate 1a & b, 11a-c). This is unlike highly keratinized epithelia which have 4-5 easily distinguishable cell layers and are particularly delineated by the presence of granular and cornified cells or squames (Plates 4 & 5) (Selby, 1957; Breathnach, 1971; Toner and Carr, 1982; Sternberg, 1992). In vestibular epithelium there is a large population of intermediate cells which alter in size, filament distribution, and organelle density as they migrate to the surface layers during maturation, a less noticeable change than that which occurs in highly keratinized epithelia. It is possible to divide the intermediate cell population into two groups; the upper intermediate cells have few organelles and large glycogen deposits, and are similar to superficial cells, and lower intermediate cells which have numerous mitochondria consistent with the ultrastructure of basal cells (Plates

5&7). Changes which occur in vestibular and vaginal epithelium include an increase in dimensions of cells and accumulation of glycogen, accompanied by a decrease in the electron-density of cytoplasmic filaments and the number and diversity of organelles. In comparison, keratinised cells undergo a more dramatic transformation with the formation of keratohyalin granules, and later squames of keratin, with no nuclei or organelles present in these "dead" cells (Plate 4). In comparison with perineal tissue, which is a keratinized surface found in close proximity to vulvar epithelium (Plate 4), the overall characteristics of the two epithelial surfaces show some similarities, but ultrastructurally the cytoplasmic detail is very different. Basal cells of perineal tissue have few microvillous projections and numerous, more electron-dense, cytokeratin bundles than observed in vestibular epithelium. Cytokeratin bundles present in the intermediate cells of the vestibular epithelium, are pale staining and less numerous, not giving the spiny, 'prickle' cell, appearance characteristically seen in true skin. Vaginal epithelium showed a similar arrangement and staining intensity of cytokeratin filaments as was evident in vestibular epithelium.

Consistent with changes reported in the vagina, the epithelium of the vulva is affected by hormonal status, the thickness of various cell layers being reported to be dependant on estrogen and progesterone levels (Friedrich, 1983a). Other similarities to vaginal and oral epithelia include: a homogeneous population of cells in the intermediate layer, characterised by prominent intercellular bridges; surface cells often with large deposits of glycogen; and no obvious thickening of the surface cell membranes (Squier *et al.*, 1976; Friedrich, 1983a & b). Women participating in the present investigation were pre- / peri-menopausal consistent with vestibulitis patients. However, women in both the control and VVS study groups were at various stages of their menstrual cycle. The effect of cyclic hormonal status was taken into consideration when assessing samples using EM. Variations in glycogen content and the thickness of various cell layers were not investigated, due to this complicating factor.

In this investigation vaginal epithelium was often characterised by extremely large surface cells which were not evident in normal vestibular tissue. It is possible that these surface cells were previously packed with glycogen which had leaked out of the cells prior to desquamation, leaving the intercellular space empty. Frequently, there were areas of the epithelium where surface cells appeared more flattened, consistent with superficial cells of the vestibule. It is possible that at different stages of the hormonal cycle, surface cells of the vagina and, to a lesser extent, the vulva, become swollen and contain large aggregates of glycogen. This would account for the variation in surface cell size in vaginal epithelium. Although variation in cell surface volume was evident in vestibular tissue, this was not as pronounced as the variation seen in vaginal tissue. King (1983), depicts ultrastructural changes in the vagina during the menstrual cycle and pregnancy. He describes a keratinised epithelium of varying thickness with numerous surface microridges and adherent bacteria. In the present investigation a partially thickened epithelium with microridges was evident. However, King (1983), reports the presence of electron dense granules, similar in appearance to those found in keratinised epithelia, which were not visible in vaginal epithelium. A dramatic transition from intermediate to superficial cells was also reported, a much more definite change than was seen in either vagina or vestibular epithelium in this study (King, 1983). Consistent with the present investigation, King (1983), describes the presence of swollen and electron lucent superficial cells. In the luteal phase, large numbers of lymphocytes, often surrounded by a dilated cytoplasmic space, were reported in the upper cell layers of vaginal epithelium (King, 1983). This is similar to observations of vestibular and vaginal epithelia in the present investigation, which often demonstrated leukocytes interposed between superficial cells.

During the investigation of normal vulvar tissue it became apparent that SEM of the surface morphology would be a useful addition to the study. Using TEM it was possible to determine that cytoplasmic projections, which were similar in appearance to microvilli, varied in length

and distribution in both vestibular and vaginal epithelia. In comparison, the surface of perineal tissue appeared undulant using TEM but was not characterised by the same type of cytoplasmic projections as were evident on the surface of vaginal and vestibular epithelia. As the SEM investigation concentrated on the surface cells of each epithelia, no information was available on the surface characteristics of lower cell layers. Keratinised vulvar epithelium is reported to be similar in appearance to keratinised skin, having flattened microridges similar to perineal epithelium (Ferenczy & Richart, 1974) The epithelia of the vagina and cervix has been described on many occasions as mucosal-like, becoming keratinised during the menstrual cycle (Hackeman *et al*., 1969; Parakkal, 1974; Davina *et al.,* 1981; Odor *et al.*, 1989). Despite several reports on the vagina and cervix of various species, there is no information on the surface ultrastructure of the vestibule of the vulva.

Vestibular samples processed by routine and LTSEM were perplexing. Variations in the surface morphology were expected using an alternative technique, however, the presence of a virtually flat feature-less surface was not anticipated. Variations in the number and density of microridges have also been described in bladder epithelium processed using LT techniques. Very different structures were visualised when LTSEM was used instead of routine CPD to study the bladder surface. In bladder epithelium, folds and microridges which were seen using traditional methods of processing were absent using LTSEM. Cell surfaces were smooth and intracellular spaces were evident where desmosomal junctions would be situated (Hopkins *et al.,* 1989). Similar findings were echoed in the present investigation. Despite poor surface visualisation due to mucus, the surface of vestibular epithelium appeared almost completely flat when processed using LTSEM. Microridges seen covering the surface of vestibular epithelium using routine processing, were absent when processed by this method. LTSEM is designed to maintain ultrastructural preservation in as natural a state as possible. It would therefore be expected that the surface ultrastructure observed using routine processing techniques may be

due to shrinkage artifacts. However, as suggested by Hopkins *et al.* (1989), it is unlikely that microridges are produced solely due to redundant membrane from shrinkage, which is consistent with the findings of Davina *et al.* (1981), who reported no change in the ultrastructural appearance due to chemical processing and CPD. During LTSEM factors such as surface ice formation and etching of the surface may affect the surface appearance, it is possible that factors such as these prevented an accurate visualisation of the surface ultrastructure during this investigation.

Several researchers have described the development of microridges in non-keratinised epithelia. Reports include similar structures in the bladder, ectocervix, oral mucosa, oesophagus, corneum of skin, cornea and also in fish skin (Murphy *et al.*, 1975; Sperry & Wassersug, 1976; Odor *et al.*, 1989). Rabbit epithelium, according to Saito & Itoh (1993) is covered with 'mesh-like microridges'. Barberinni *et al.* (1991), completed a study of rabbit vagina, including tissue from the cervix to the 'vestibulum'. This study indicated that cyclic changes in the epithelium do occur in relation to hormonal status. Reports include the development of mucin, keratin and the appearance of microridges in response to ovarian hormones. Other studies by Davina *et al.* (1981), show cyclical changes in the epithelium of the human ectocervix, investigated using freeze fracturing techniques. Studies of the Rhesus monkey during the menstrual cycle and during pregnancy, demonstrated that the surface of the vagina changed considerably with hormonal status. During pregnancy the surface of the vagina was reported to become thinner, and develop microvillus projections rather than microridges (Davina *et al.*, 1981). Similar cyclical changes may be evident in human vestibule and vaginal epithelium. However, details of menstrual cycle, and the use of oral contraceptives was not obtained from control patients, and consequently surface ultrastructure could not be correlated with hormonal status. It was expected that the surface ultrastructure of the vagina may vary between samples due to hormonal differences, however, the epithelium of the vestibule is reported to be endodermal in

origin, and unresponsive to ovarian hormones, and was not therefore expected to vary much in appearance (Wilkinson, 1992).

Vaginal epithelium did exhibit short microvillous projections in some areas, generally however, microridges were seen to characterise the surface of the vagina. Corbeil *et al.* (1985) describe microvilli and microrugae on the vaginal surface. In the present investigation, the surface of both the vestibular and vaginal epithelia were covered by microruggae rather than microvilli. Microrugae as described by Corbeil *et al.* (1985), appear to be similar the microridges on the surface of the vestibular epithelium. These structures are not the same as the microridges evident on the surface of keratinised perineal epithelium. Microrugae are described as long raised projections with no organisation, rather than evenly distributed, stubby surface projections (microvilli). In the vagina the length of microvilli has been correlated with the level of estrogenic activity. This may be linked to absorption and cell adhesiveness in uterine cells to promote blastocyst implantation (Lamb *et al.*, 1978 as cited by Corbeil *et al.*, 1985).

The development of microridges may be an expression of the estrogen status, or alternatively, microridges may be linked to the stability, protection or lubrication of the epithelial surface (Sperry & Wasersug, 1976). The vestibule does not contain true mucus secreting glands, therefore relying on mucus from the cervix for lubrication and protection. Microridges on the vestibule were anticipated, as these structures are reported to trap mucus and maintain a moist protective covering, to prevent dehydration and penetration of pathogenic organisms. The surface of the vagina was more difficult to visualise than that of the vestibule and perineum. The presence of large amounts of mucus did not permit the visualisation of vaginal surface cells. The maintenance of mucus on this surface, despite treatment to remove the mucus, may indicate the effectiveness of microridges.

Using TEM the pattern of microridges in the perineum appeared more regular than in vestibular epithelium. Microridges were short and stubby in appearance with thickened cytoplasmic membranes (Plate 4a) consistent with the SEM findings in this investigation. The superficial cell layers of vestibular and vaginal epithelium were not as compacted as those characteristic of keratinised skin (Plate 7a & 11a). By TEM, surface cells of the vestibule and vagina were electron-lucent in comparison with their counterparts in perineum, which are keratinised and compressed and have retained many electron dense tonofilaments (Breathnach, 1971; Toner and Carr, 1982; Sternberg, 1992). The cytoplasmic membrane in perineal surface cells is heavily thickened, a process which was not evident in vestibular surface cells (Plate 4b). Fully keratinised squames of true skin do not have organelles, nuclei or glycogen deposits (Odland, 1958; Breathnach, 1971; Nakano *et al.*, 1987; Sternberg, 1992), all of which were evident in vestibular and vaginal surface cells, and which have been reported in oral epithelia (Squier, 1976; Landay and Schroeder, 1977). In vestibular and vaginal epithelium, superficial cells retain surface folds of the cytoplasm whereas in perineal epithelium the surfaces become smoother (Plate 4a and Plate 7a). This is particularly evident on the underside of surface cells in vestibular epithelium. The cytoplasmic membrane is thrown into folds, similar in appearance to cytoplasmic processes visible in other cell layers of vestibular epithelium. King (1983), reports changes in the vaginal epithelium during pregnancy, which result in an epithelial surface similar in appearance to the vestibular epithelium observed in this investigation. Vaginal tissue is reported by King (1983), to have a keratinised surface, only becoming un-keratinised during pregnancy. This was not observed in the present investigation, where the vaginal epithelium did not appear keratinised in any of the biopsies examined (Plate 11a). None of the control patients were pregnant, or had recently given birth. However, the variation in appearance may be due to the sample site, or the hormonal status of the patients. Vaginal biopsies were removed from the entrance of the vagina and not near to the cervix as in Kings' investigation (1983), which may account for the variation in keratinisation.

The presence of vesicles close to the cytoplasmic membrane of surface cells was particularly intriguing as these structures were not evident in all of the vestibular samples. Electron lucent, spherical, membrane -bound vesicles, were often seen fusing with the cytoplasmic membrane, or seen in clusters in close contact with the cytoplasmic membrane (Plate 7a, b & d). Occasionally an electron-dense vesicle was present, these structures may be membrane coating granules (MCG) associated with keratinisation and thickening of the cytoplasmic membrane of surface cells. However, the vesicles visible in this study were generally electron lucent in appearance, inconsistent with the characteristic 'cored' appearance of MCG in keratinised and non-keratinised epithelia (Breathnach, 1971; Squier, 1977; Landay & Schroeder, 1977, 1979: Schroeder, 1981). Typical MCG in non-keratinising epithelia are membrane bound with an electron dense core from which fine filaments radiate out towards the trilaminar membrane (Squier, 1977). The major difference between MCG in keratinising and non-keratinising epithelium is the absence of internal lamellae in non-keratinising MCG (Squier, 1977). Landay and Schroeder (1978), describe numerous MCG in the upper intermediate cell layer of oral epithelium; these granules are non-laminated, with a dark core characteristic of MCG found in non-keratinising epithelia (Hayward & Hackemann, 1973, as cited by Landay and Schroeder, 1978). In vestibular epithelium, a small percentage of vesicles were electron dense, however, the majority did not exhibit electron dense cores, characteristic of true MCG. The fate of electron lucent vesicles in vestibular epithelium appears similar to MCG. As in keratinised epithelia, vesicles were frequently seen in close contact and fusing with the cytoplasmic membrane of surface cells, possibly releasing their contents into the intracellular space. However, there is no evidence that these vesicles have the same properties or contents as MCG found in keratinised epithelial surfaces. The function of the MCG has received much attention, there is evidence that they contribute to intercellular permeability , and produce an intracellular cement, holding superficial cells together (Squier, 1978). Contents of granules in non-keratinising epithelia have been identified as muco-substances and glycoproteins (Hayward &

Hackemann, 1973, as cited by Squier, 1978) which would not provide the same permeability barrier as keratinised squames. It has been suggested by Squier (1978), that modification of the intercellular substance by the contents of MCG may take place to produce an intercellular permeability barrier in non-keratinising epithelia. Similar structures have been reported in the non-keratinising epithelium of rumen mucosa (Lavaker, 1969, Lavaker *et al.,* 1969). The presence of numerous electron lucent vesicles in some of the vestibular biopsies may suggest that the contents have been discharged, or alternatively, that the contents have been removed during processing. It is possible that the use of chemical fixation may influence the visualisation of these structures, as they were present in all three of the samples processed using LTFS. The absence of vesicles in a majority of the vestibular samples may suggest a hormonal influence on the formation of these structures. The influence of ovarian hormones is well documented to affect the thickness of superficial and intermediate cell layers, and deposition of glycogen. The maturation of vestibular epithelium and the deposition of glycogen may be linked to the formation of vesicles which appear to have a similar function to MCG, which would suggest why these structures were only evident in a minority of the control samples.

Vestibular epithelium processed by LTFS proved particularly interesting. The general structure was found to be identical to chemically fixed tissue using light microscopy. However, low temperature processing did not give comparable results when samples were examined using electron microscopy. Light chemical fixation, rapid freezing and UV polymerisation of the resin was expected to provide good ultrastructure and ensure antigen preservation (Robertson *et al.*, 1992). Despite this, results achieved using LTFS were disappointing, however, it was possible to detect several significant differences between tissues processed by using chemical fixatives or by LTFS. Using LTFS, the ultrastructural preservation was poor in the basal and lower intermediate cells, suggesting that during rapid freezing the full thickness of the tissue was not frozen rapidly. The cells of the basal layer were electron dense and shrunken in appearance,

with no organelles visible (Plate 13a-d). In comparison superficial and upper intermediate cells, which were in direct contact with the pre-cooled mirrored surface, were well preserved (Plate 12a-c). A larger sample number would allow variations in speed and force of slamming to be investigated. Increasing the speed of slamming may result in increased penetration of the freezing process and maintenance of ultrastructure to a greater depth in the tissue. Another factor which may influence the depth of freezing is the length of time which the specimen is left in contact with the pre-cooled surface, increasing this time from 30 to 60 seconds may benefit ultrastructural preservation.

Glycogen was evident in large amounts in vulvar and vaginal epithelium, particularly in the surface cell layers (Plates 7 & 11). Other authors have reported similar amounts of glycogen in rabbit vaginal epithelium, oral epithelial surfaces (Squier, 1976; Landay and Schroeder, 1977), and in developing embryonic skin, where it is seen in large aggregates, rosettes, or as individual particles (Breathnach 1971). Unlike skin, which does not contain glycogen in its normal state, the mucous membranes of the oesophagus, oral cavities and vagina are rich in glycogen (Falin, 1961). Deposition of glycogen in these tissues has been associated with regeneration and proliferation of epithelia (Vassiljeva, 1955, as cited by Falin, 1961); and in the vagina, glycogen released as surface cells degrade may help to maintain the acid pH. In this investigation surface cells of vaginal epithelium appeared swollen, with large aggregates of glycogen, in greater quantities than seen in vestibular epithelium. Not all of the vaginal samples investigated had this appearance, which suggests that the development of large, glycogen filled superficial cells is probably linked to hormonal status.

Glycogen, usually found in large aggregates in superficial cells, was absent in vestibular samples processed using LTFS (Plates 12 & 13). In the place of extensive glycogen deposits, large empty areas were visible, possibly where glycogen had been removed during processing.

The use of methanol as a substitution medium may affect glycogen storage in superficial cells, possible leaching glycogen from these cells. These samples were not fixed with strong aldehyde fixatives, or secondary fixed in osmium tetroxide, which may result in the removal of glycogen deposits from superficial cells. Vesicles, seen in several of the vestibular samples were visible in superficial cells in all three of the samples processed by LTFS. This may be coincidence, however, chemical processing may affect the presence of these vesicles or alter their contents, resulting in electron-lucent vesicles in some samples, and the absence of vesicles in other samples. As in the chemically fixed tissue, vesicles were seen in close contact with the cytoplasmic membrane of the surface cells, often fusing with the membrane (Plate 12 b & c). One of the benefits of LTFS is that the antigenicity of the tissue is maintained during processing, and secondly, the true nature of these vesicles could be determined using immuno-gold labelling techniques for MCG. (Bailly *et al.*, 1992).

Although little additional information was obtained using LTFS, the trial of this technique proved promising and may be important in the study of vestibular tissue from VVS patients, especially if labelling of specific antigens is required. Unfortunately, low sample numbers dictated that this method of processing was used on only three samples of normal vestibular tissue, and therefore the process was not well developed. Possible refinements of this technique for future studies of vestibular epithelium would include; increased sample numbers, variations in speed and force of slamming, and a reduction in the substitution time to prevent extraction of glycogen.

The surface of both vestibular and vaginal epithelium is moist due to the presence of mucus from the cervix, and therefore considerable transepidermal water loss from these epithelia is reported when compared with skin (Lawrence, 1993). In comparison keratinised epithelia retain

water due to the presence of keratin which acts as an barrier to excessive water loss. The rate of cell removal must be matched by the mitotic activity of the basal cells. Factors affecting the rate of turnover of cells include adrenalin and other hormones, stress and inflammation. Buccal cells are usually renewed at a rate of between 5-16 days, much more rapidly than some types of skin which renew at rates of 12-75 days (Squier *et al.*, 1976). Buccal mucosa is often slightly inflamed, the presence of a small inflammatory cell population being said to correlate with the mitotic rate and consequently the rate of cell renewal (Squier, 1976). Vestibular epithelium, which has a significant number of inflammatory cells present in its normal state and which has similarities in structure with oral epithelium, consequently, may have a similar rate of cell turnover, although there is no direct evidence to support this.

Oral mucosa may fall into one of three categories, keratinized, para-keratinised and non-keratinised. Keratinised areas have a distinct granular layer which is hard to recognise in para-keratinised epithelia and completely absent in non-keratinised epithelia (Squier *et al.*, 1976). It may be the case that a similar situation occurs in vestibular epithelium. Passing from the hymen towards Hart's Line, the degree of keratinisation gradually increases, accompanied by changes in tissue structure from sub-mucosal to true keratinised skin. This would agree with some researchers findings that the epithelial surface of the vulva is slightly keratinised and pigmented (Friedrich, 1985), contrary to the findings of this study which showed no evidence of keratinisation or pigmentation. Although cells of the upper intermediate layer do not have keratohyalin granules as in perineum and true skin, cells at a similar distance from the surface of the vulvar epithelium do have characteristic features which allow them to be distinguished from cells of the lower intermediate and supra basal layers. Differences include an accumulation of large amounts of glycogen, an increase in the size of individual cells which is not accompanied by an increase in organelle density, and scattered individual cytokeratin filaments not organised into large bundles. These features were also seen in vaginal epithelium,

and are consistent with reported changes seen in oral mucosa (Squier, 1976; Landay and Schroeder, 1977, 1979).

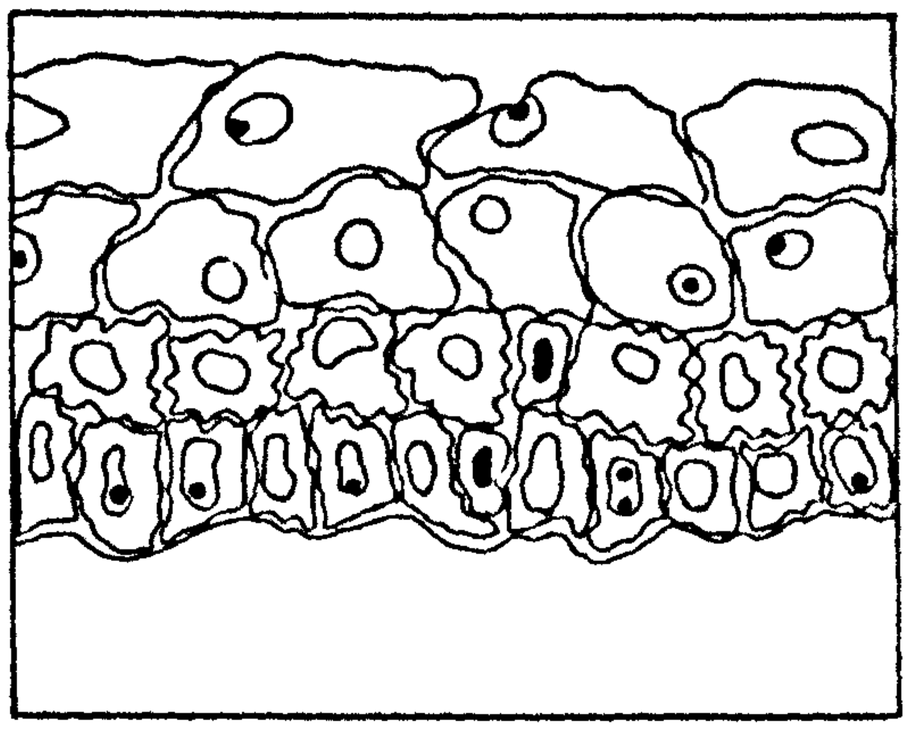
Some darkened, possibly apoptotic cells, were present in vestibular and vaginal epithelium. Visible in all cell layers, these cells were seen infrequently in both vestibular and vaginal epithelia, in isolated cases. These epithelial cells had the appearance of apoptotic cells, being shrunken in appearance, often with vacuolated cytoplasm and condensed cytokeratin filaments. Organelles, when visible, did not appear damaged or swollen. These apoptotic-like cells were barely visible using light microscopy. However, cells which stained more intensely using methylene blue for light microscopy, correlated with the electron dense apoptotic-like cells obvious using electron microscopy.

In summary, the presence, and incidence of apoptotic-like cells, although not a significant feature of the control tissue, proved to be extremely important in the ultrastructural study of pathological tissue from vestibulitis patients (see Chapter 5). The presence of apoptotic cells in vestibular epithelium has not previously been described. The use of LTFS proved to be a useful addition to the investigation, but needed refining to ensure good ultrastructural preservation. Vestibular epithelium was found to share many ultrastructural characteristics with vaginal epithelium, but several differences included flattened surface cells, more defined cytoplasmic processes and frequent inflammatory cells in vestibular epithelium. Vestibular samples processed using LTFS illustrated the presence of vesicles similar in appearance to MCG, which were not evident in perineal or vaginal epithelium. These structures may be investigated in future studies using immunocytochemistry (Bailly *et al.*, 1992). Characteristics distinguishing vestibular epithelium from keratinised perineal epidermis included: the absence of keratohyalin granules and granular cells; the presence of pale staining cytokeratin filaments; large glycogen deposits in surface and intermediate cells; close cytoplasmic processes between

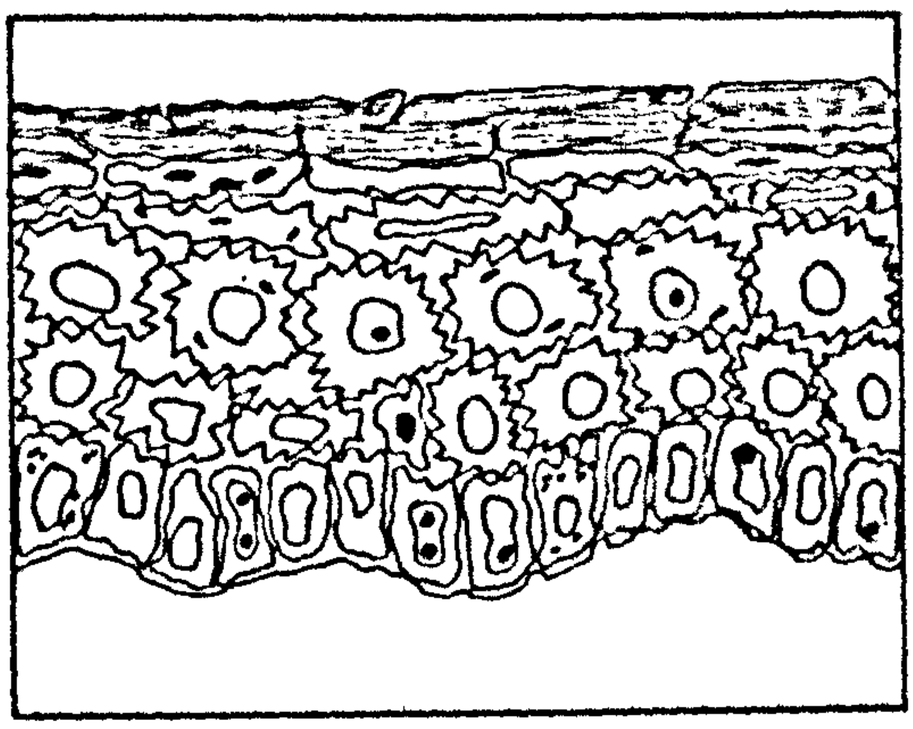
cells of all layers and the presence of prominent, rounded microridges on the surface of this unique, and highly specialised epithelium.

Fig.4.4 Line drawings of the overall appearance of a) vaginal, b) perineal and c) vestibular epithelia

A.VAGINA



B.PERINEUM



C.VULVA

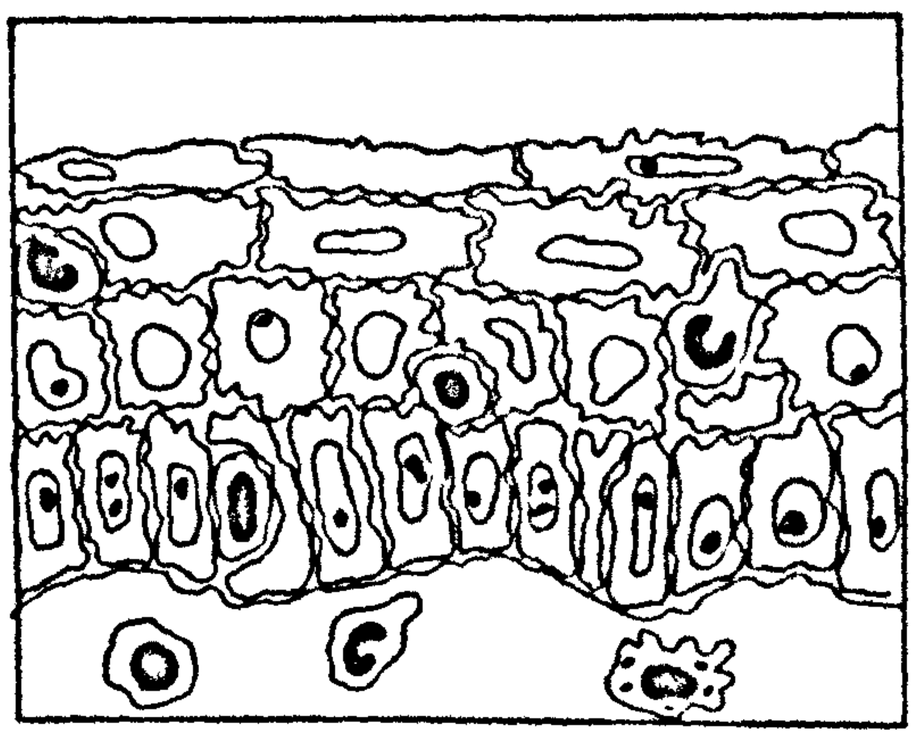
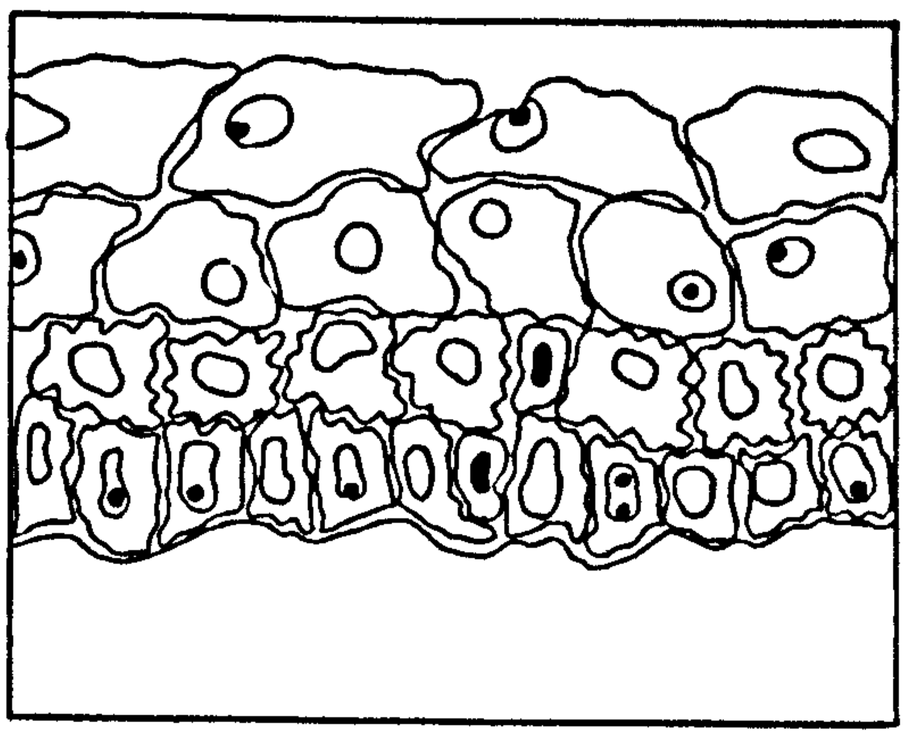
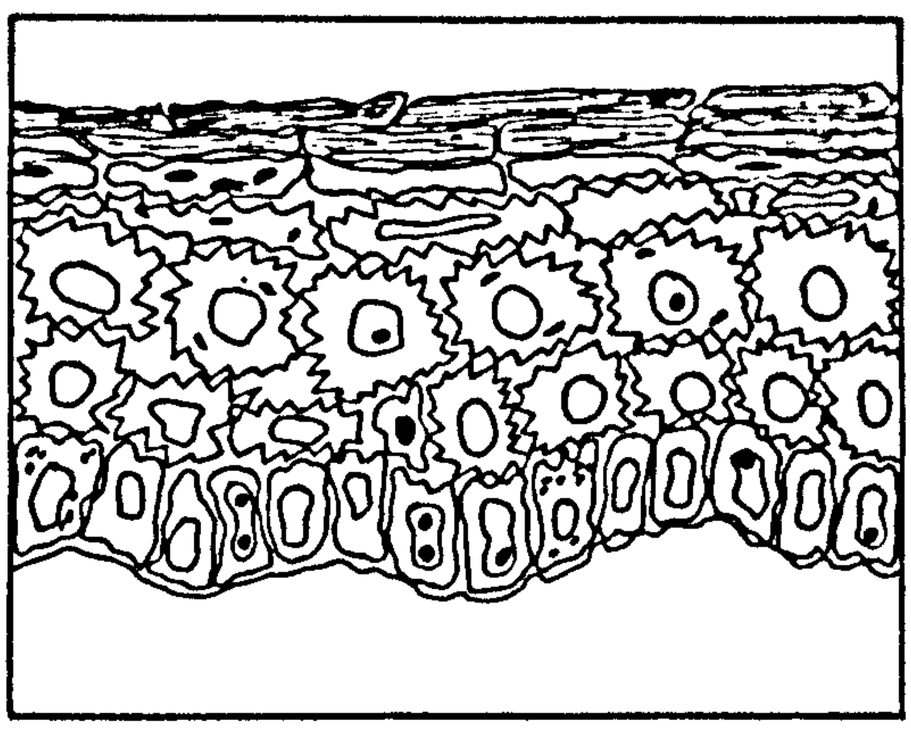


Fig 4.4 Line drawings of the overall appearance of a) vaginal, b) perineal and c) vestibular epithelia





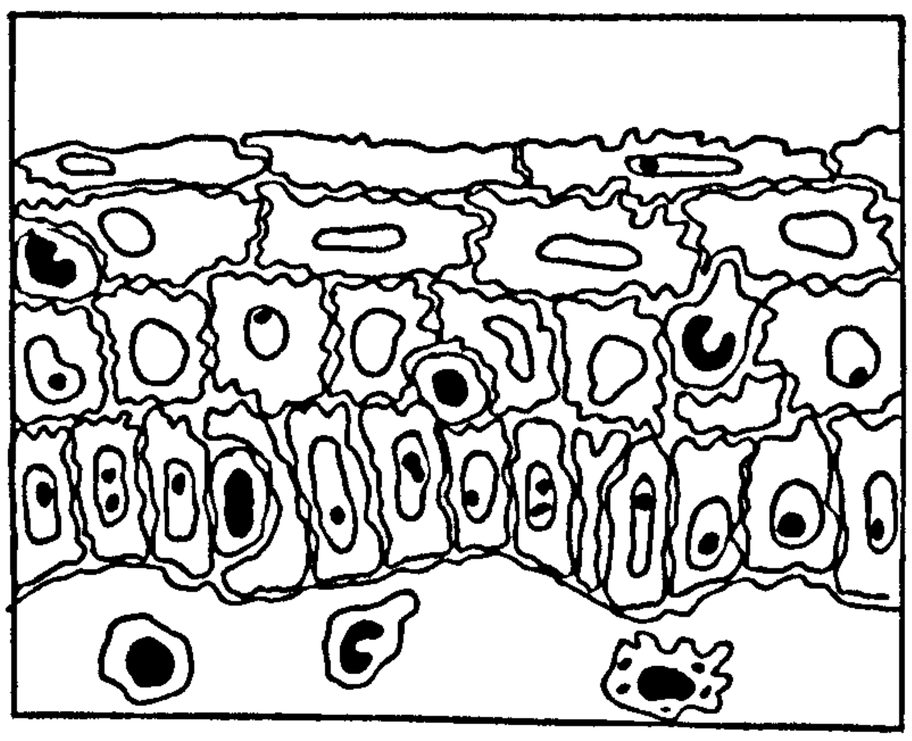


Fig.4.5 Schematic representation of perineal epidermis, illustrating the presence of granular cells (Gc), keratinised squames (Sq), and spiny cells (Sp). Granular cells have patch like keratohyalin granules (Kg). Spiny cells are characterised by intensely staining cytokeratin filaments (F) and numerous desmosomes (De). Note the undulant basement membrane (Bm), and leukocytes (Le) in the dermis.

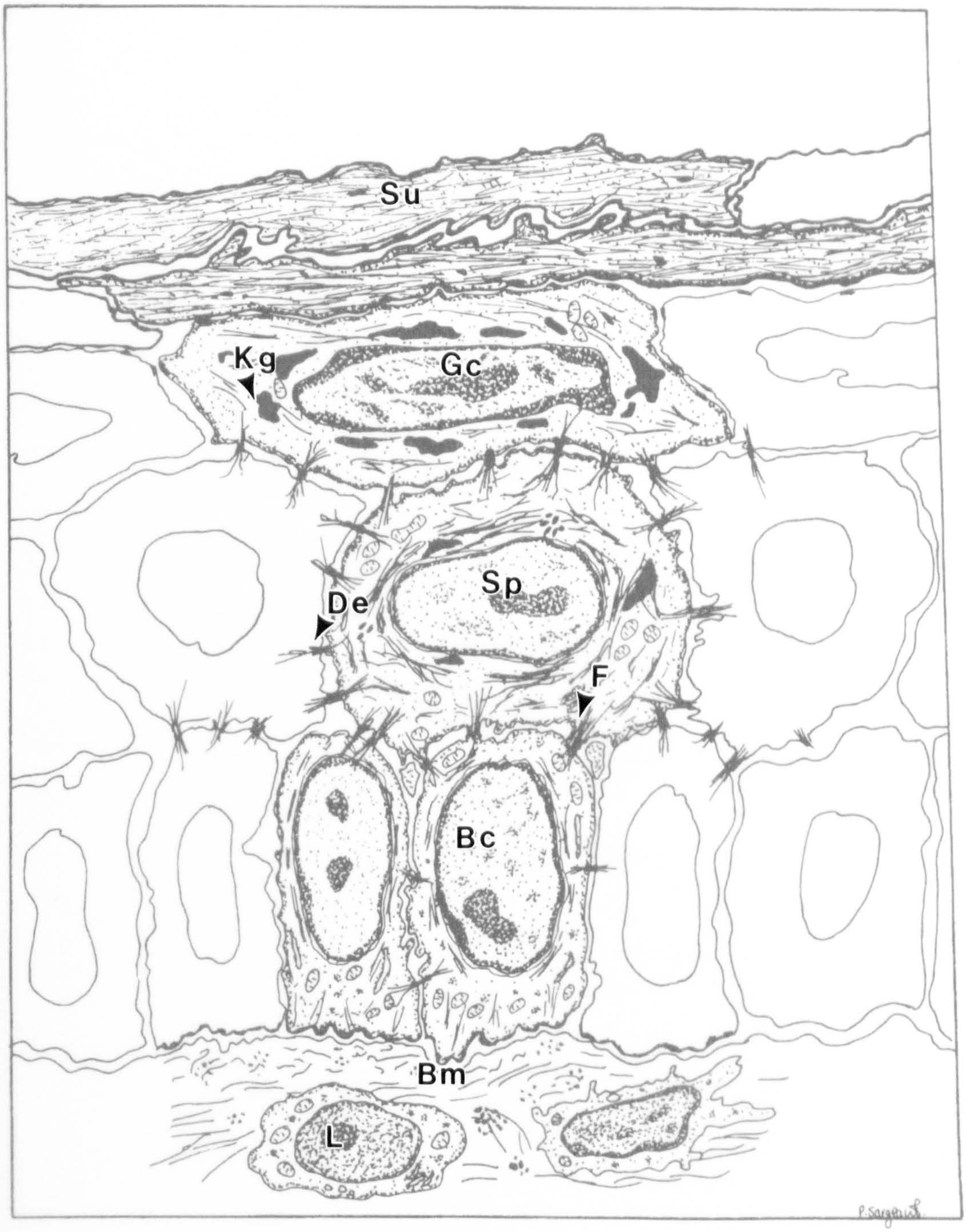

Su
Kg
Gc
Sp
De
F
Bc
Bm
L

Fig.4.6 Schematic representation of vestibular epithelium, illustrating the presence of pale staining cytokeratin filaments (F) in intermediate cells (Ic). Microvilli (Mv) are seen on non-keratinised superficial cells (Su). Glycogen (G) is seen in large aggregates in the intermediate and superficial cells. Desmosomes (De) are infrequent; and mitochondria (M) are common in basal (Bc) and intermediate cells (Ic). The basement membrane (Bm) undulant, below which diffuse connective tissue and capillaries (Ca) are evident.

Mv

Su

G

Ic

De

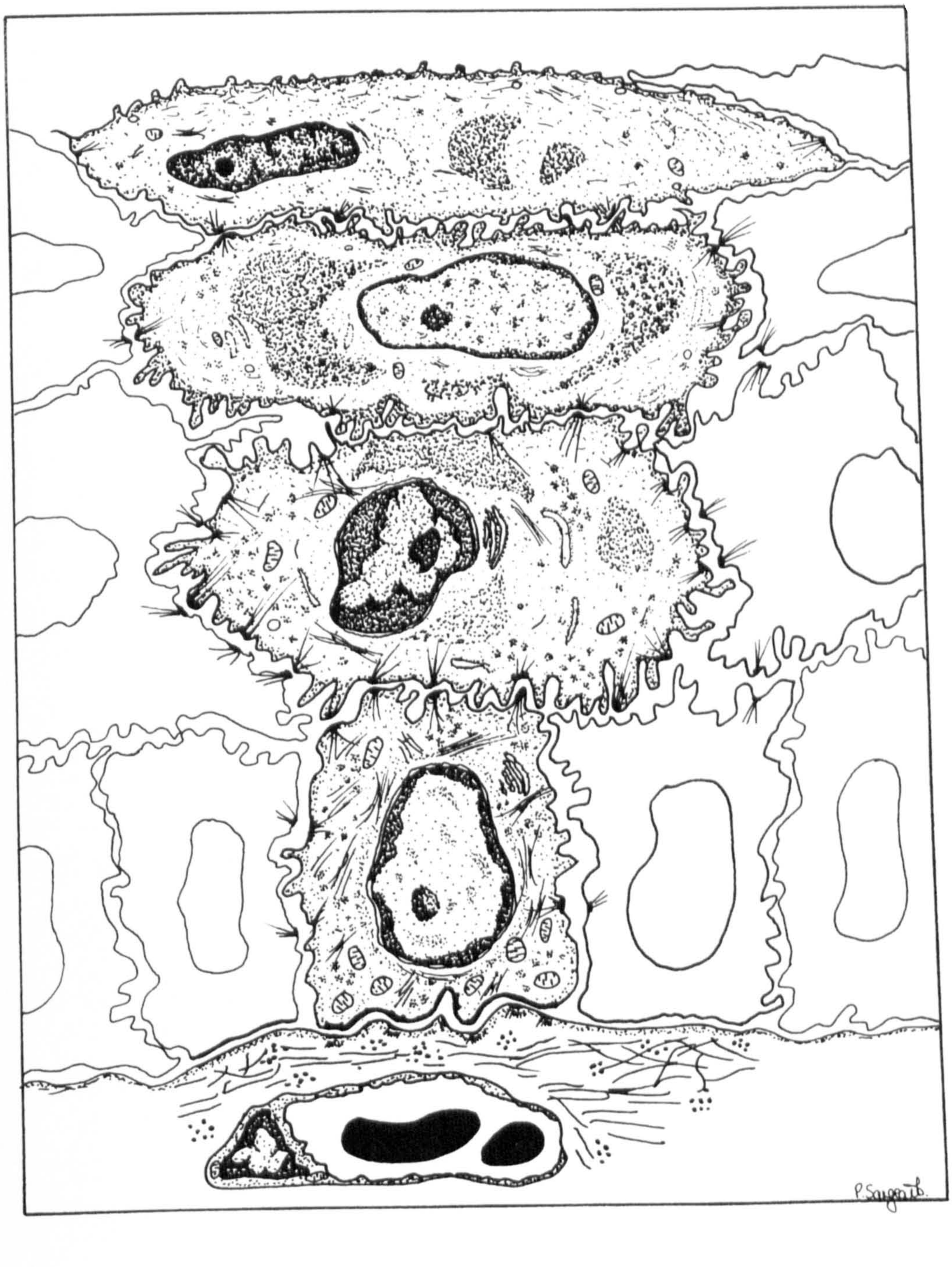
M

F

Bc

Bm

Ca



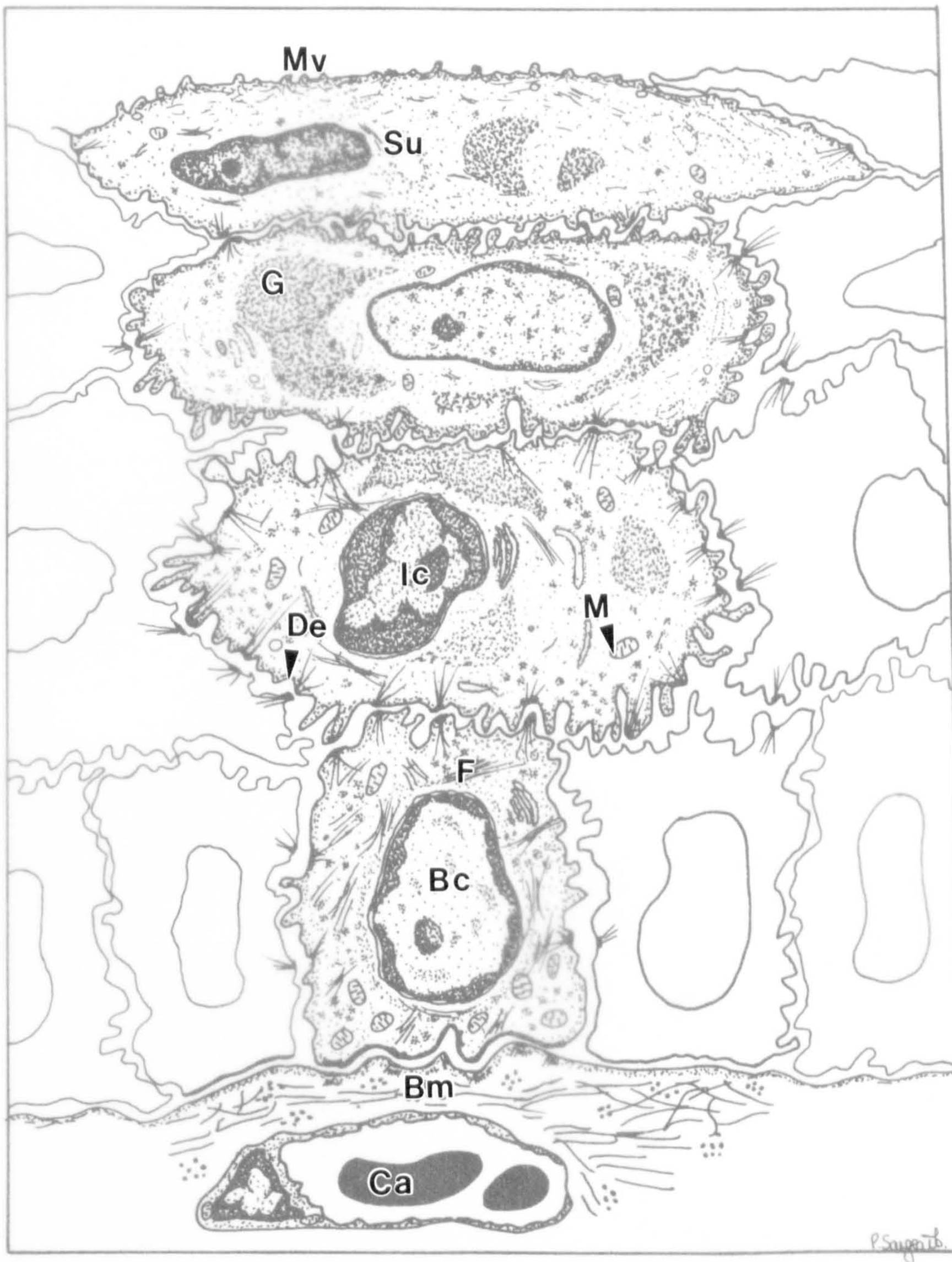
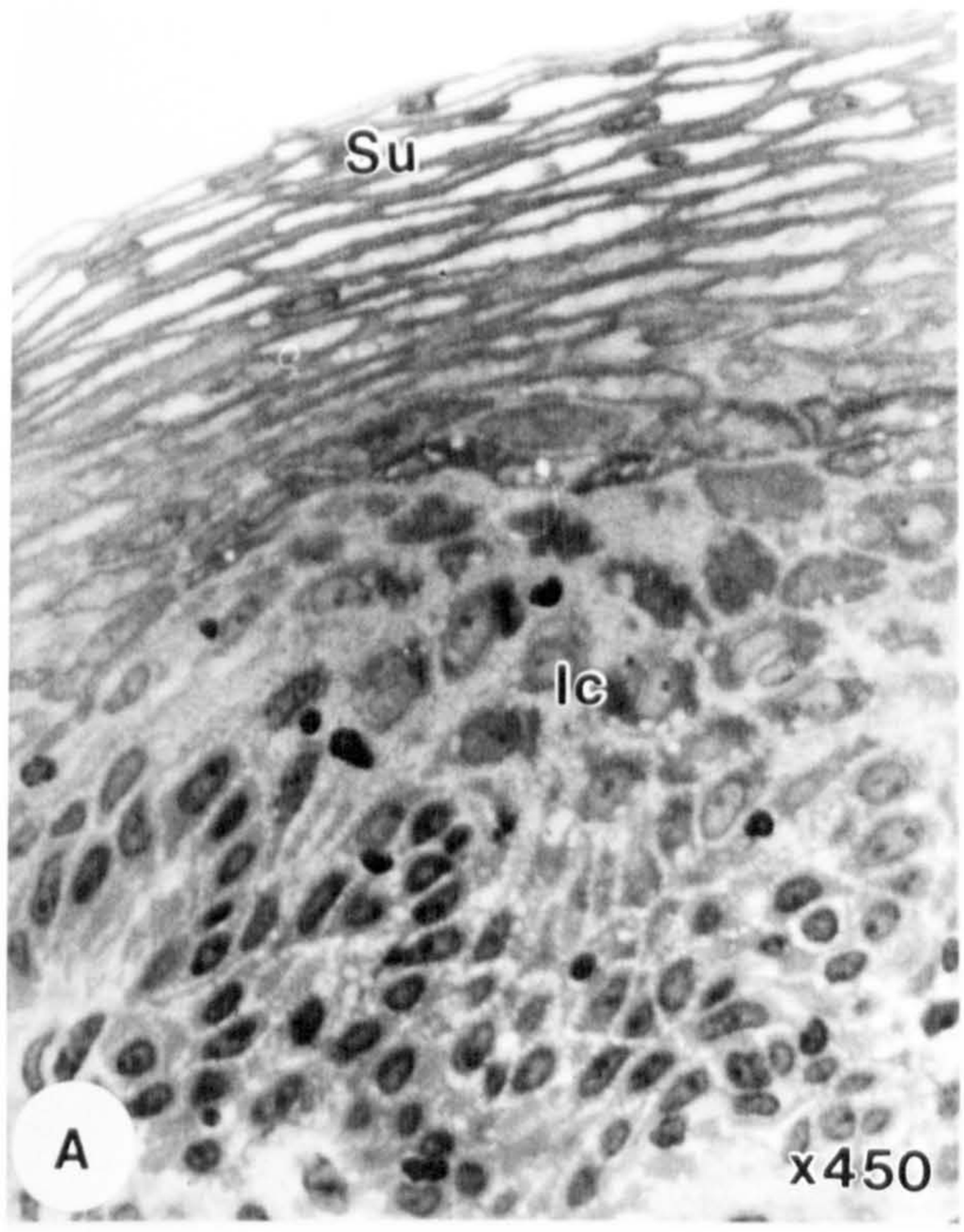

Mv
Su
G
Ic
De
M
F
Bc
Bm
Ca

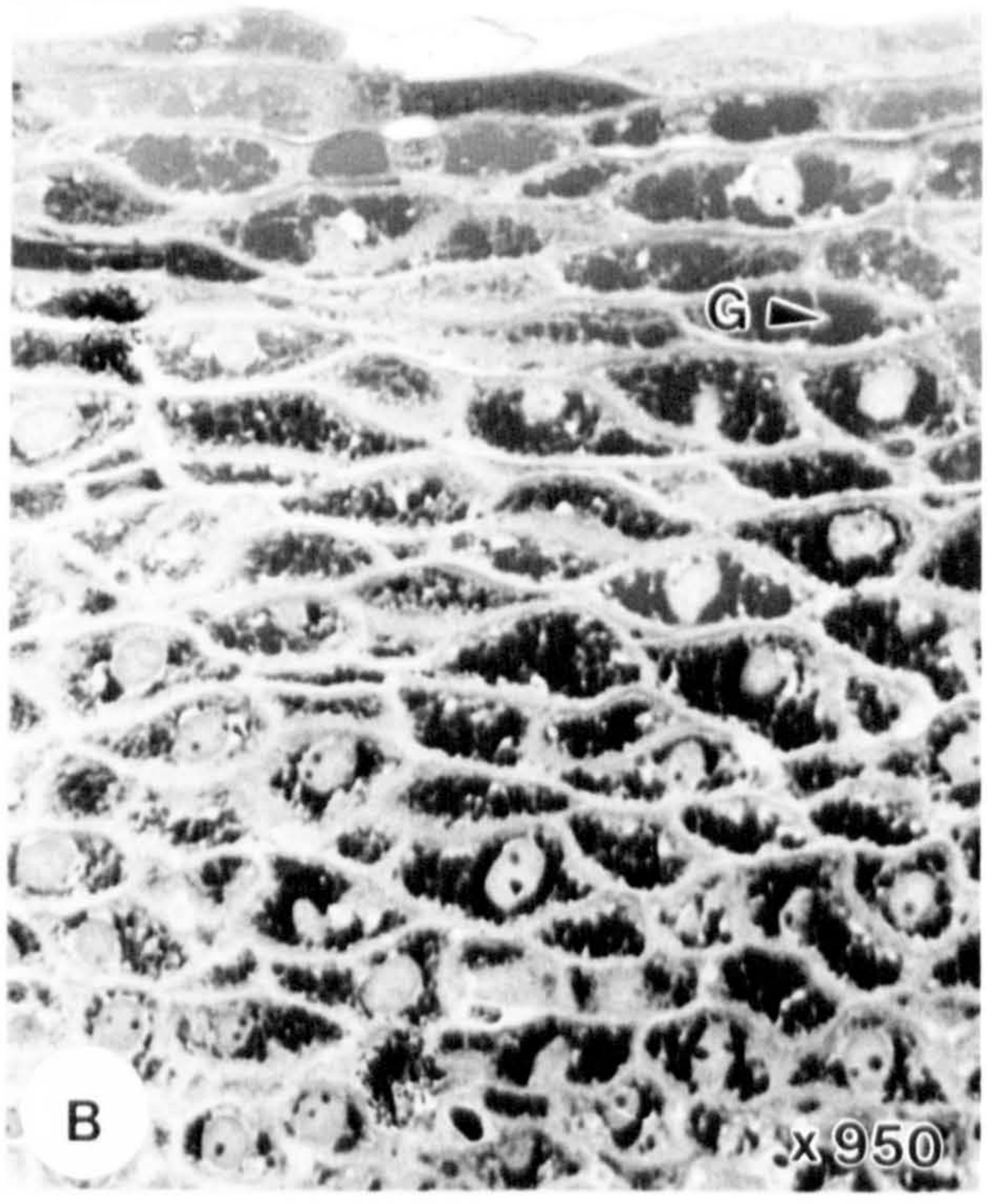
Plate 1a LM photograph of vaginal epithelium stained with 1% methylene blue, showing an epithelial surface of 15 -20 cells thick. Surface cells are flattened (Su), and have displaced nuclei. Intermediate cells form a large proportion of this epithelium (Ic) x 450

Plate 1b LM photograph of surface cells of vaginal epithelium packed with glycogen (G). x 950

Plate 1c LM photograph of vulvar epithelium stained with 1% methylene blue, demonstrating slightly thickened surface cells (Su). A largely homogeneous intermediate cell (Ic) population make up the majority of this epithelium. Leukocytes (Le) are seen penetrating this epithelial surface, and are visible in the dermis (Der). x 450

Plate 1a LM photograph of perineal epidermis stained with 1% methylene blue, illustrating keratinised surface cells (Sq), a thin granular cell layer (Gc), a prominent spiny cell layer (Sp) and basal cells. The basement membrane and connective tissue (Der) are also clearly visible. x 450


Su
Ic
A
x450


G
B
x 950


Su
Le
Ic
C
Der
x 450

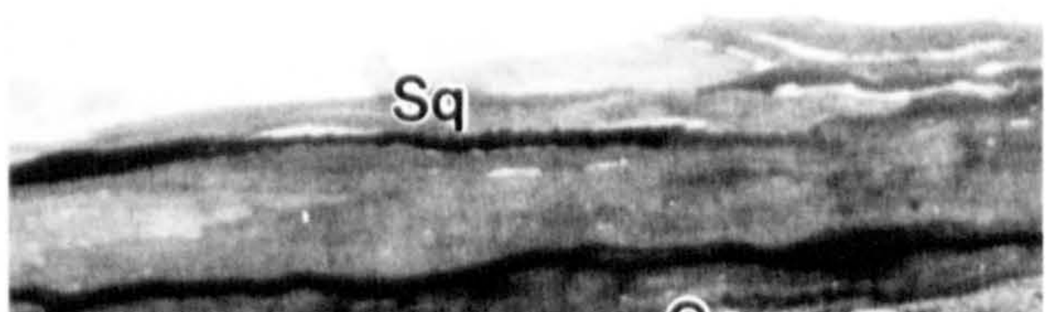
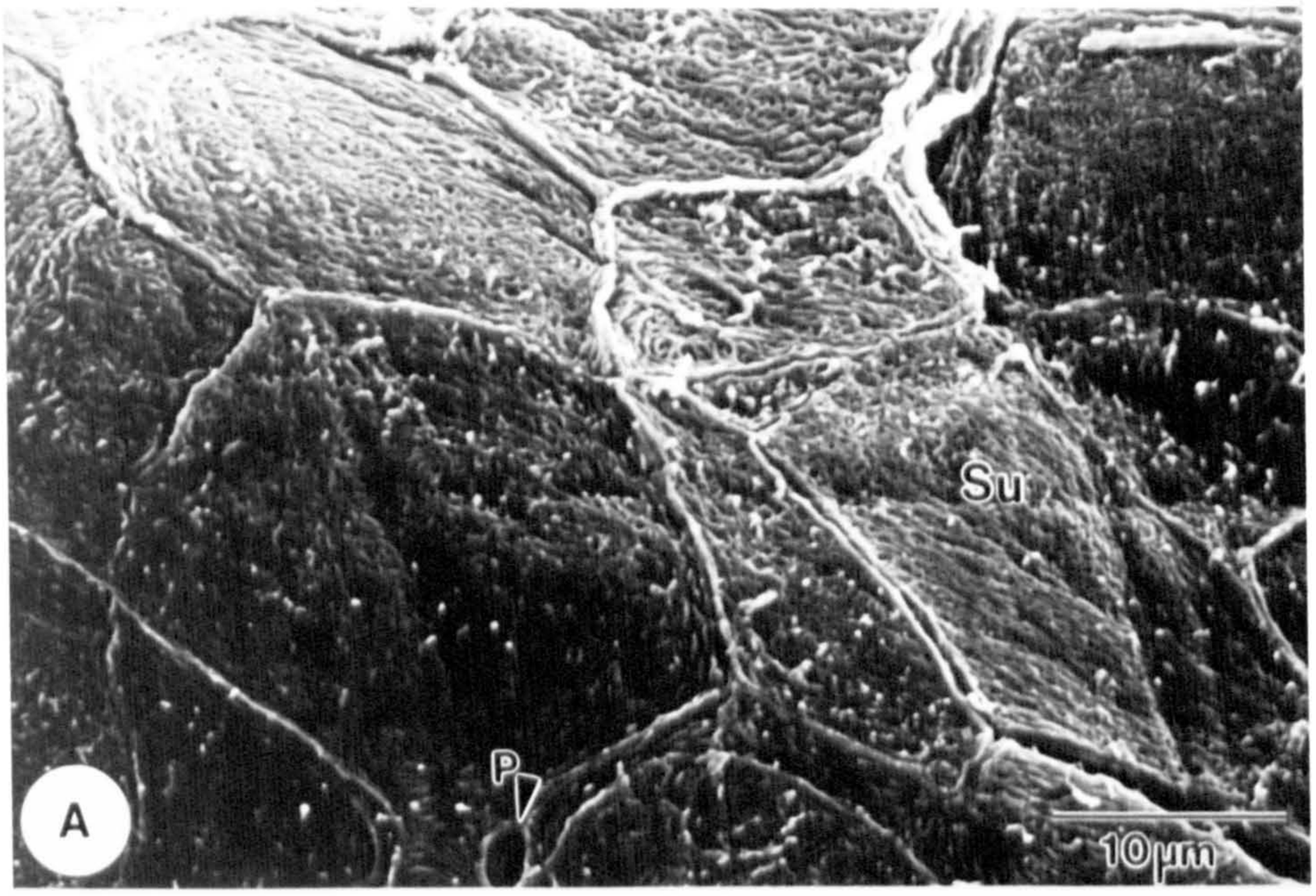
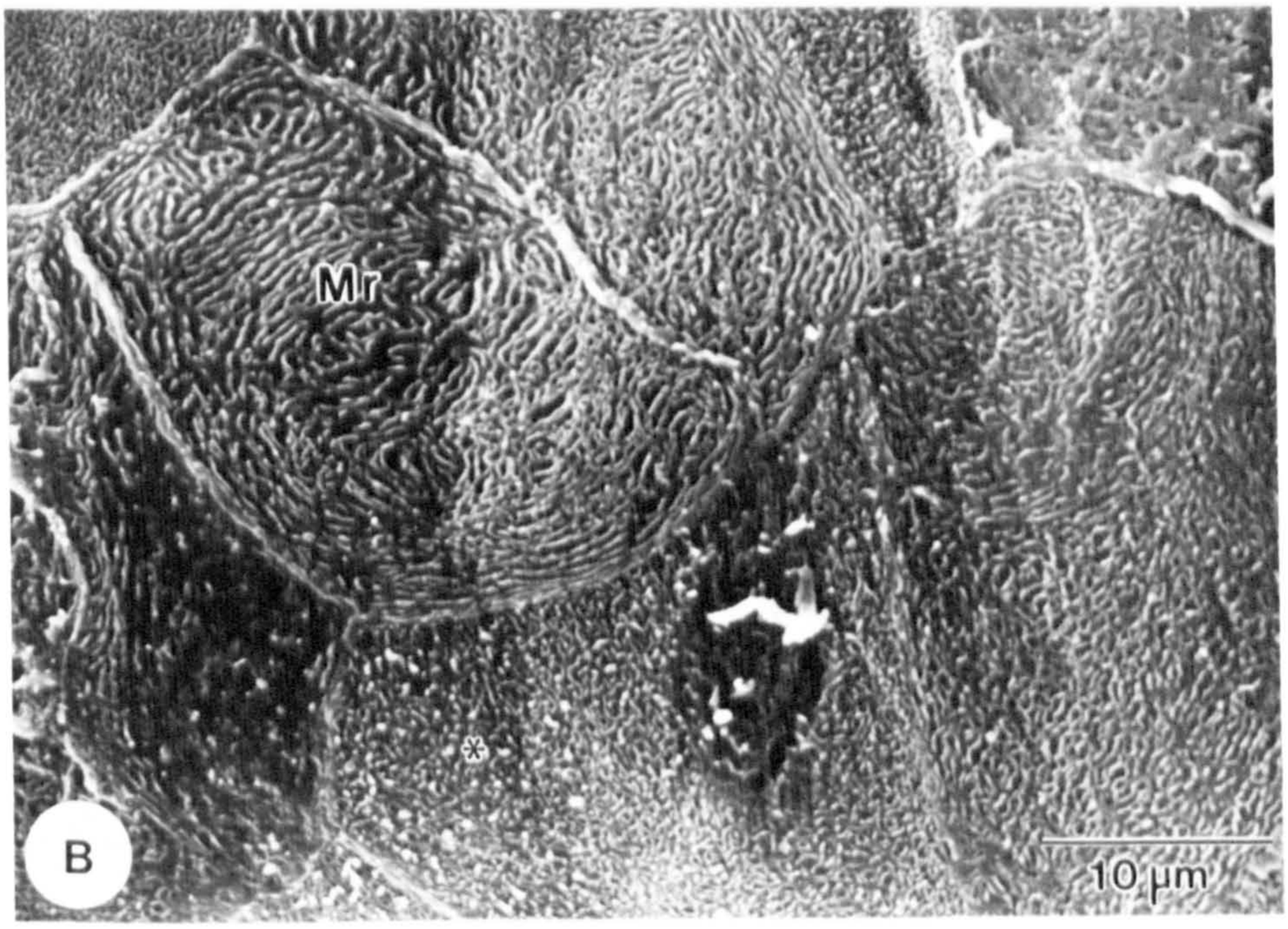

Sq
Gc
Sp
Der
D
x450

Plate 2a Scanning electron micrograph of perineal surface cells (Su). Cell junctions are prominent, thickened and raised. Surface cells (Su) are polygonal in shape. A pore is visible (P) which may be a mucus secreting gland or sebaceous gland opening. Bar =10μm

Plate 2b Polygonal surface cells of perineal epidermis, characterised by a network of flattened microridges (Mr). The cell at the bottom of the micrograph has a slightly different surface appearance (*). This cell has prominently raised surface microridges. Bar = 10μm

Plate 2c High power micrograph of surface ridges. In some areas the surface cells appear completely flat (Fl) and structure less. Some microridges are flattened and worn in appearance (Mr). Bar = 5μm


Su
P
A
10μm


Mr
B
10 μm

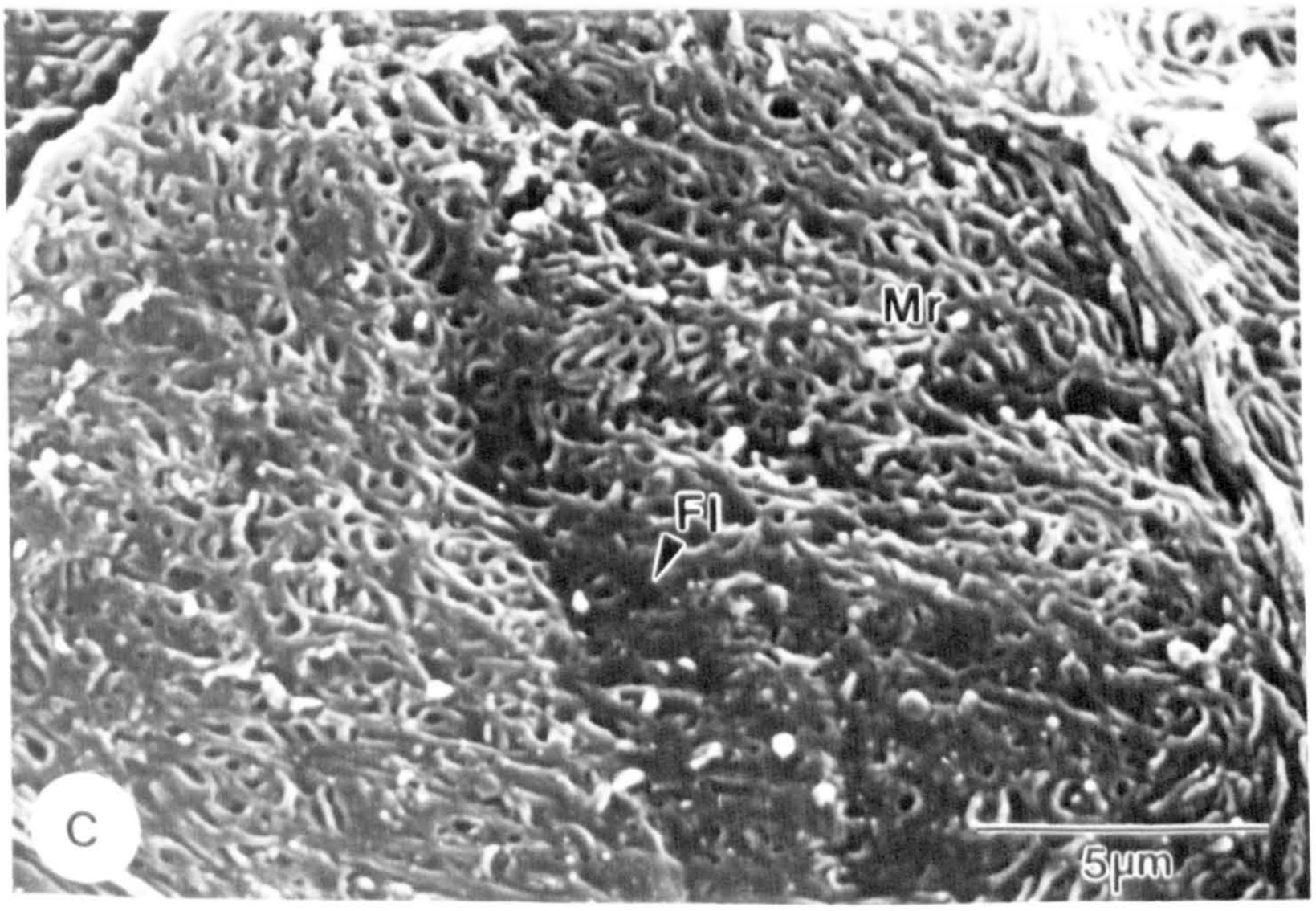

Mr
Fl
C
5μm

Plate 3a Scanning electron micrograph of normal vestibular epithelium. The surface cells in this micrograph appear undulant, with prominent cell junctions (Cj).

Bar = 5μm

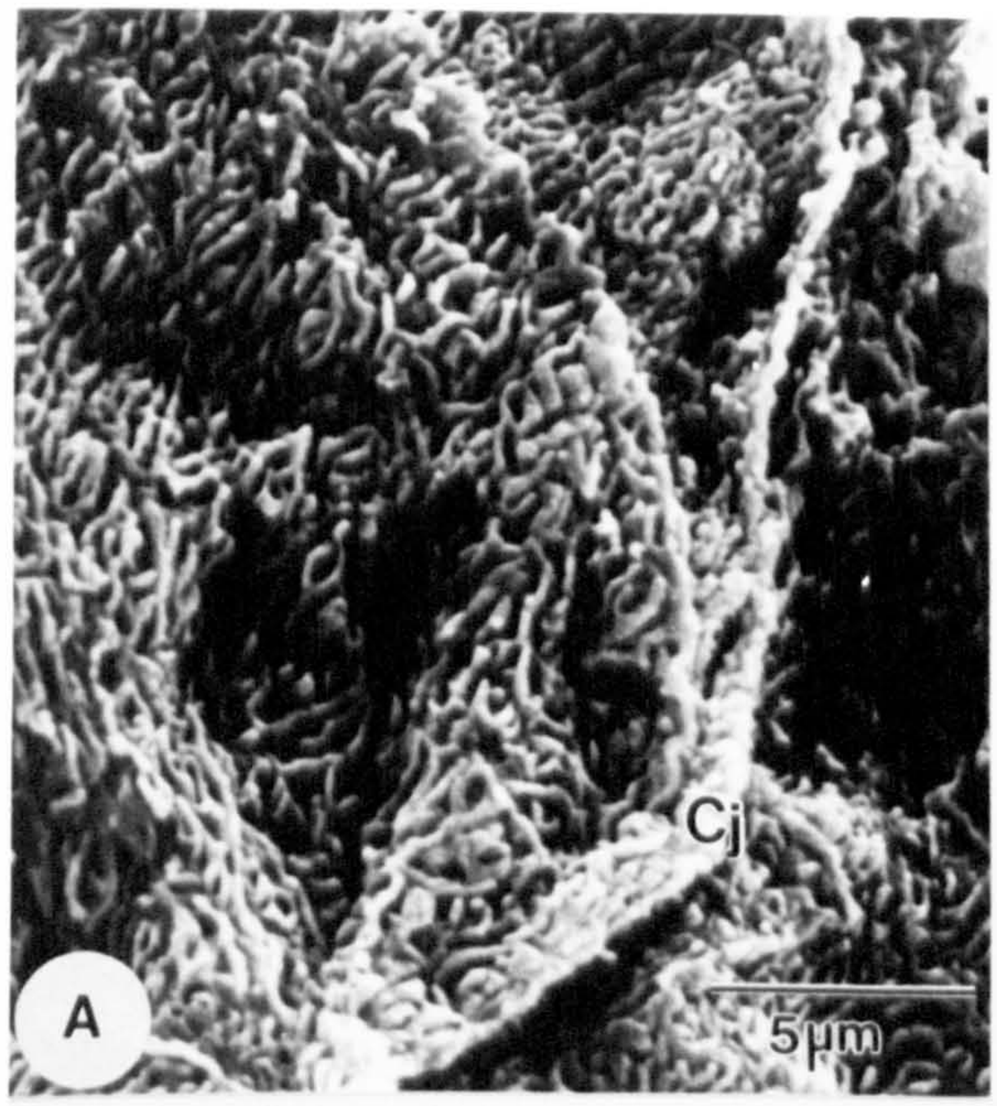
Plate 3b & c The surface microridges of vestibular epithelium are raised and rounded in appearance. Microridges (Mr) of varying lengths and dimensions form an interlacing network covering the surface of superficial cells.

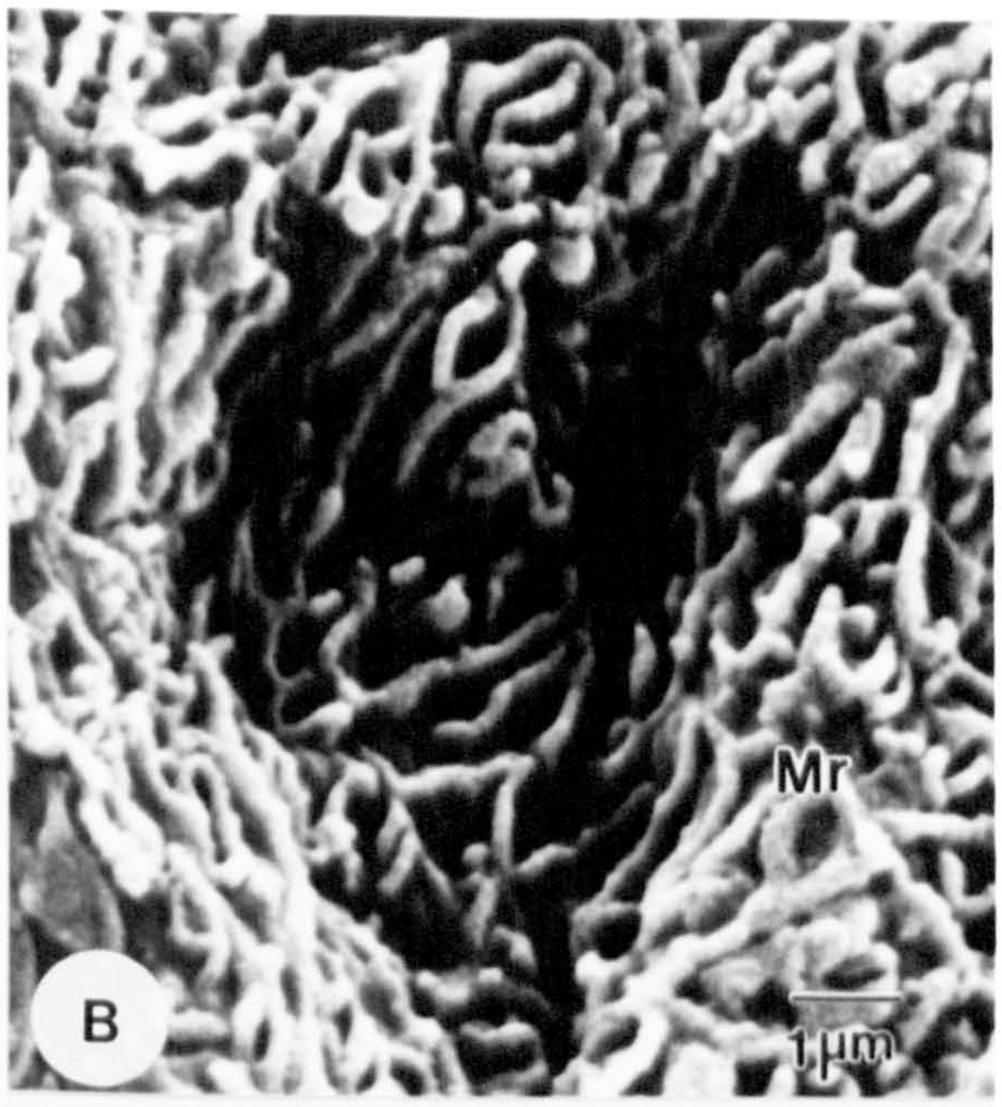
b) Bar = 1μm

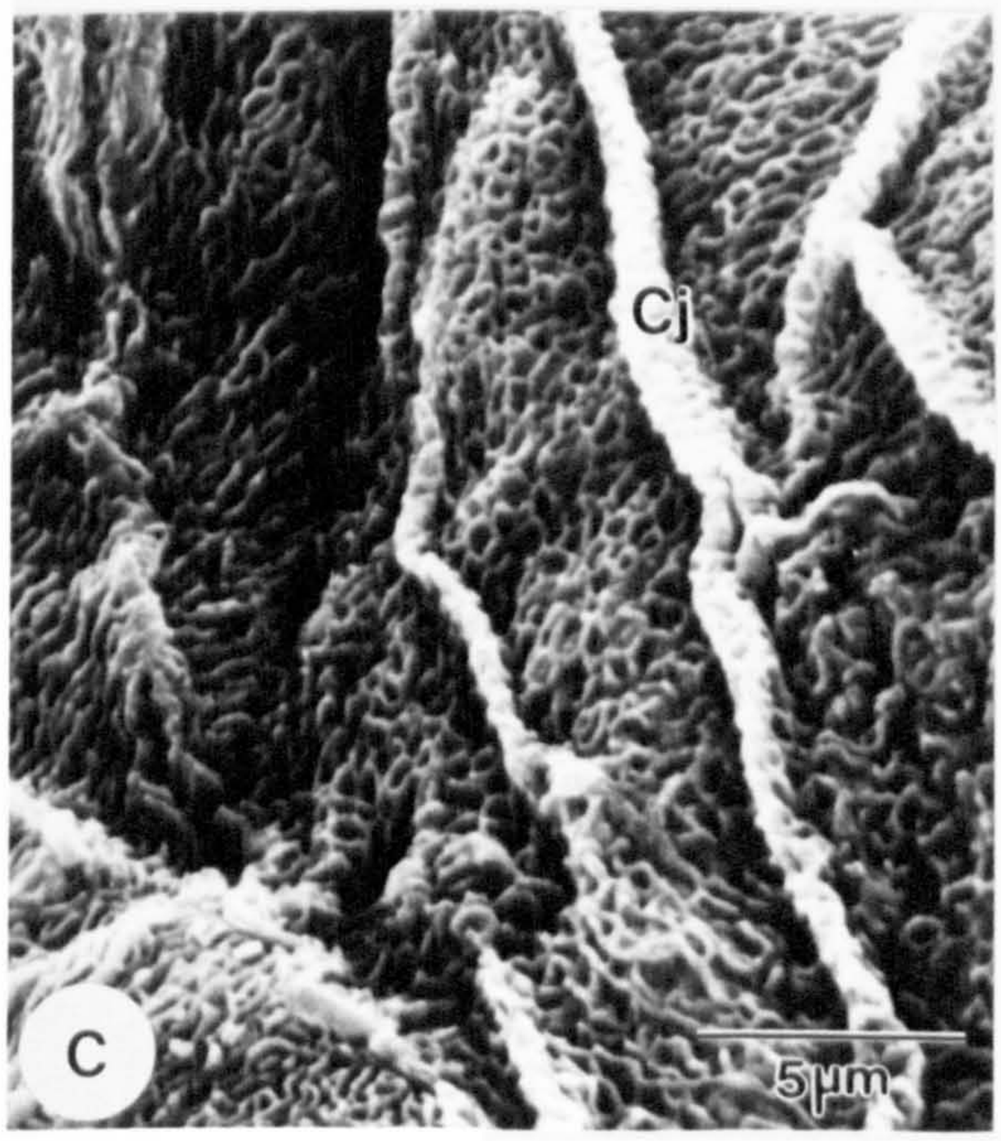
c) Bar = 5μm

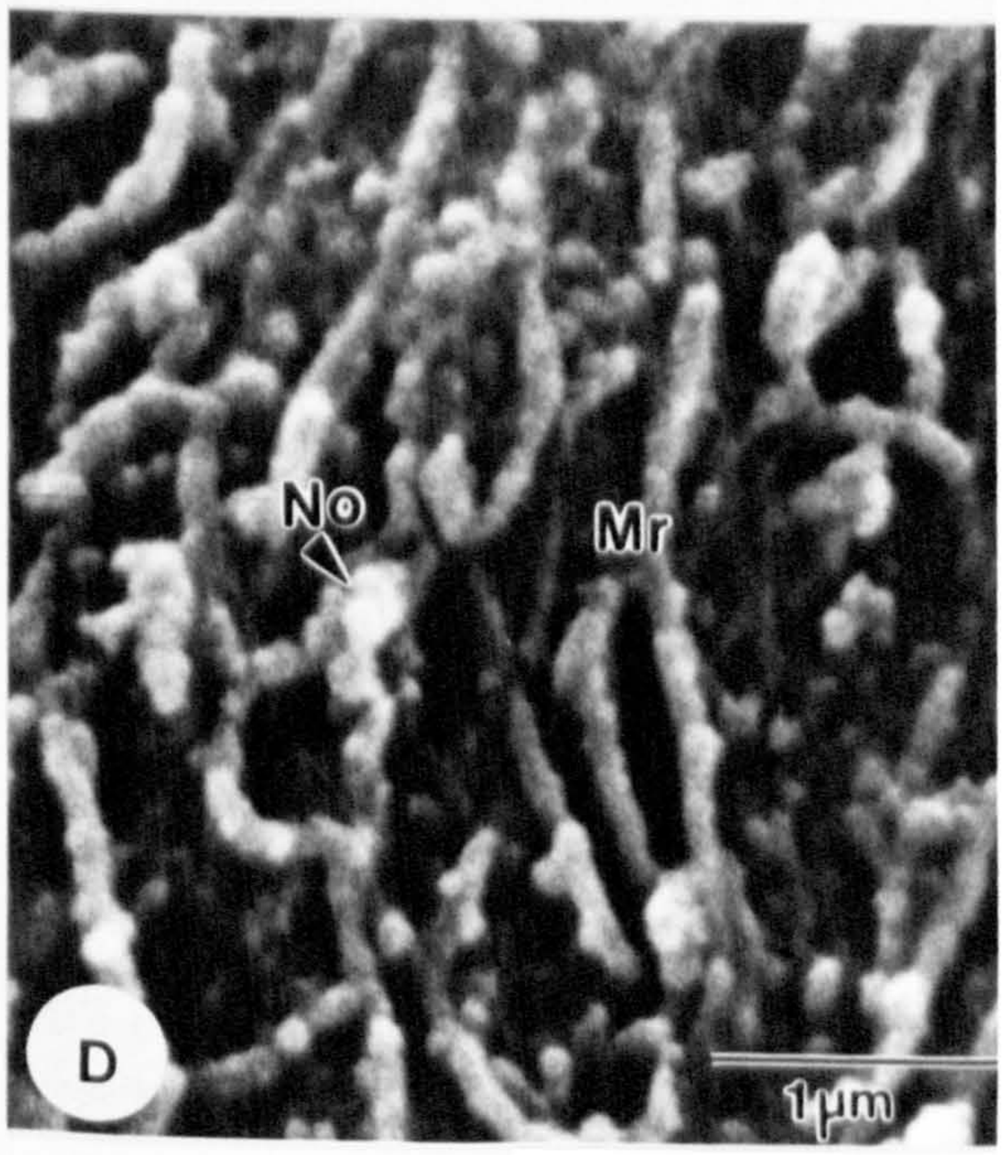
Plate 3d At high magnification the microridges (Mr) appeared nodular (No). Bar = 1μm

Plate 3e A flat area of epithelium, indicating the fine, slightly thickened cytoplasmic junction (Cj) and interlacing, raised microridges (Mr). The orientation of microridges varies on individual cells and between surface cells. Bar = 5μm


Cj
A
5μm


Mr
B
1μm


Cj
C
5μm


No
Mr
D
1μm

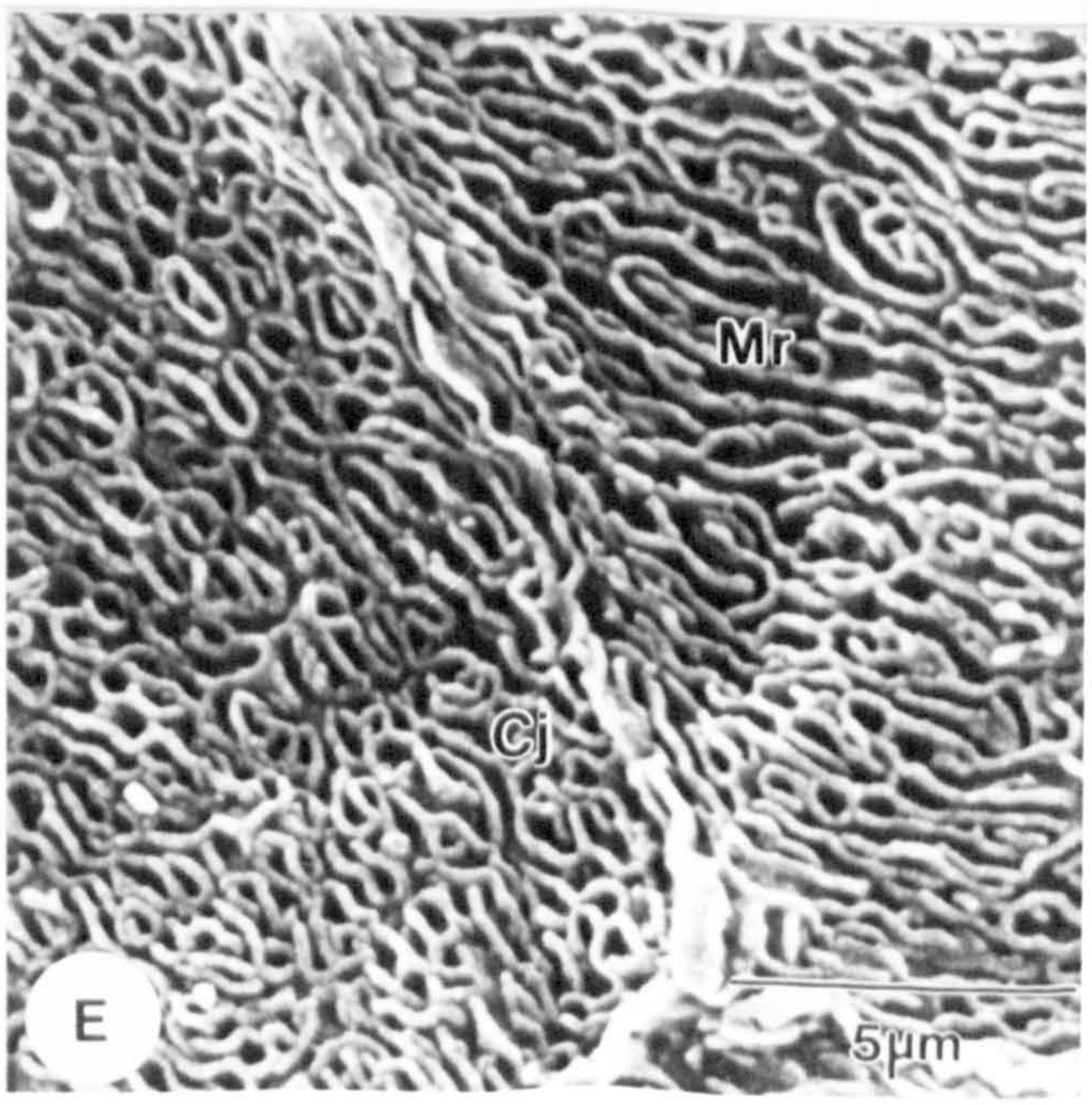

Mr
Cj
E
5μm

Plate 4a Surface squame (Sq) of perineal epidermis; the paler squame is desquamating leaving the remains of cellular junctions (De) on the exposed surface of the underlying darker staining cell. Cytokeratin filaments (F) are still visible in these keratinised cells. x 8,500

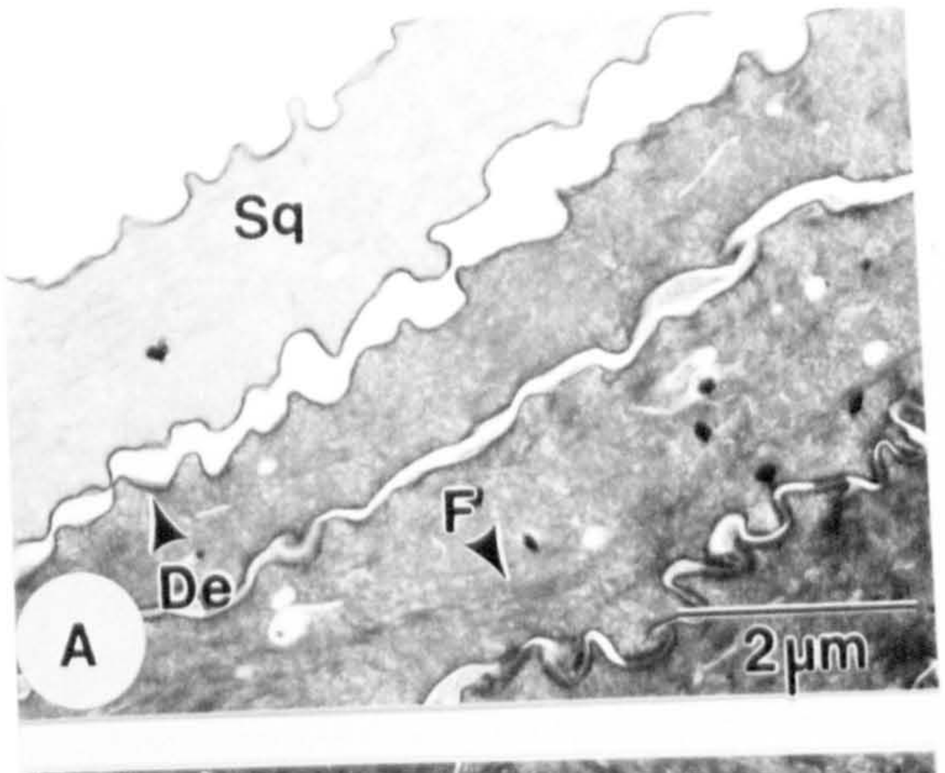
Plate 4b Desmosome junction (De) from an area similar to Plate 4 a, showing thickened cytoplasmic membranes, dark staining cytokeratin filaments (F) and the absence of cellular organelles in the surface cells. x 41,000

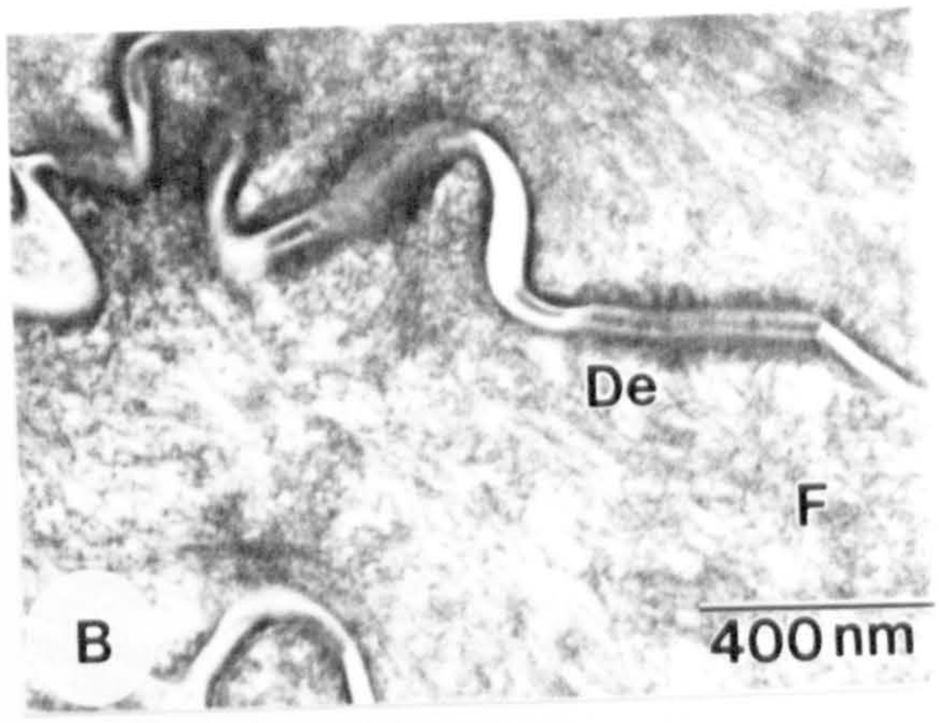
Plate 4c Transition zone in perineal epidermis from granular cells (Gc) to surface squames (Sq) of the stratum corneum. Note the absence of organelles in both cell layers, and the presence of cytokeratin filaments (F) and globular and patch-like keratohyalin granules (Kg) in the granular cells. x 5, 500

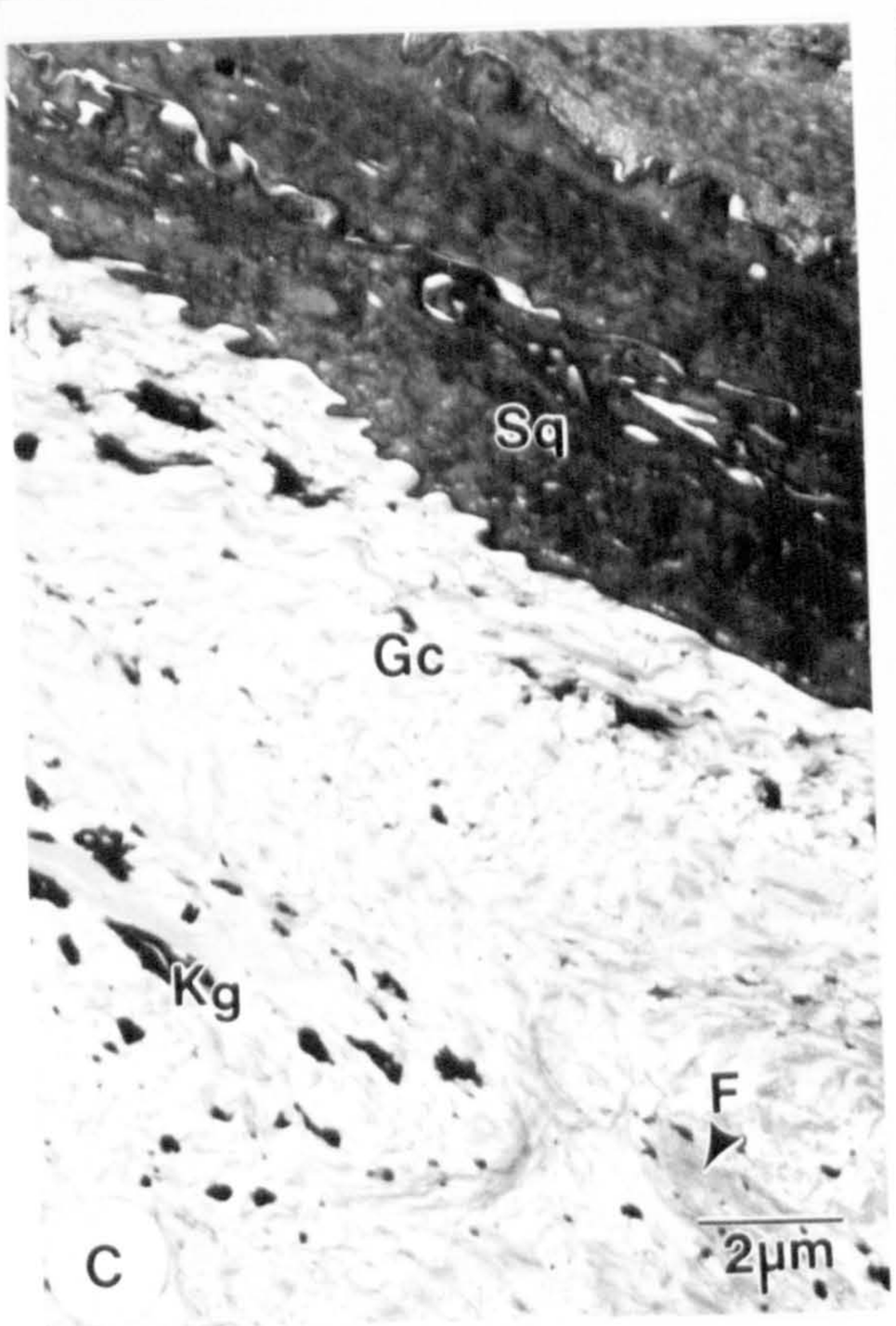
Plate 4d Globular keratohyalin granules (Kg) in the transition zone from granular to corneal cells. x 17,000

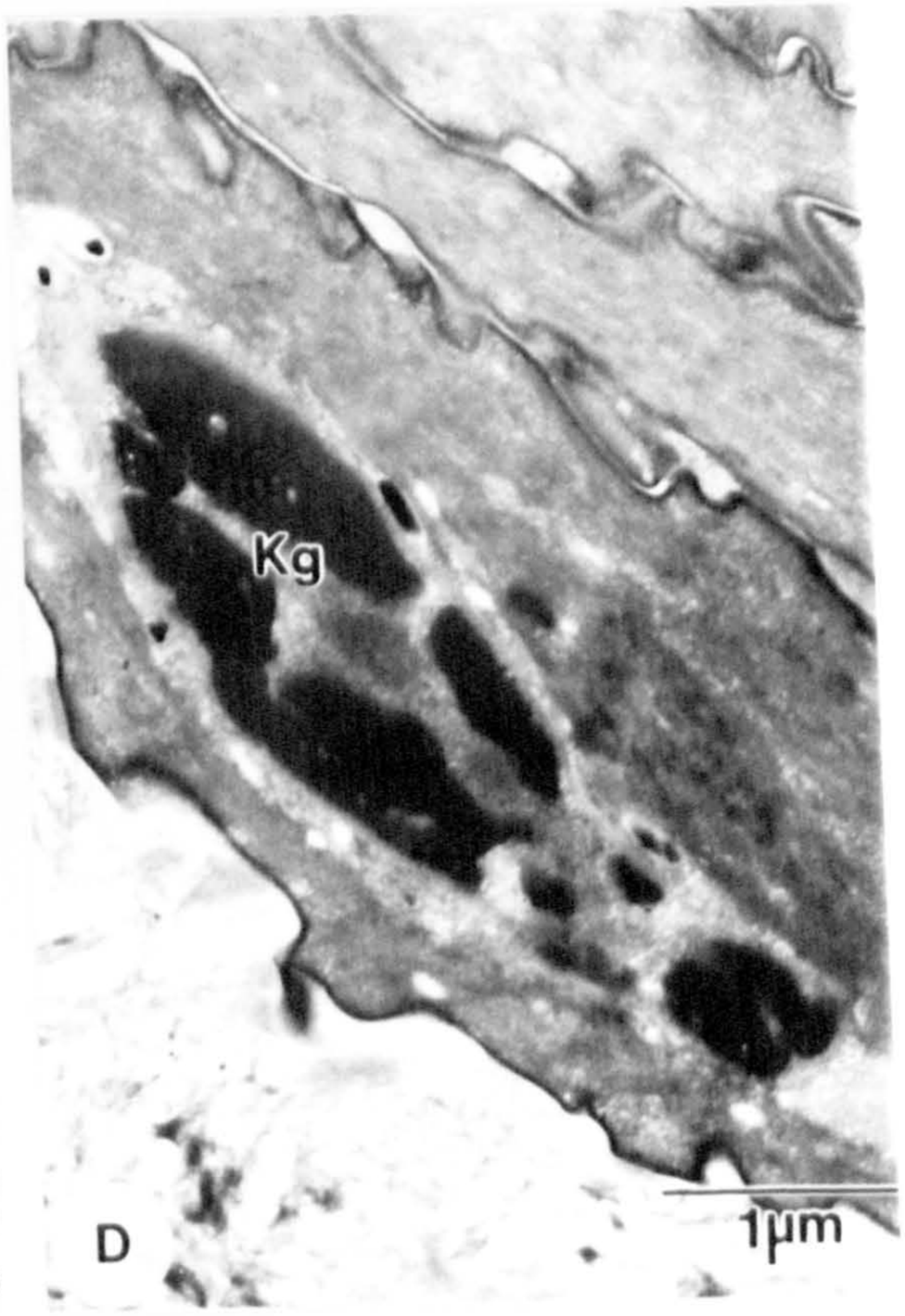
Plate 4e Low power micrograph of a flattened cell in the granular cell layer (Gc), which has retained its nucleus. Note the dark staining cytokeratin filaments (F) and patch-like keratohyalin granules (Kg). x 5,200

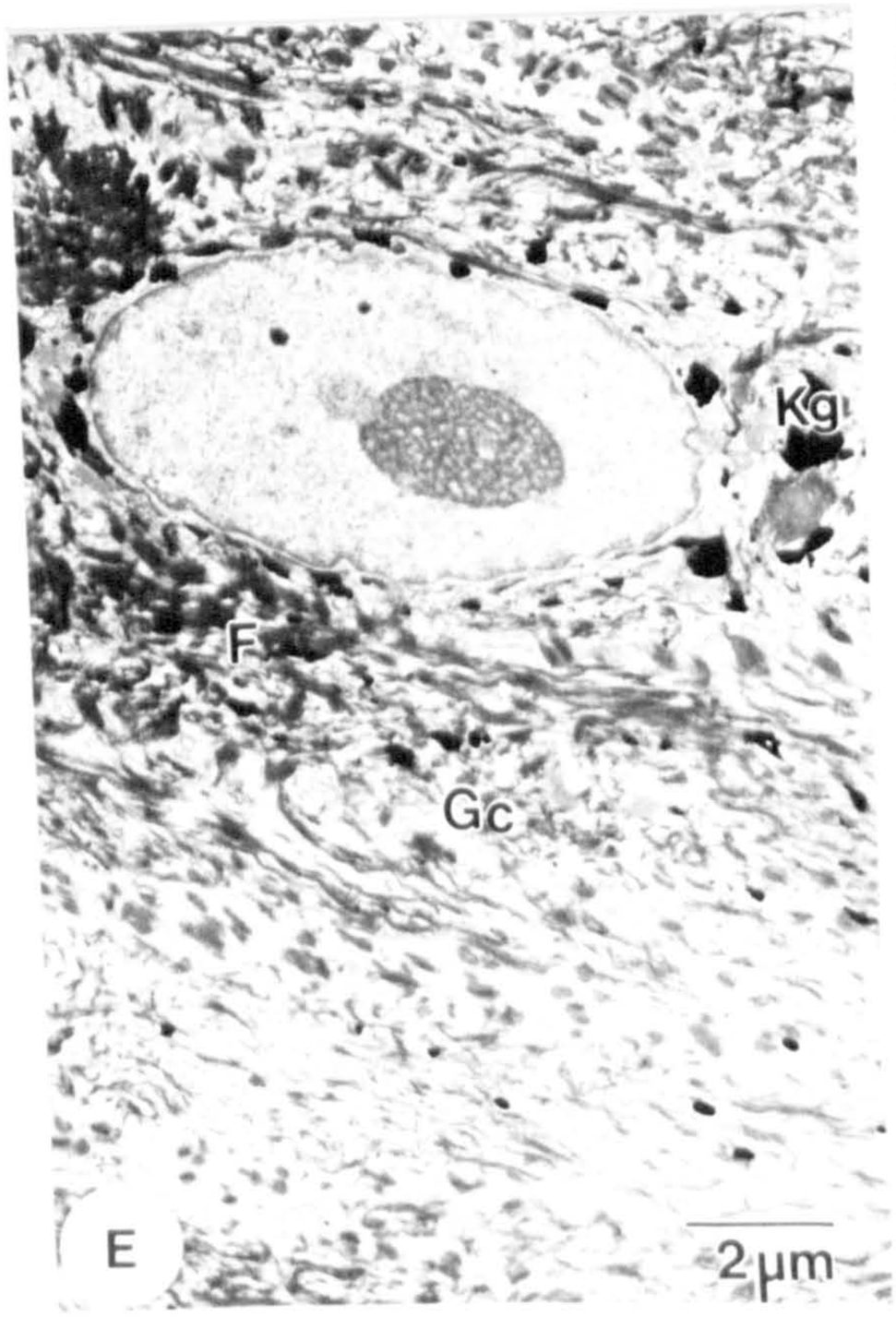
Plate 4f High power of Plate 4e, illustrating intensely staining cytokeratin filaments (F), melanin granules (Me), keratohyalin granules (Kg), and desmosomes (De). x 10,500


Sq
De
F
A
2µm


De
F
B
400 nm


Sq
Gc
Kg
F
C
2µm


Kg
D
1µm


Kg
F
Gc
E
2µm

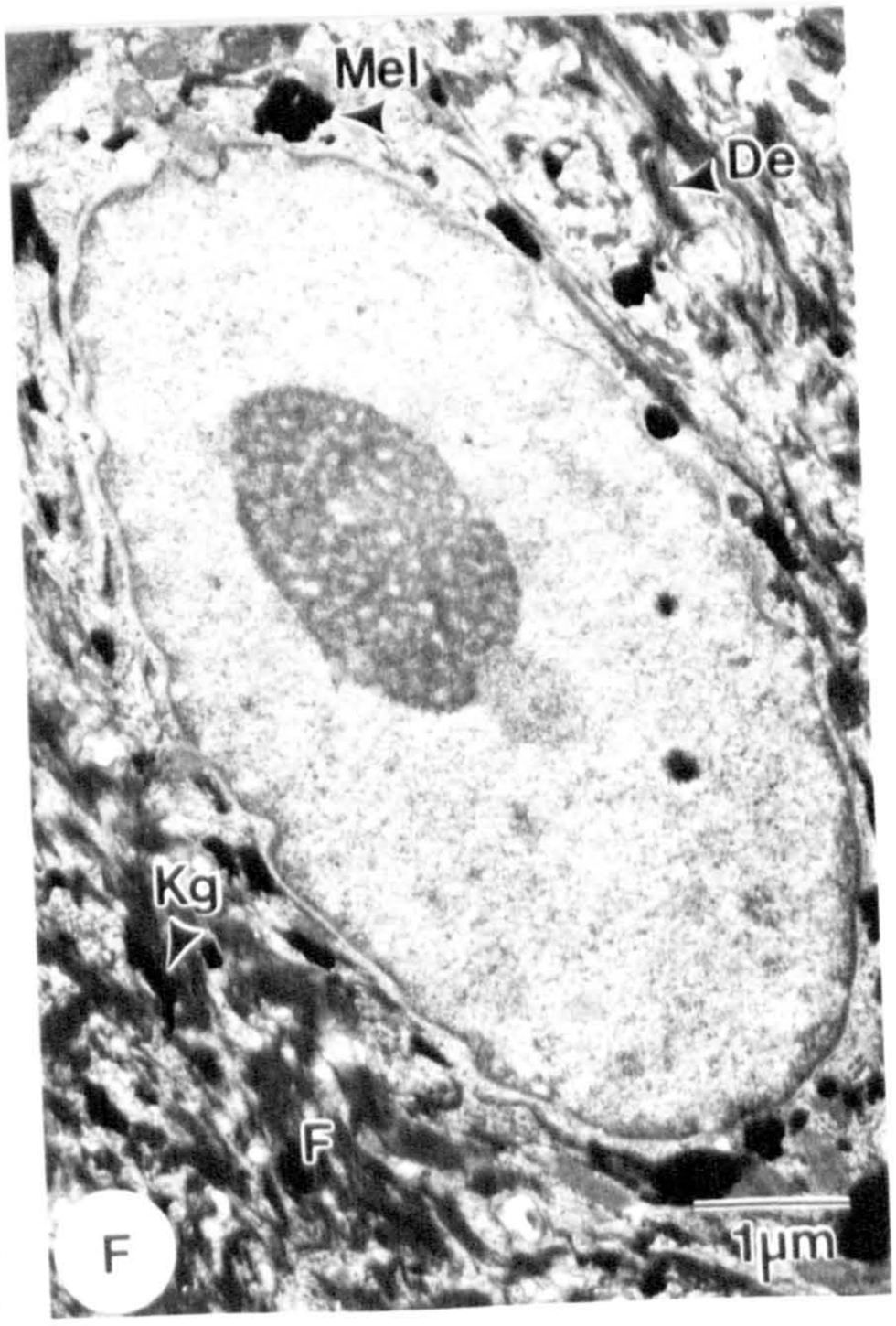
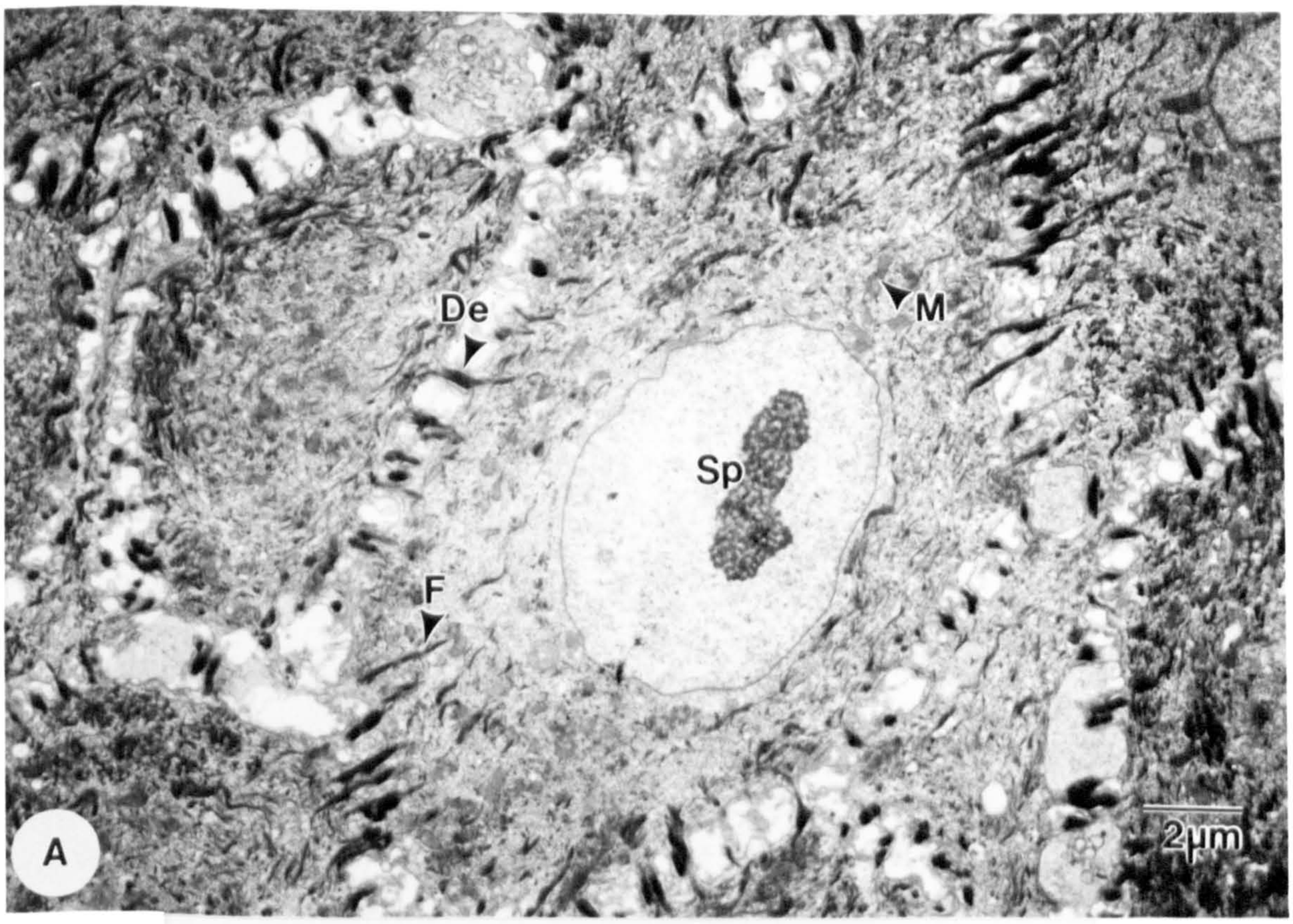

Mel
De
Kg
F
F
1µm

Plate 5a Spiny cells of perineal epidermis (Sp). Note the density of staining of the cytokeratin filaments (F) associated with desmosomes (De), and absence of these filaments in the perinuclear region. Desmosomes are numerous (De), studded at frequent intervals around the periphery of the spiny cells. Folding of the cytoplasmic membrane is negligible, mitochondria are common (M).

x 5,000

Plate 5b Basal cells of perineal epidermis (Bc). Note the presence of: significant numbers of mitochondria (M) in the peri-nuclear region, particularly near the basement membrane (Bm) and cytoplasmic folds (Cf) at the lateral cell surfaces. x 9,000


De
M
Sp
F
A
2µm

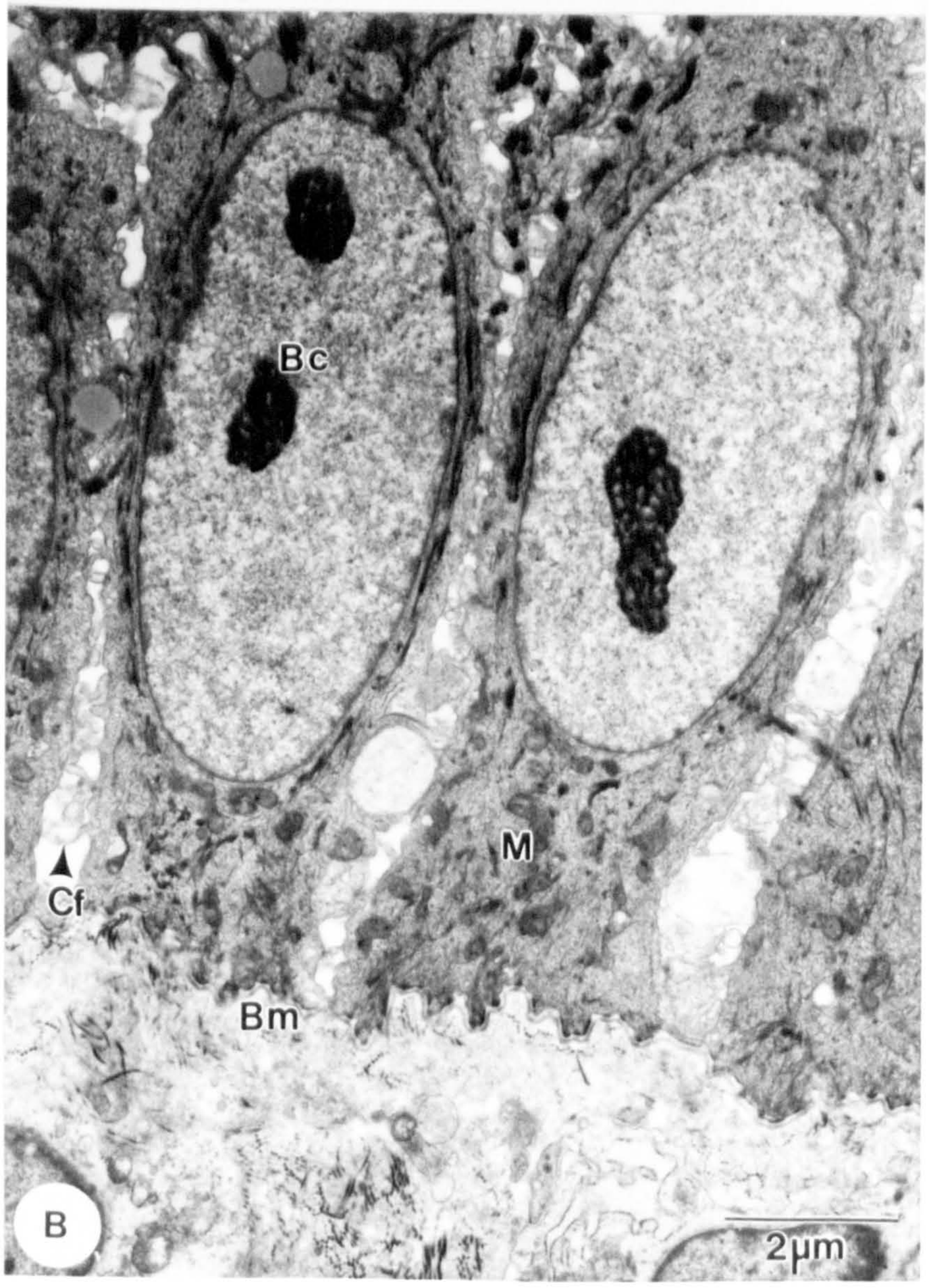

Bc
M
Cf
Bm
B
2µm

Plate 6 Low power electron micrograph of perineal epidermis, showing the differences in staining of cytokeratin filaments in basal (Bc) and spiny cells (Sp). Note also numerous desmosomes (De), and the presence of a leukocyte (Le) which has just penetrated the basement membrane. A mitotic basal cell is also visible (Mi).

x 6,300

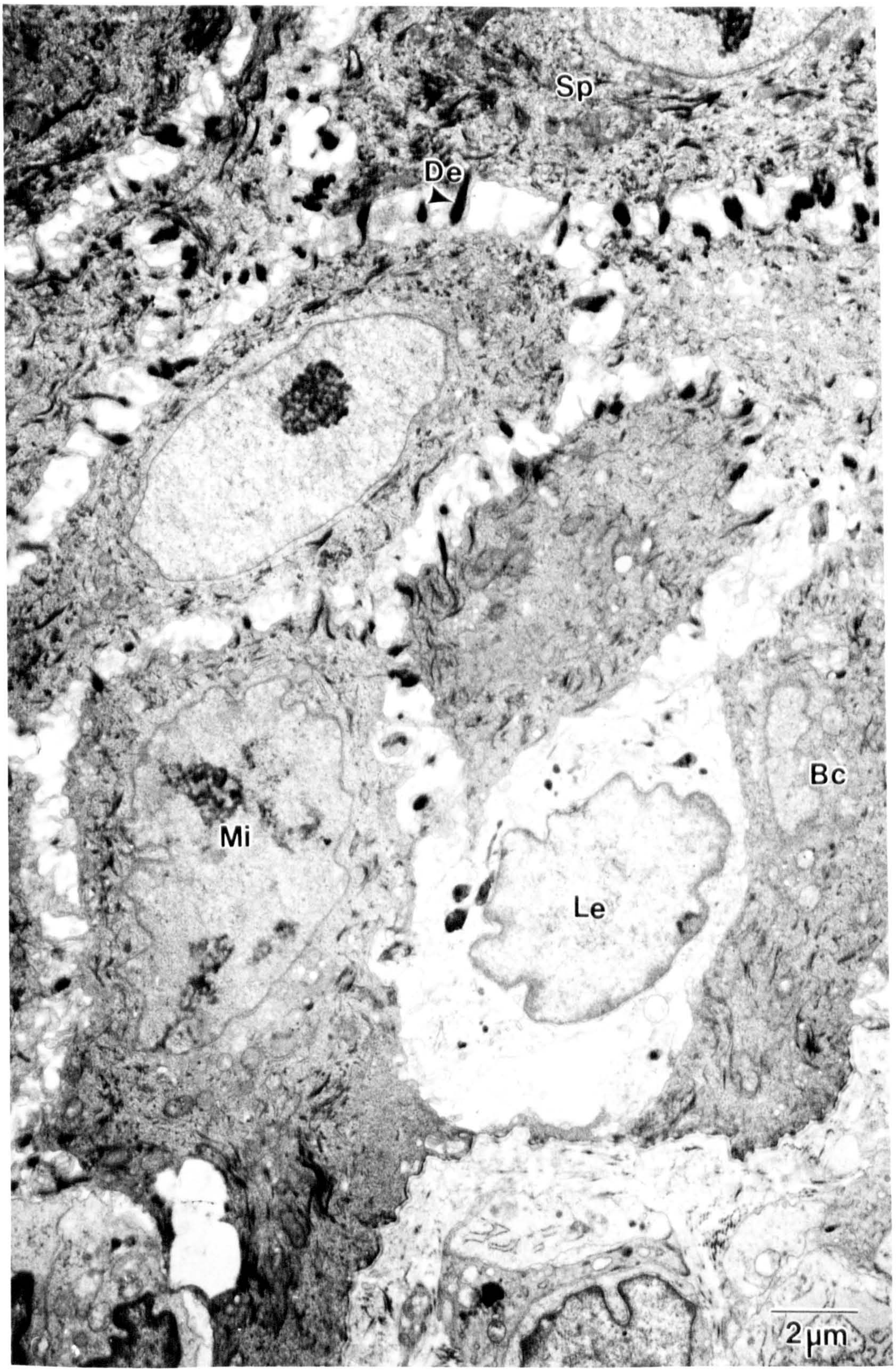
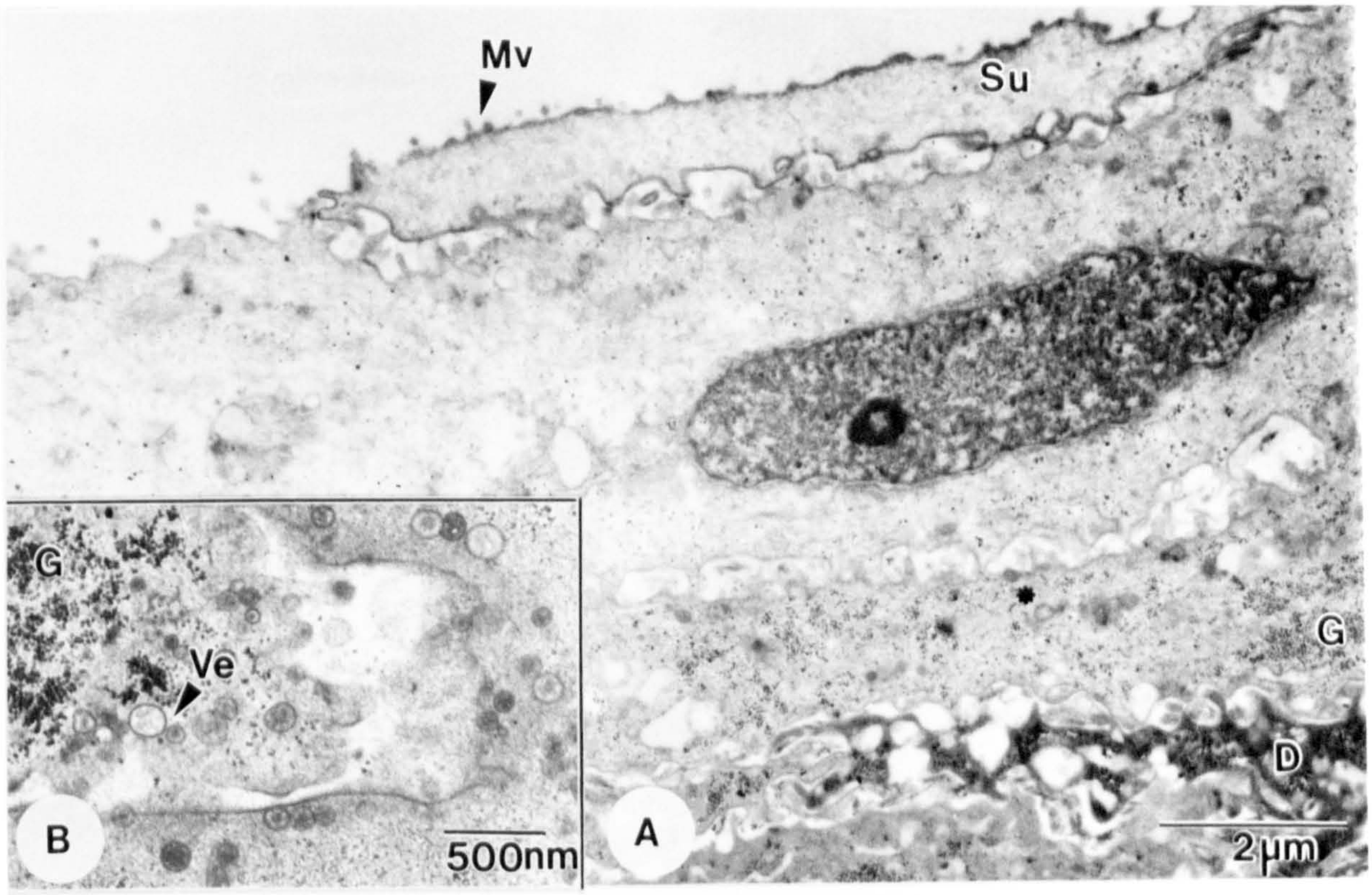

Sp
De
Mi
Le
Bc
2 µm

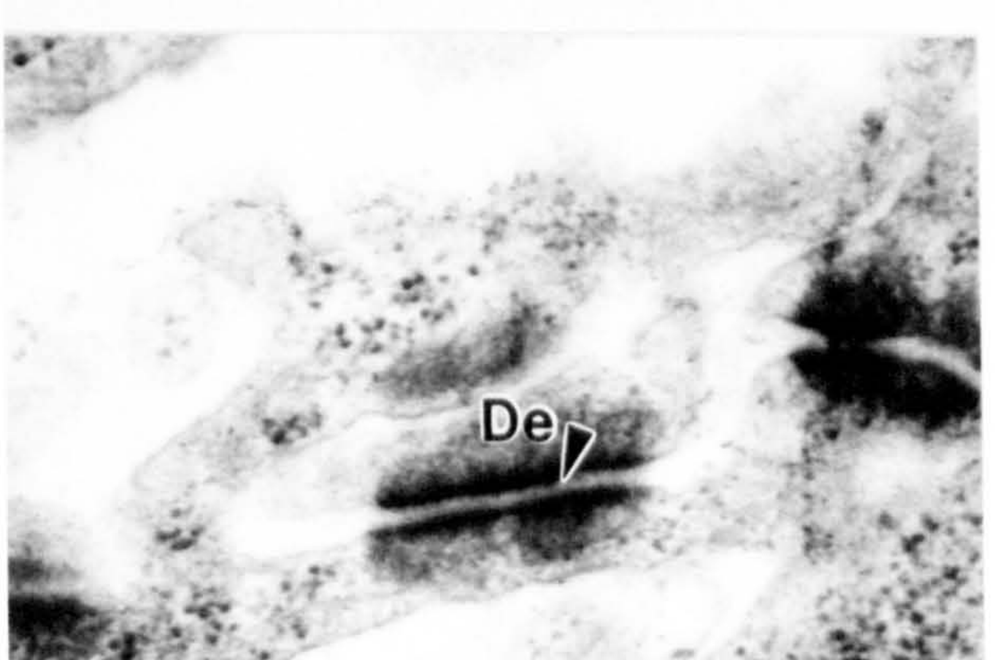
Plate 7a Surface cells of vulvar epithelium (Su). Note the partially thickened appearance of the membranes of one surface cell (Su), the presence of large deposits of glycogen (G), flattened nuclei (N), and a darkened, possibly apoptotic, cell (D). An area similar to * is shown in Plate 7c. x 9,500

Plate 7b High power micrograph of a superficial epithelial cell. Vesicles (Ve) are visible in close contact with the cytoplasmic membranes of the surface cells. Vesicles are commonly electron lucent, occasionally having electron dense cores. Note also the presence of glycogen aggregates (G). x 25,000

Plate 7c Desmosome junctions (De) from a region similar to that in Plate 7a.(asterisk). Note the slightly thickened cytoplasmic membranes, pale staining cytokeratin filaments (F), thickened plaques at desmosomal junctions (De), but particularly the numerous cytoplasmic folds (Cf). x 43,000

Plate 7d A superficial cell of vulvar epithelium, the nucleus of the cell has ben retained (Nu). Note the presence of pale-staining cytokeratin filaments (F), mitochondria (M), small electron lucent vesicles (Ve) and microvillous ridges (Mv). The remains of a desmosomal junction (De) is visible on the surface of this flattened cell. x 37,500


Mv
Su
G
Ve
B
500nm
A
G
D
2µm


De
Cf
F
C
400 nm

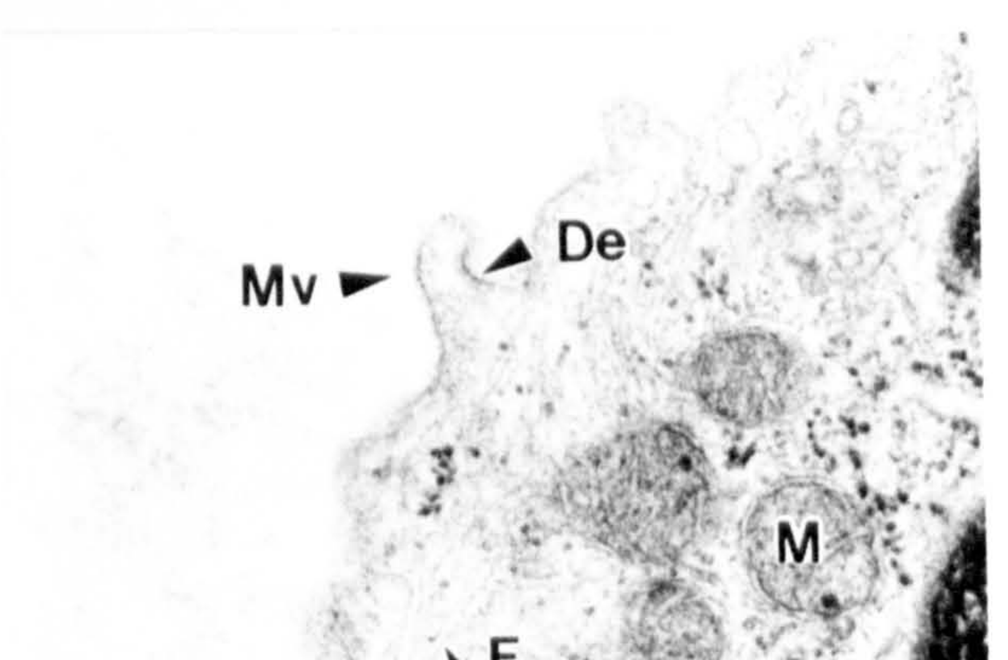
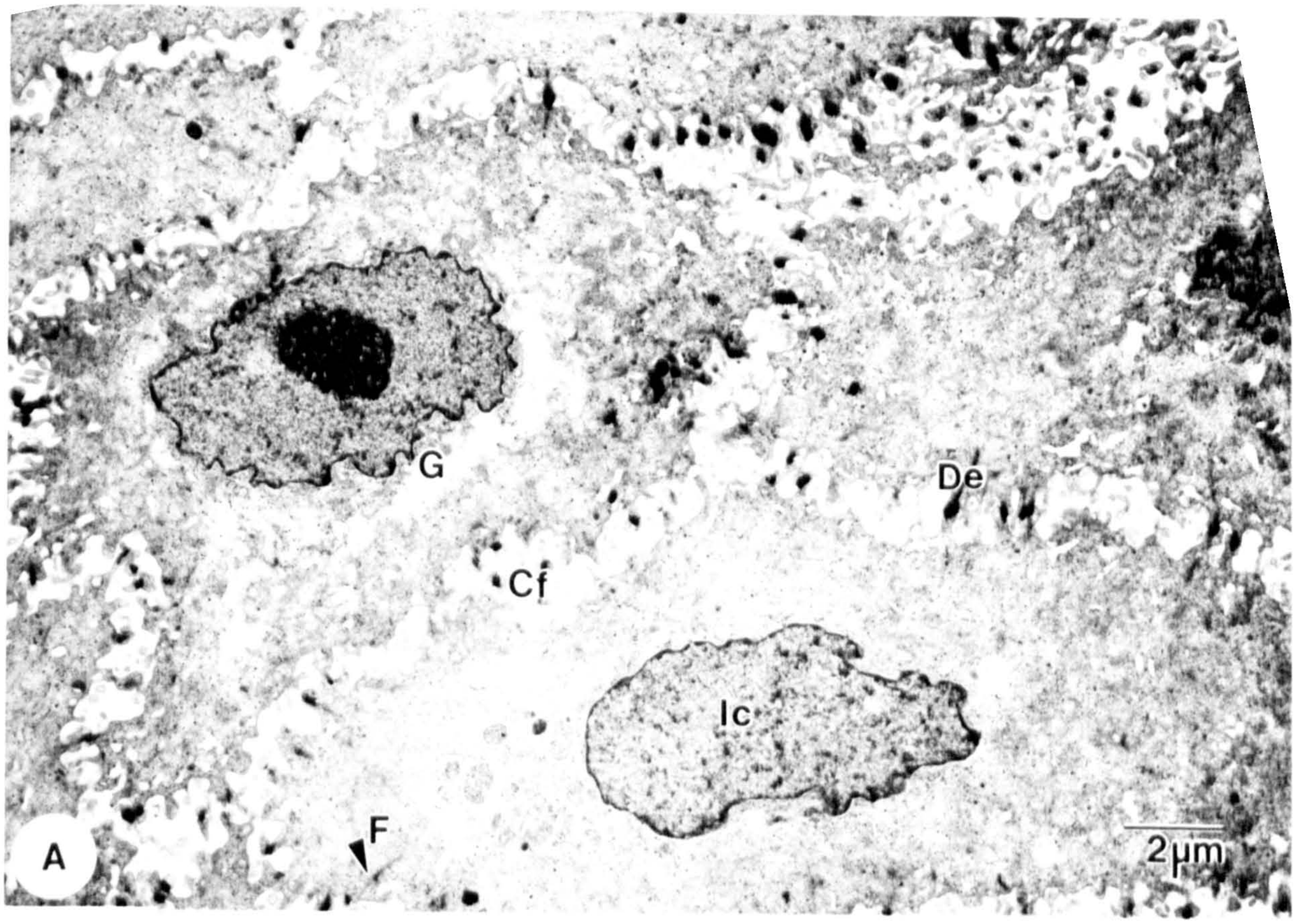

Mv
De
M
F
Ve
D
400 nm
Nu

Plate 8a Upper intermediate cells (Ic) of vulvar epithelium. Note particularly: the presence of large deposits of glycogen (G) in the perinuclear region, forming a pale halo around the nucleus in some cases; the density of cytokeratin filaments (F) at desmosomal junctions (De); and the presence of numerous interdigitating cytoplasmic folds (Cf). x 5,300

Plate 8b Intermediate cells layer (Ic) of vulvar epithelium. Two leukocytes (Le) of unknown origin are visible between cells of the upper intermediate cell layer (Ic). The nucleus of the larger leukocyte is lobed, and the cytoplasm is rich in organelles. x 8,000


G
De
Cf
Ic
F
A
2µm

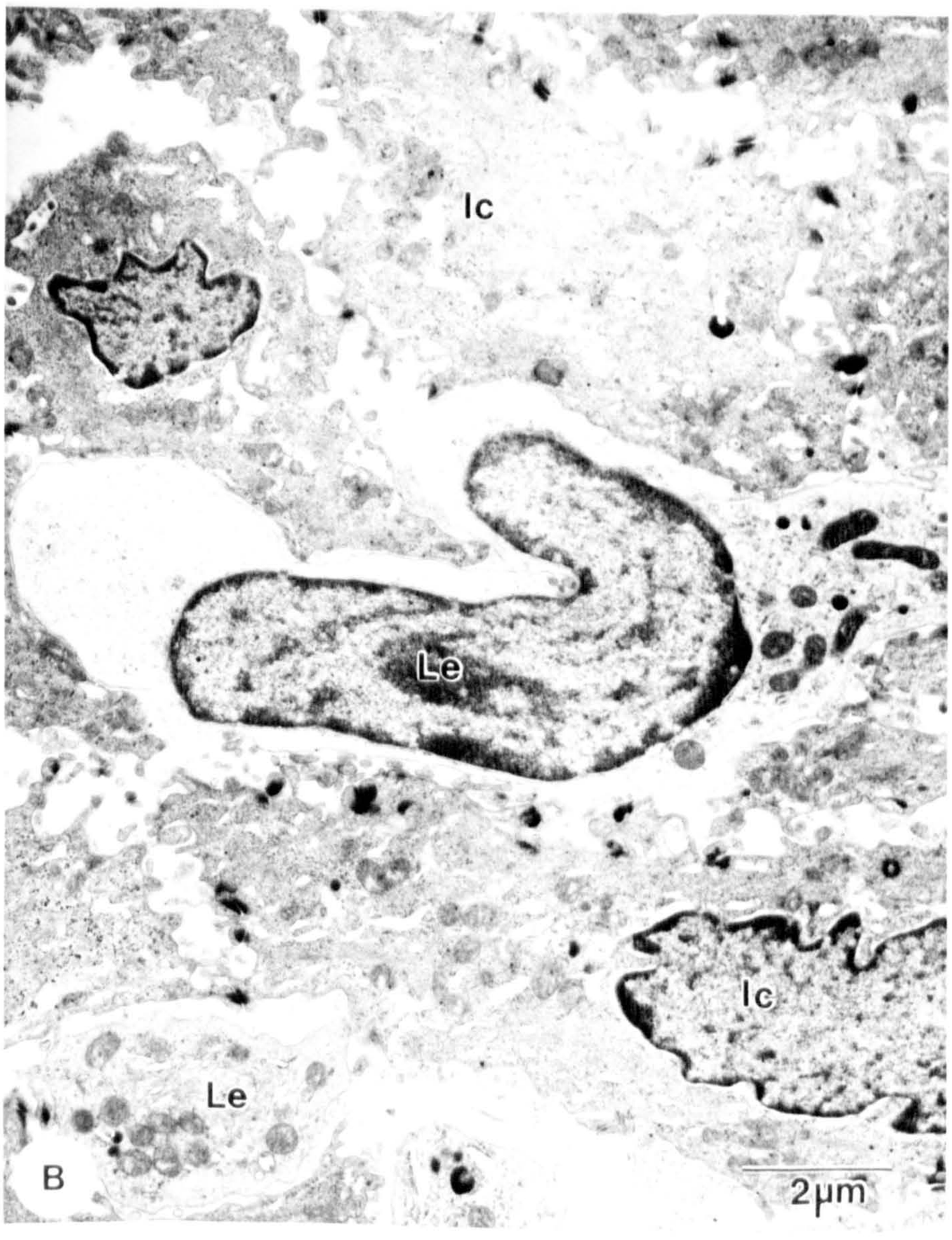

Ic
Le
Ic
Le
B
2µm

Plate 9 Low magnification electron micrograph of the vulva, illustrating the presence of: lymphocytes (L); an dark staining, apoptotic-like cell (D); and intermediate cells (Ic) with many mitochondria (M) and endoplasmic reticulum (Er). Note also, the basement membrane (Bm) in bottom right corner of the micrograph, cytoplasmic folds interdigitating between epidermal cells and infrequent desmosomal junctions (De). x 6,300

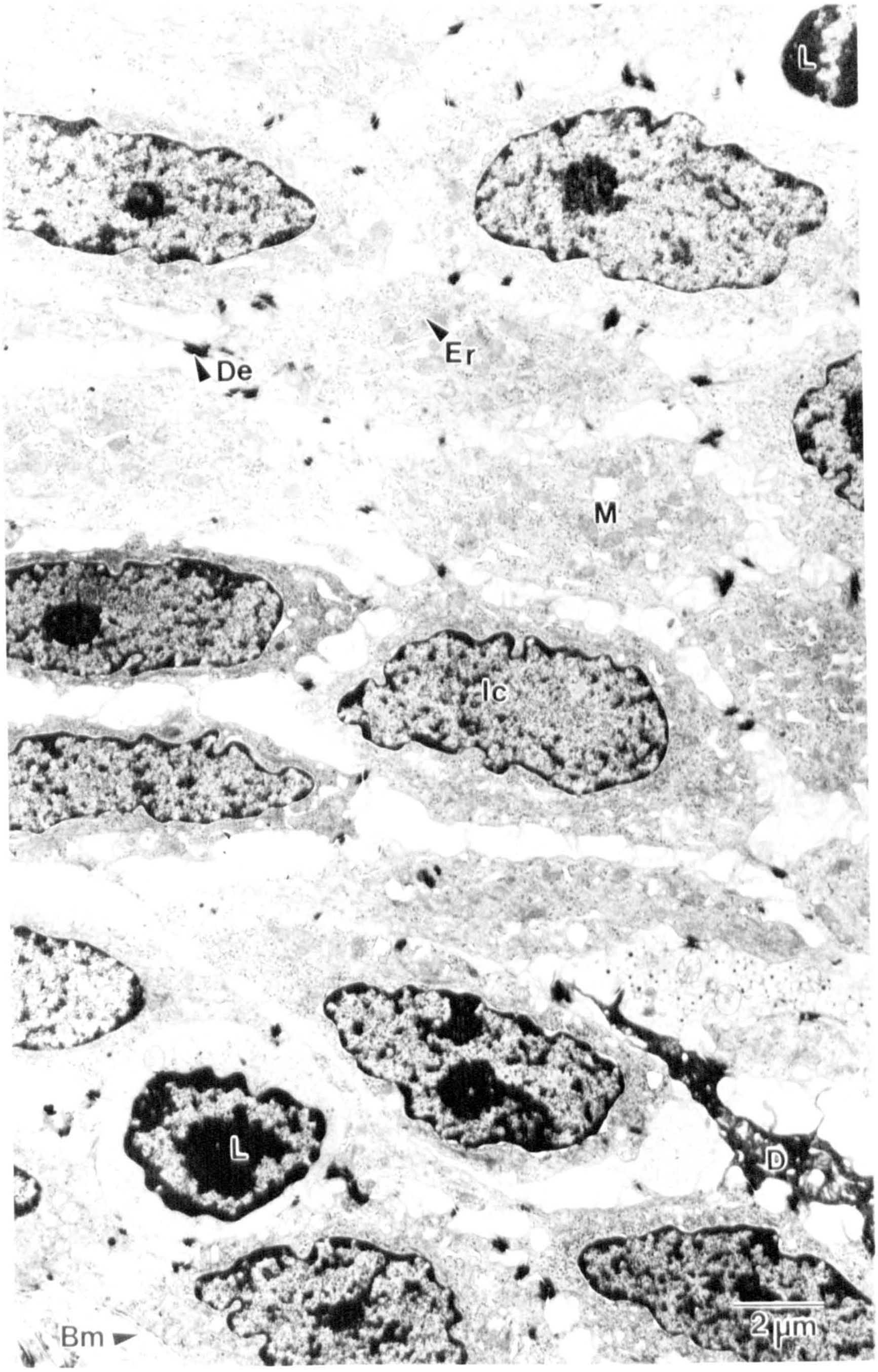
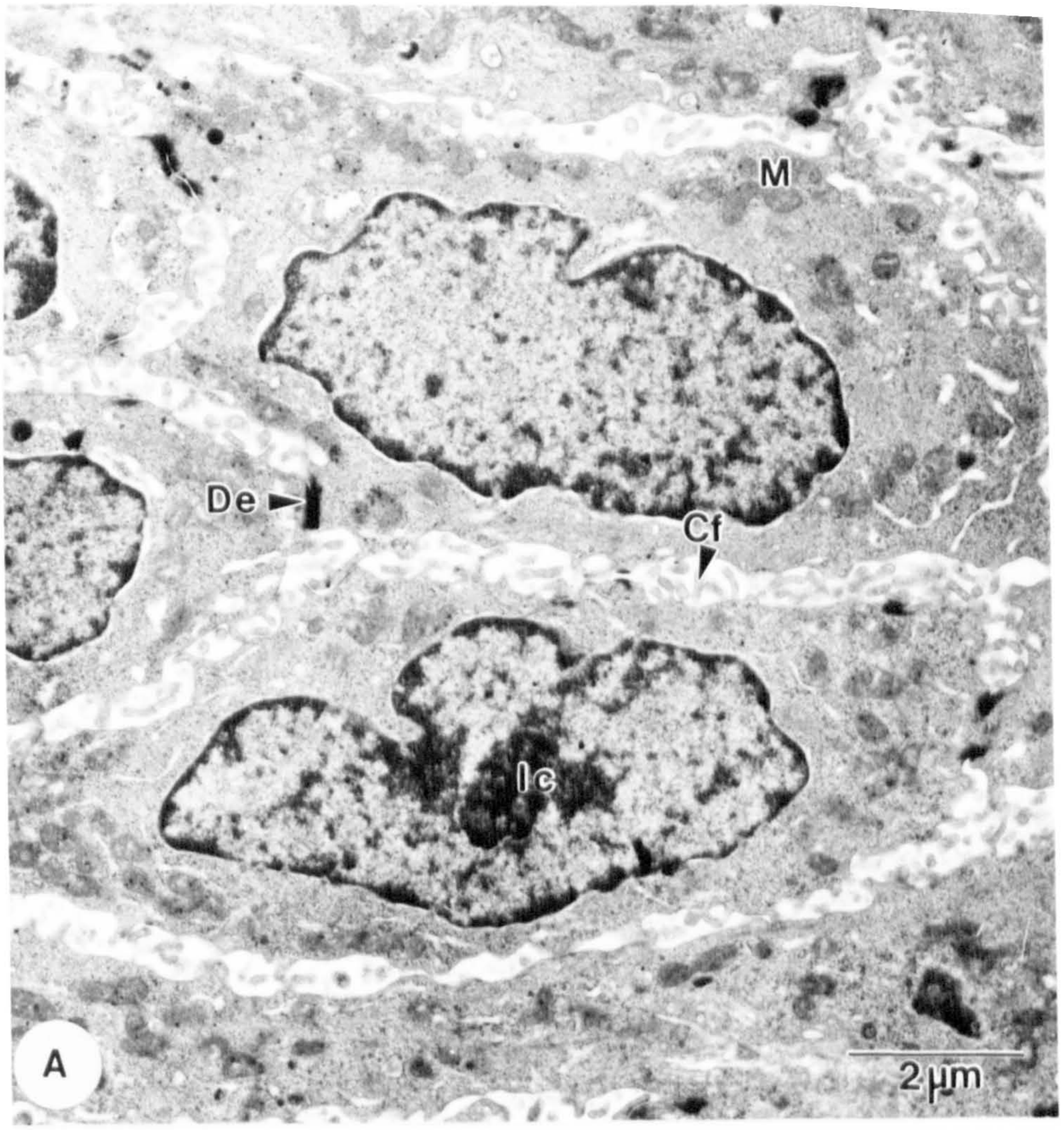
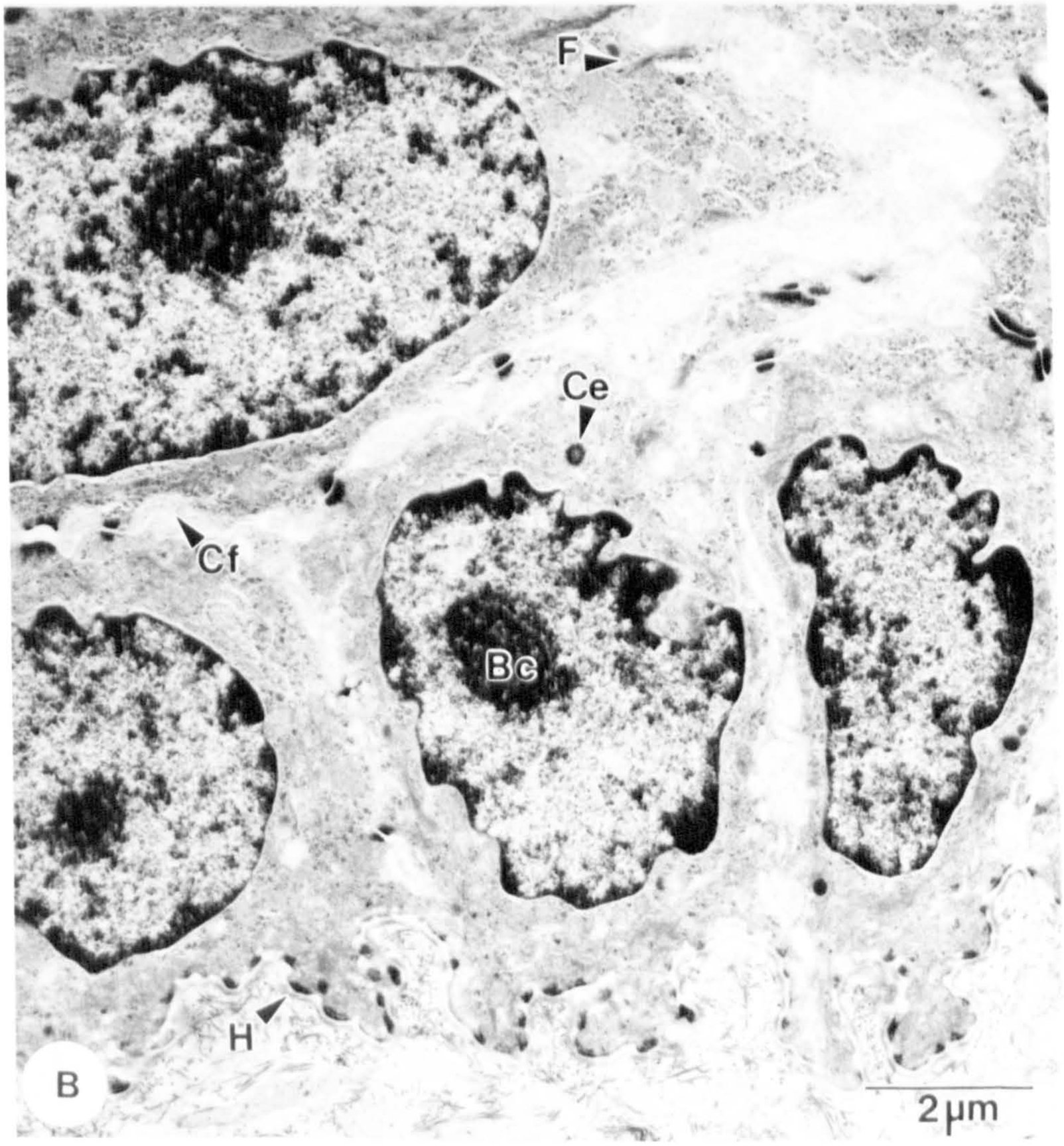

L
Er
De
M
Ic
L
D
Bm
2 µm

Plate 10a Lower intermediate cells (Ic) with abundant folding of the cytoplasmic membrane (Cf) and numerous mitochondria (M). Desmosomes (De) are not common in this cell layer.

x 8, 800

Plate 10b Basal cells (Bc) of vulvar epithelium. Cytokeratin filaments are pale-staining, forming small bundles (F). Desmosomal junctions (De) are sparse, and cytoplasmic folds are numerous between basal cells. Hemi-desmosomes (H) are visible at the basement membrane. A centriole (Ce) can be seen in one of the basal cells. x 8,600


M
De
Cf
Ic
A
2 µm


F
Ce
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Bc
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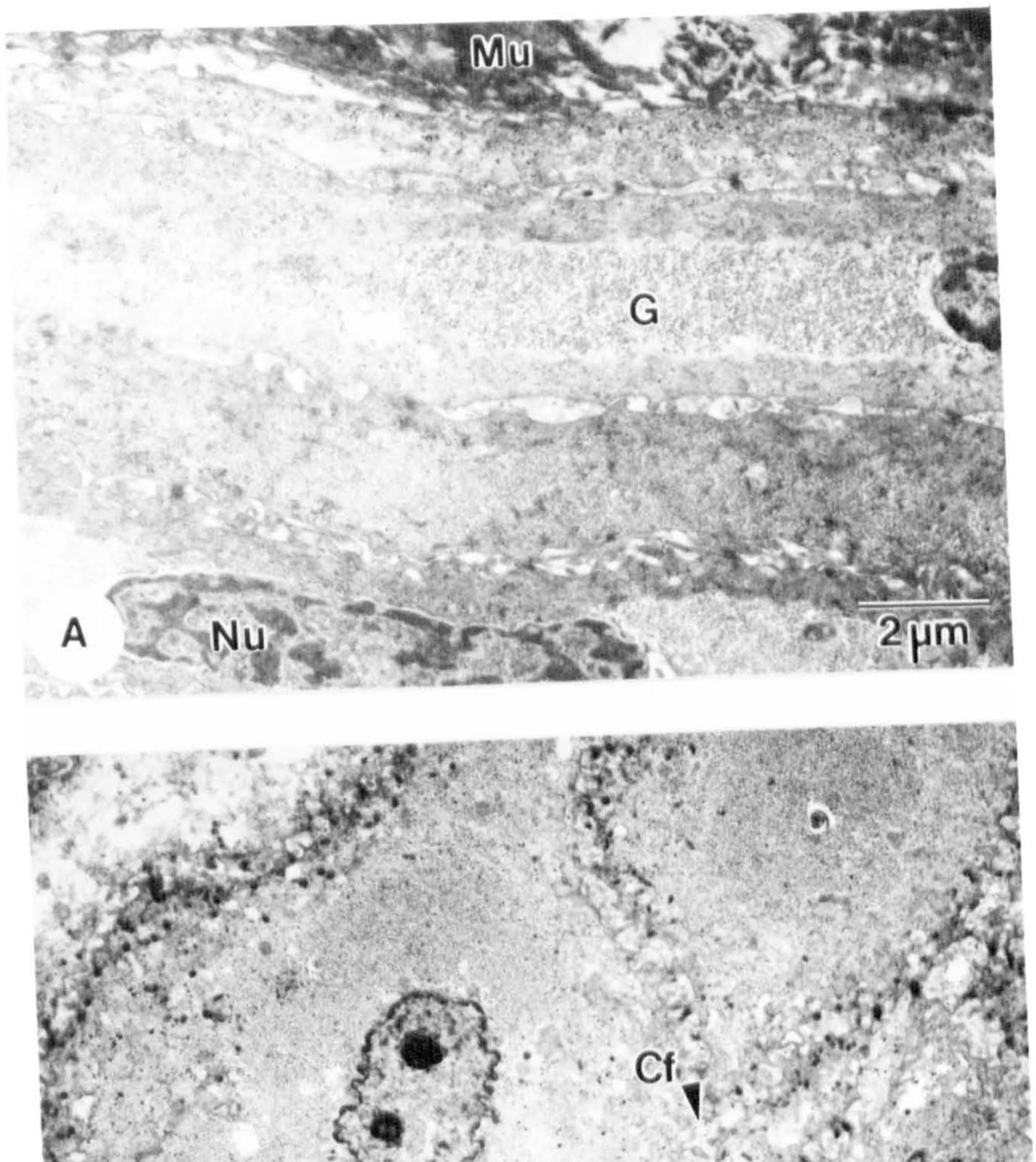
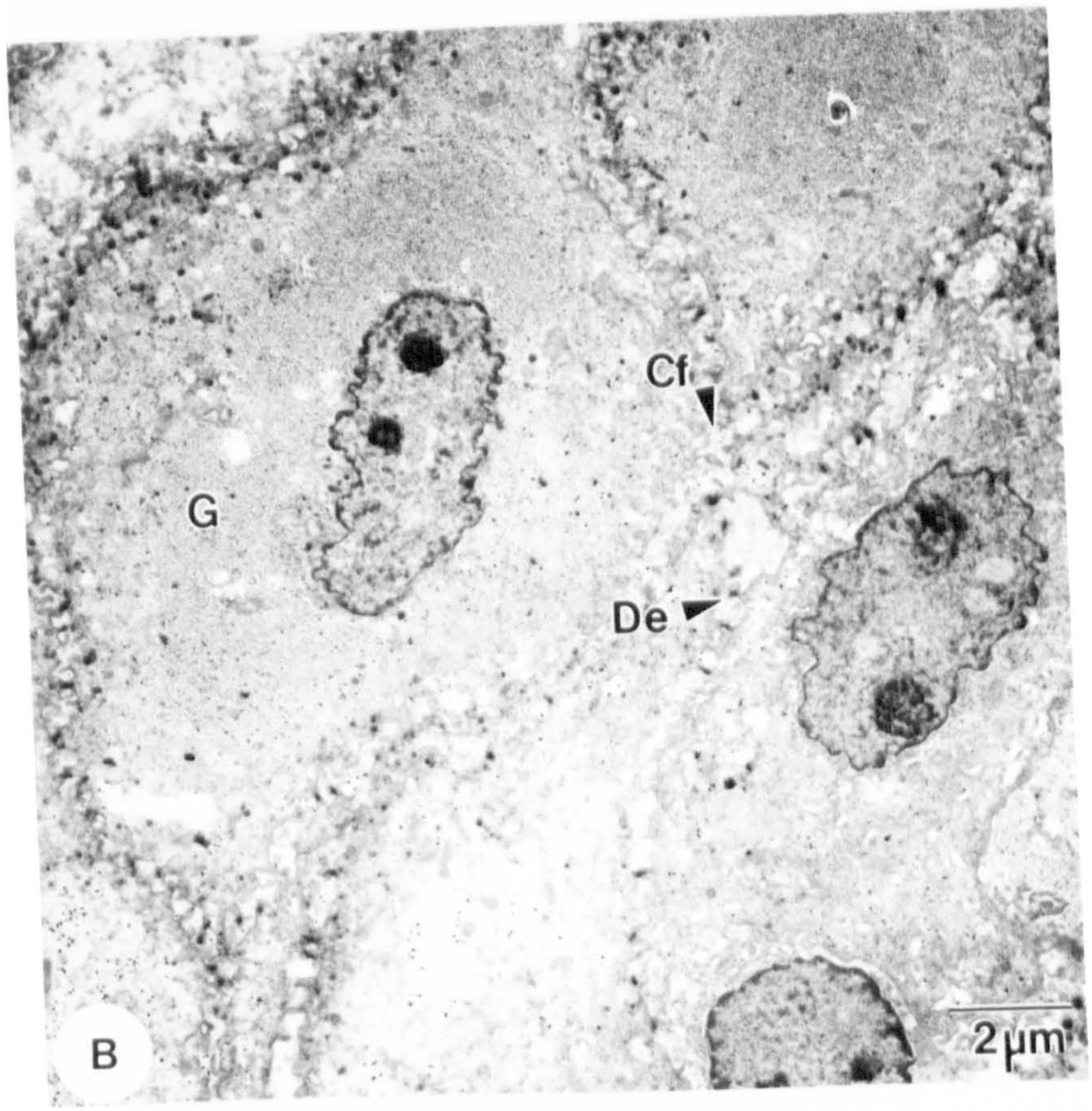
Plate 11a Surface cells of vaginal epithelium. Surface microvilli are masked by the presence of electron dense mucus (Mu). Surface cells have large deposits of glycogen (G) and flattened nuclei (Nu). There are few organelles in this cell layer. x 6,300

Plate 11b Upper intermediate cells of vaginal epithelium. Cells appear swollen with large amounts of glycogen (G) present in a halo around the nucleus. Cytoplasmic folds (Cf) are common, and desmosomes are short (De). x 5,700

Plate 11c A lower intermediate cell, with glycogen deposits (G), close cytoplasmic folds (Cf), and few organelles. Note the leukocyte in the top right of the micrograph (Le). x 9,000


Mu
G
A
Nu
2 µm


G
Cf
De
B
2 µm

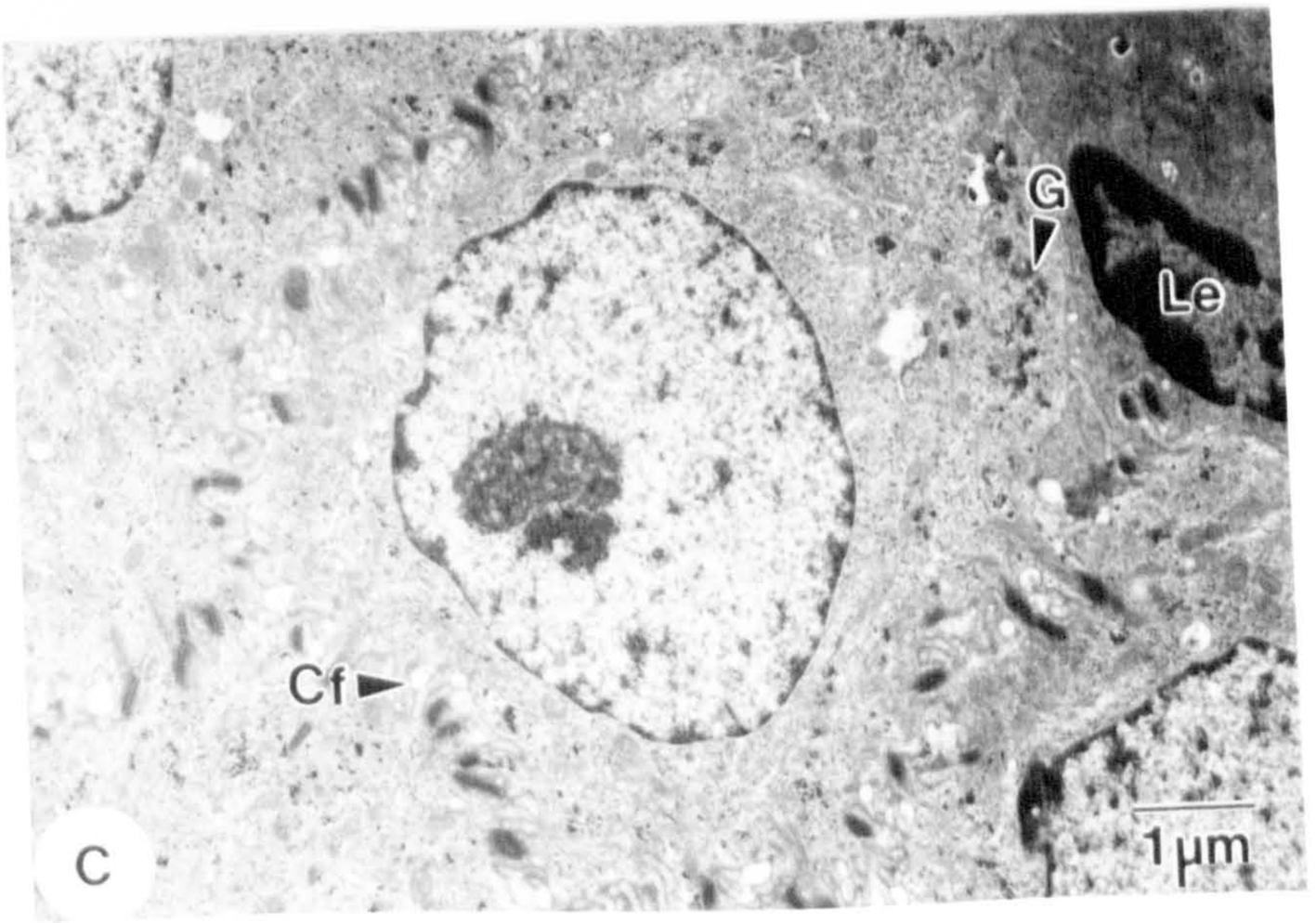

G
Le
Cf
C
1 µm

Plate 12a Vulvar epithelium processed by LTFS. Illustrating an upper intermediate cell (Ic), with large voids where glycogen has been removed (*). The nuclear membrane and cytoplasmic membrane are intact, however, mitochondria appear to be damaged (M). Membrane coating granules are visible (Mcg). x 7,800

Plate 12b Surface cells of vestibular epithelium processed by LTFS. Microvillous processes are visible (Mv), as are electron lucent vesicles (Ve). Mitochondria appear to have lost their internal membrane structure (M). x 26,000

Plate 12c A high power micrograph of the surface cell shown in Plate 12b. Cytokeratin filaments (F) are clearly visible, the cytoplasmic membrane appears slightly thickened. Electron dense, membrane coating granules appear to have an internal lamellae structure (Mcg). Vesicles are electron lucent, with no internal structure (Ve). x 52,000

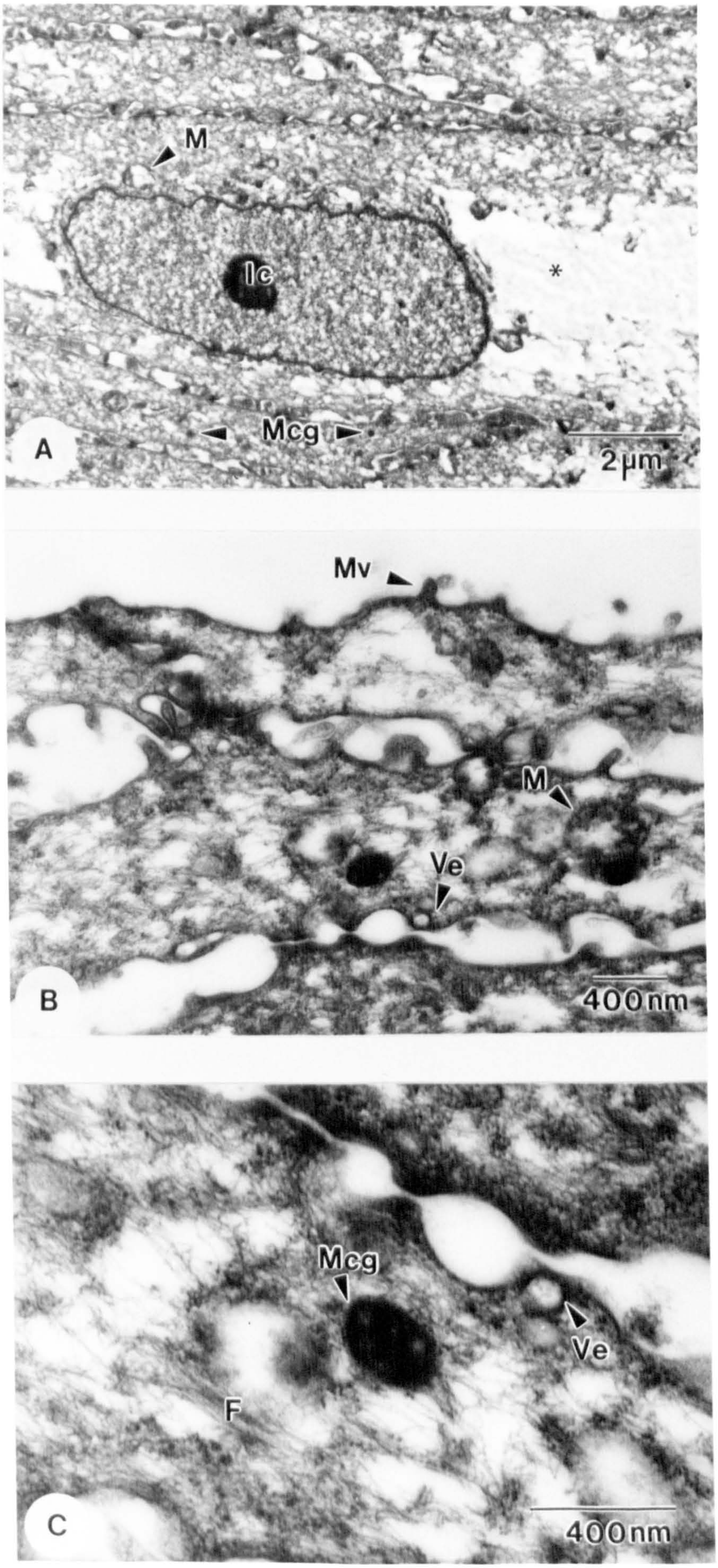
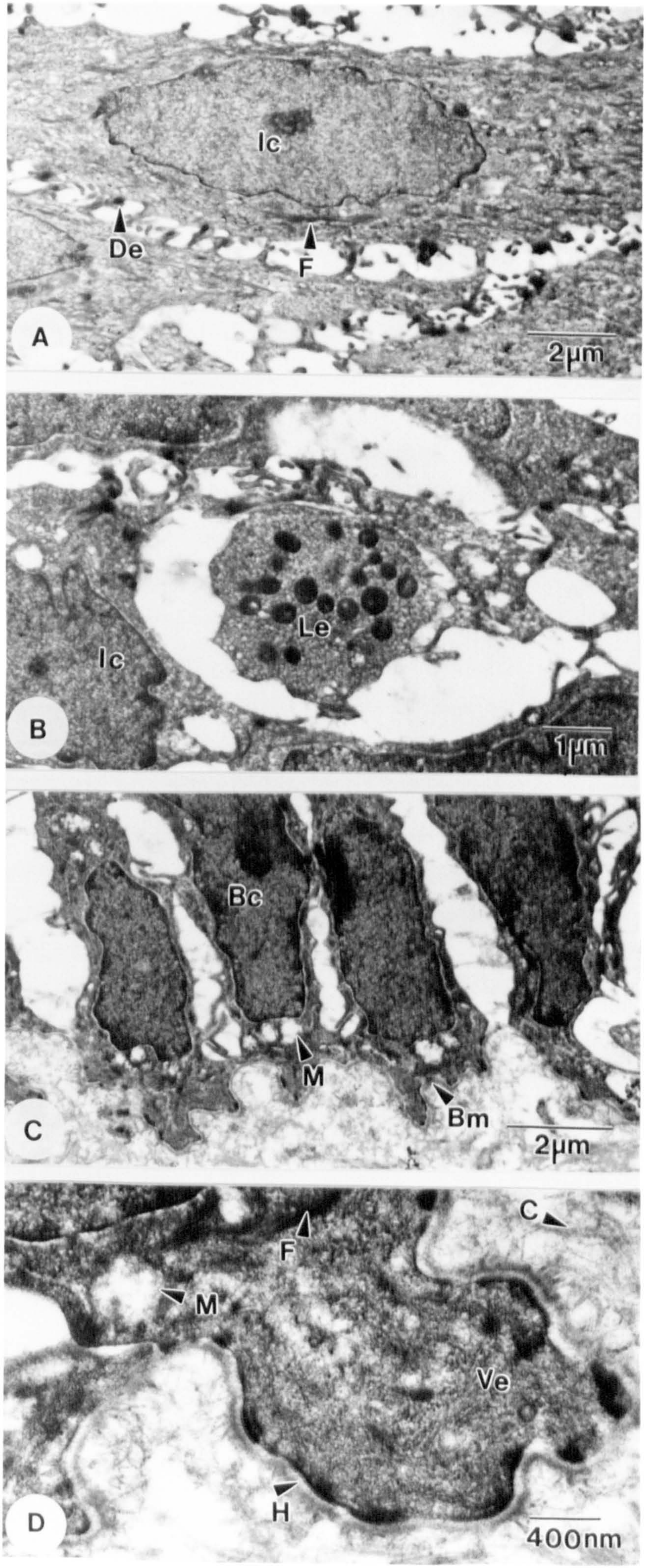

M
lc
*
Mcg
2 µm
A
Mv
M
Ve
400nm
B
Mcg
Ve
F
400nm
C

Plate 13 Lower intermediate cells (Ic) from vestibular epithelium processed using LTFS. Cytokeratin filaments are visible arranged in small bundles (F). Desmosomal junctions are infrequent (De). There are no intact organelles in these cells. x 6,500

Plate 13b Illustrating a leukocyte (Le) interposed between intermediate cells (Ic). Mitochondria are not present in this plane of section, however this leukocyte is characterised by electron dense granules and small vesicles. x 9,750

Plate 13c Basal cells (Bc) appear extremely shrunken with few organelles; mitochondria have no internal structure (M), however the nuclear membrane is still visible. The basement membrane appears intact (Bm). x 7,800

Plate 13d High power micrograph of the dermo-epidermal junction. The basement membrane does not appear damaged, hemi-desmosomes are distinct (He). Cytokeratin filaments (F) and small vesicles (Ve) are evident in these cells. Collagen (C) in the dermis shows characteristic banding. x 26,000


Ic
De
F
A
2µm
Le
Ic
B
1µm
Bc
M
Bm
C
2µm
C
F
M
Ve
H
D
400nm

CHAPTER FIVE

The ultrastructural characteristics of vulval periductal epithelium from patients with Vulvar Vestibulitis Syndrome

5.0 Abstract

The term vulvar vestibulitis syndrome (VVS) was introduced by Friedrich (1987), to describe a constellation of symptoms which result in idiopathic vulvar pain. Using transmission electron microscopy, it was concluded that the normal vestibular epithelium was ultrastructurally similar to mucosal-like surfaces (see Chapter 4). Examination of vulvar, periductal epithelia from VVS patients using TEM, revealed several distinct differences not previously described, and which were not obvious using light microscopy. Intensely staining cell profiles were seen in foci, particularly along the basement membrane. Shrunken in appearance, with vacuolated cytoplasm, intact organelles and condensed cytokeratin filaments, these cells appeared similar to cells undergoing apoptosis. Leukocytes were commonly interposed between epidermal cells, particularly in contact with foci of intensely staining epidermal cells. Membrane bound lobules of cytoplasm, associated with the apoptotic-like cells, were numerous. These cytoplasmic bodies may be processes of Langerhans cells, or byproducts of the process of apoptosis. Vesicles, not commonly seen in control tissue were frequently visible in close contact with the cytoplasmic membrane of superficial cells. Using SEM, the epithelium from VVS patients showed a modification of the normal microridge structure. Microridges appeared shrunken and flattened in appearance, and pore like structures were visible. These structures varied in dimensions, distribution and frequency and were often localised in groups of three or four. These pore-like structures may be the openings of minor vestibular glands, or mucous secreting glands, the characteristics of which have not previously been described using SEM. The visualisation of apoptotic cells may be important in future studies of VVS, as the presence of these cells may affect the onset, or be a result of the syndrome. The presence of an inflammatory cell infiltrate in VVS samples, in conjunction with apoptotic-like cells may suggest a cell signalling defect which is resulting in epidermal cell death.

5.1 Introduction

As outlined in the general discussion, vulvar pain has been documented for over a century, and was first described by Skene in 1889, described a 'hyperaesthesia' of the vulva. At the 1983 meeting of the International Society for The Study of Vulvar Disease the term 'vulvodynia' was introduced, and defined as chronic vulvar discomfort. Thorough diagnosis has indicated that there are several subsets of vulvodynia, one of which is vulvar vestibulitis syndrome. The term Vulvar Vestibulitis Syndrome (VVS) was introduced by Friedrich in 1987, to expand the terminology associated with vulvar disease, and to replace inaccurate and ambiguous terms previously used to describe this constellation of symptoms.

According to Friedrich (1983), and Woodruff & Friedrich (1985), the vestibule is that area of the vulva which extends laterally from the hymenal ring to a line of more keratinised skin on the labia minora , termed Harts' line (Hart, 1893). Anteriorly the vestibule reaches upwards to the frenulum of the clitoris and posteriorly downwards to the fourchette. The vestibule is endodermal in origin, as is the urethra and the trigone of the bladder; the remainder of the external female genitalia are ectodermal (Woodruff & Friedrich, 1985). The epithelial surface of the vestibule has not been studied in detail, but has often been described as a mucosal-like surface with similarities to both skin and true mucosal surfaces. It can be surmised that the epithelium of the vestibule has all of the sensitivity of a mucosal surface with little of the resistance offered by keratinised skin. There are no mucous secreting glands in the vulva as in other mucosal surfaces, however, the surface of the vestibule is bathed in mucus from the cervix, and the vestibular glands are reported to provide some lubrication of the vestibular surface (see Chapter 2). The glands present in the vulva, which open onto the surface of the vestibule, appear to be the sites of inflammation in this syndrome. There are two main types of glands in the vestibule; the major vestibular glands (Bartholins' and Skenes' glands); and the minor vestibular glands, which may be found all over the vestibular surface and which vary

considerably in number (Robboy *et al.*, 1978).

It has been proposed by many workers, that the aetiology of Vulvar Vestibulitis Syndrome is multifactorial, however, an association with a particular aetiological agent is yet to be conclusively proven. Human papillomavirus (HPV) and recurrent *Candida* infections have been shown to be important in some cases, as have therapeutic and destructive chemical agents, allergic reactions to cosmetic products, and psychosexual factors (Koblenzer, 1983; Lynch, 1986; Mann *et al.,* 1992; Schover *et al.,* 1992; Abramov *et al.,* 1994; Kehoe & Luesley, 1995; Marks *et al.*, 1995). Other authors have suggested that VVS is a 'urogenital sinus-derived epithelium disorder' (Fitzpatrick *et al.,* 1993), or that is has developed as a consequence of interstitial cystitis (Foster *et al.,* 1993). A considerable amount of research has concentrated on the incidence of HPV associated VVS. The association of HPV with VVS has been supported by both histological and virological data (Horowitz , 1989; Pyka *et al.,* 1988; Turner & Marinoff, 1988; Kent & Wisniewski, 1990; Bergeron *et al.*, 1994). However, the diagnostic criteria used for HPV detection in these studies have been proved unreliable. A majority of studies, particularly those completed prior to 1990, relied on a histological diagnosis for the detection of HPV in tissue biopsies. Typically the biopsy area is selected by the application of acetic acid. Areas of thickened epithelia become whitened on application of dilute acetic acid, indicative of HPV infection. However, aceto-whitening is not indicative of just HPV infection, but any condition which causes thickening of the epidermis. Histological examination of vestibular biopsies for the detection of HPV is also fraught with difficulties. Koilocytic cells, characterised as having pyknotic and displaced nuclei are notoriously difficult to distinguish from normal epithelial cells (Bergeron *et al.,* 1994). This is supported by the findings of several VVS studies which have detected koilocytic cells in 16-71% of VVS samples (Pyka *et al.,* 1988; Horowitz, 1989; Umpierre *et al.,* 1991; Wilkinson *et al.,* 1993). Cells infected with HPV have a similar appearance to normal superficial cells in the vulva. Normal cells commonly have

displaced nuclei as a consequence of large glycogen deposits situated in a halo around the nucleus (see Chapter 4.). Frequent reports of koilocytosis associated with VVS have led to more detailed investigations using PCR, Southern blotting and *in situ* hybridization to determine the true incidence of HPV in cases of vestibulitis. Two recent studies using PCR gave contradictory results, the study by Umpierre *et al.* (1991) indicating an 85% incidence of HPV in VVS cases, where as Wilkinson *et al.* (1993) found the incidence in their investigation to be only 10% (see Chapter 2).

The pathology of VVS is non-conclusive. There is almost always a mild to moderate inflammatory cell infiltrate present, particularly beneath the basement membrane (Pyka, *et al.*, 1988; Michelwitz *et al.*, 1989 Furlonge *et al.*, 1991; Marinoff & Turner, 1991; Bergeron *et al.*, 1994; Wilkinson *et al.*, 1993 and Prayson *et al.*, 1995). However, on characterisation of the cells present, the pattern of distribution is not indicative of a particular type of immunological reaction (Pyka *et al.*, 1988). Although a reasonable amount of research has been completed using light microscopy, investigations of this type have failed to make any progress in the understanding of the pathology of VVS. The most comprehensive study was completed by Pyka *et al.* (1988); however, since this significant paper very little work has added to the literature on the pathology of VVS (see Chapter 2). The lack of knowledge of the inflammatory process and etiological agents, has resulted in a 'hit or miss' approach to treatment. In early papers on VVS researchers investigated the effects of many different topical and systemic drugs for the treatment of VVS with very limited success (Friedrich, 1987). The use of interferon, and the development of surgery to remove the affected area of the vestibule, has proved more successful than the use of topical treatments (Woodruff & Parmley, 1983; Peckham *et al.*, 1986; Hatch, 1988; Horowitz, 1989; Umpierre *et al.*, 1991; Waltzman & Wade, 1991; Mann *et al.*, 1992; Bornstein *et al.*, 1993; Larsen *et al.*, 1993; Marinoff & Turner, 1993). Unfortunately, both of these pathways are expensive, require specialised techniques, and are only completely

successful on a carefully selected subgroup of patients. Both interferon injections and surgical removal of the vestibule are particularly invasive treatments for VVS, and do not have a 100% success rate. Non-invasive treatment of VVS is essential; however in order to establish an appropriate treatment, the pathology of the condition must be understood.

Initially, it was hoped that suitable patient numbers would allow the use of low temperature processing for transmission electron microscopy. This technique would maintain the antigenicity of the tissues, enabling immunocytochemical detection of antigens possibly linked to VVS. However, during the early stages of the investigation it became apparent that sample numbers would not enable this type of study. Obtaining biopsies from other hospitals was pursued, however, it was not always possible to gain ethical committee approval in time to complete the investigation. The aim of this investigation was primarily to describe the surface ultrastructure of the normal vestibular epithelium in comparison with related epithelia using TEM and SEM (see Chapter 4). Secondly, with some understanding of the ultrastructure of this unique epithelial surface, an analysis of possible differences between control and VVS affected vestibular tissue would be completed. Bacteria and, or fungal spores on the surface of the epithelia would also be investigated. It was anticipated that some advance would be made in the understanding of the inflammatory response occurring in this syndrome, with the long term aim of encouraging non-invasive treatment regimes.

5.2 Materials and Methods

5.21 Light microscopy

Samples of vestibular epithelium were obtained from Birminghams' Dudley Road Hospital. Samples were removed from patients with VVS as routine during vestibulectomy. This operation, carried out under general anaesthetic, involves removing the part of the vestibule affected by VVS. Samples were fixed in 10% formal saline, dehydrated in a graded series of ethanol and embedded in paraffin wax. Section of 4μm, were cut and collected on glass slides. Sections were stained using Mallorys trichrome.

5.22 Scanning electron microscopy

Blocks retained for SEM were fixed in the primary fixative for 2 hours, subsequently washed in sodium cacodylate buffer (0.1M, pH 7.2), and post fixed in 1% osmium tetroxide for 1 hour. After washing in buffer, tissue blocks were dehydrated in a series of graded ethanol, (30%, 50%, 70%, 90%, absolute ethanol), and propylene oxide, for 10 minutes in each mixture, and subsequently critical point dried for SEM. The intermediate fluid was amyl acetate and the transitional fluid liquid carbon dioxide. Dry samples were mounted on brass stubs and gold coated in a Polaron E1500 sputter coating unit and examined in a Jeol JSM 5300 scanning electron microscope operated at 15kV.

5.23 Transmission electron microscopy

Patients attending the GU Medicine clinics at Freedom Fields Hospital (Plymouth Health Trust) and Addenbrookes Hospital (Cambridge) were assessed for the presence of VVS using the criteria established by Friedrich (1987) (see Chapter 2). Patients diagnosed as having VVS, were asked to participate in the study. Patients were informed that they could withdraw from the study at any time, and that their treatment would not be affected in any way. Ethical committee approval was obtained for the study from Plymouth Regional Health Authority

Ethical Committee, in February 1994 (see Appendix 1). Samples from Addenbrookes GUM were part of biopsies taken for routine histological investigation. Informed consent was obtained from each patient in writing prior to a biopsy being taken. Twenty patients in total were diagnosed as having VVS, of which all consented to participate in the investigation.

Biopsies of vestibular epithelium were taken under local anaesthetic. The right Skenes' gland was examined colposcopically and the periductal tissue infiltrated with Citanest (4% Prilocaine HCl). A 3mm Keyes punch biopsy sample was removed from the lateral border of the right Skenes' gland (Fig. 4.0, Chapter 4) and transferred immediately to a vial containing the primary fixative, 4% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.2) at 4°C. Bleeding was controlled with silver nitrate, and the lesion was then treated with an antibiotic powder. After 30 minutes the biopsies were subdivided into tissue blocks of 1mm x 1mm whilst immersed in primary fixative. The tissue blocks were returned to fresh primary fixative for a further 90 minutes. Blocks were subsequently washed twice in 0.1M sodium cacodylate buffer (pH 7.2), 10 minutes each wash, and post fixed in 1% osmium tetroxide for 60 minutes (4°C). At this point several tissue blocks were retained in buffer for scanning electron microscopy. Tissue blocks for TEM were stained *en bloc* with 2% uranyl acetate in the dark, for 30 minutes, washed twice with distilled water, and subsequently dehydrated in a series of graded ethanol, (30%, 50%, 70%, 90%, absolute ethanol), and propylene oxide for 10 minutes in each mixture. Infiltration of resin was carried out over several days. A series of graded mixtures of propylene oxide and Spurr resin (Spurr, 1969) was used (3:1, 1:1, 1:3, Pure x 2). Blocks were polymerised at 70°C for 8 hours. Ultrathin sections (0.1μm), cut on a Reichert ultracut ultramicrotome, were collected on copper grids, stained for 10 minutes and 20 minutes with uranyl acetate and lead citrate respectively, and subsequently viewed using a Jeol 1200 transmission electron microscope. Semi-thin sections, of 0.5μm, were cut and stained with 1% methylene blue.

5.3 Results

5.31 General structure of vestibular epithelium from VVS patients

Using light microscopy, the general appearance of vestibular epithelium, from patients with VVS was found to be comparable to the control tissue. However, in periductal epithelia from VVS patients, a mild to moderate inflammatory cell infiltrate was seen in the dermis, some cells penetrating the upper layers of the epithelia. The predominant intrinsic cell type was the lymphocyte, however, there were numerous plasma cells visible beneath the basement membrane. Intensely stained foci of cells, just visible using light microscopy, were seen distributed throughout the epithelia. Clustered particularly along the basement membrane, these cells stained moderately with methylene blue, having a similar staining appearance to leukocytes (Plate 14a). Staining with Mallorys did not demonstrate these dark staining cells, however, using this stain, the epithelium of the vestibule was shown to vary considerable in terms of keratinisation. Within vestibular samples from VVS patients obtained from vestibulectomy operations, areas of keratinised and partially keratinised epithelia were found, as well as non-keratinised vestibular epithelium (Plate 15 & 16). Wax sections of vestibular epithelium also demonstrated structures which were similar in appearance to goblet cells (Plate 16). These structures were not seen in any of the vestibular samples processed for TEM.

5.32 Scanning electron microscopy of vestibular epithelia from VVS patients

At low magnification cells appeared polygonal in shape, 10-30μm in diameter. Microridges appeared shrunken in VVS samples. However, individual cells and the epithelium as a whole did not appear to be shrunken. Microridges were thin, and did not appear to be interlacing. Often straight ridges were evident (Plate 17). No bacteria or fungi were evident on this epithelial surface. Pores appeared to be common in samples from VVS patients (Plate 17). Frequently seen in small groups between the surface epidermal cells, pores were of varying size and shape and were often masked by the surface topography.

5.33 TEM of vestibular epithelia from VVS patients

Schematic representations of the differences between control and VVS affected vestibular tissue are shown in Fig. 4.6 (Chapter 4) & Fig.5.1. Examination using TEM revealed several distinct differences between control and VVS affected periductal epithelia. The intensely staining cells just visible using light microscopy, were found to correspond with epidermal cells which appeared electron dense when examined using electron microscopy (Plates 19, 20a & b, 23a & b). These cells were characterised by having shrunken vacuolated cytoplasm, and cytokeratin filaments condensed into bundles; organelles when present, did not appear particularly damaged. Some mitochondria appeared to have lost their cristae. Electron dense cells were numerous, and frequently observed in small foci of 3-4 cells throughout the epithelium, and were also evident along the basement membrane (Plates 19, 20a & b).These cells were visible at various stages of deterioration, which was indicated by an increase in electron density, condensation of cytokeratin filaments, and shrinkage and vacuolisation of the cytoplasm (Plate 20a & b). The nucleus of these dark cells was often shrunken in appearance and intensely staining. Occasionally the nucleus appeared to be arc shaped, the euchromatin remained in clumps around the edge of the nucleus (Plate 20c & d). The nuclear membrane remained intact. No swelling of cells or organelles was visible (Plate 19). Desmosomal junctions between intensely staining cells and normal epidermal cells were maintained, but appeared to be reduced in number (Plate 20a & b). Interdigitating cytoplasmic processes were seen less frequently surrounding the electron dense cells in VVS samples (Plates 19, 20a & b).

Electron dense cells were seen associated with leukocytes, the majority of which were lymphocytes (Plate 19a). Lymphocyte profiles were generally small with sparse cytoplasm and few organelles, and were interposed between epidermal cells of all layers. Also present, close to intensely staining cells, were leukocytes with lobed nuclei, which were possibly macrophages, neutrophils or NK cells (Plate 21a-c). Some leukocytes contained dark staining,

spherical granules which were membrane-bound (Plate 21a); others were characterised by the presence of numerous vesicles and organelles (Plate 21c). Leukocytes appeared to be activated, the cytoplasmic membranes were folded to form projections which were seen to make close contact with the cytoplasmic membrane of dark staining cells. Beneath the basement membrane (BM), a moderate inflammatory cell infiltrate was repeatedly observed (Plate 22a-c). One VVS biopsy did not have a significant inflammatory cell infiltrate, and had a similar appearance to control samples. Plasma cells, at various stages of development were frequently seen below the BM, although numerous lymphocytes and an occasional mast cell were also present (Plate 22b & c).Capillaries appeared numerous below the BM in a majority of the VVS samples. Frequently the capillary wall was seen in close contact with the BM, leukocytes which were often packed into the capillaries were seen penetrating the dermo-epidermal junction, squeezing between the basal cells. The basement membrane (BM) was not seen to be disrupted by the passage of lymphocytes, also there was no splitting of the basal laminae suggestive of other disease processes such as blistering. The basal cells appeared to have been displaced to allow leukocytes to pass through the basement membrane (Plate 19, 23a). It was not evident whether the integrity of the BM was affected in the long term by the passage of leukocytes.

Lobules of cytoplasm with no apparent origin were numerous in VVS samples, and were commonly seen in association with intensely staining cells (Plates 19, 20b & c, 23a & b). Round or oval in section, lobules of cytoplasm were pale staining, of varying size, with a well defined cytoplasmic membrane (Plate 24a-c). Intensely staining epidermal cells were frequently seen associated with several cytoplasmic lobules (Plate 19 & Plate 21). Frequently contained within these lobules were vesicles, polyribosomes, mitochondria and fine cytoplasmic filaments (Plate 24 a-c). There were neither characteristic granules nor nuclei evident in these lobules on serial sectioning. Occasionally an electron dense body, similar in appearance to a nucleus, was visible, but without a surrounding nuclear membrane (Plate 20c & d). Cellular junctions and

cytoplasmic projections were not evident. Frequently, the cytoplasmic membranes surrounding the lobules were seen in close contact with intensely staining cells and leukocytes (Plates 21 & 23). Frequently epidermal cells contained membrane bound lobules of cytoplasm (Plate 25), thus, cytoplasmic lobules appeared to be phagocytosed by epidermal cells.

Using TEM, the nuclei of superficial cells were frequently displaced giving the appearance of koilocytosis (Plate 26a). There was no evidence of viral infection in koilocyte-like cells. No bacteria or fungi were found attached to the surface cells. Surface cells were characterised by the presence of numerous microvillus projections of varying dimensions, but were generally 0.2-0.3μm in height, spaced at intervals of approximately 0.1-0.15μm (Plate 26b). The plasma membrane of the surface cells was partially thickened, but not to such an extent as in keratinised cells. Intensely staining cells were uncommon in this cell layer; if present these electron dense cells were isolated. Leukocytes, particularly lymphocytes, were occasionally seen in this cell layer. As in the control material, the superficial cells of this epithelium are characterised by the presence of large deposits of glycogen, often seen in a halo around the nucleus, or replacing the nucleus (Plate 26c). Vesicles were in close contact with the cytoplasmic membrane of superficial cells. Some of the vesicles were characterised by electron-dense cores, however, the vesicles were usually electron-lucent. Frequently vesicles were seen fusing with the cytoplasmic membrane. Vesicles of this type were found in a majority of the VVS samples (Plate 26c & d).

5.4 Discussion

The study of normal periductal tissue from women without vulvar pain was considered essential to the study of pathological tissue from women with VVS. As no work had been completed on the EM of the vulva, a base line of the tissue structure was required, and therefore, this constituted the first stage of the investigation. The general structure of normal epithelium, from the vestibule of the vulva, has been shown to resemble mucosal surfaces rather than keratinised epidermis (Sargeant *et al.,* 1996). Using light microscopy, differentiation of the epithelium into distinct cell layers was difficult. However, moving upwards, towards the surface, variations in morphology were visible. Epithelial cells increased in volume, became slightly flattened, and were less intensely staining. Surface cells were pale staining in comparison with basal cells, and in many cases had retained their nuclei. Leukocytes, visible as intensely staining cell profiles were visible in all cell layers, but were seen commonly along the dermal / epidermal junction (see Chapter 4.) Wax sections stained with Mallorys, illustrated that part of the vestibule which is removed during a vestibulectomy operation as treatment of VVS, contains areas of epithelium which are keratinised, or partially keratinised (Plate 15 & 16). However, samples obtained from VVS patients in Plymouth, which were removed from the lateral border of the right Skenes' gland, did not show any keratinisation of the surface cells.

Ultrastructurally, vestibular epithelia from control patients showed no conclusive evidence of keratinisation, although some thickening of the superficial cell walls was observed. The characteristic keratinised squames, and granular cells found in skin were absent. This was expected, as past reports on the anatomy and histology of the vulva have suggested that a portion of the vulva may be non-keratinised (Woodruff & Friedrich, 1985). The prominent cell type was the glycogenated intermediate cell, which formed a large, structurally homogenous population of cells. These cells lacked the characteristic spiny appearance of cells in keratinised epithelia; cytokeratin filaments were pale staining and loosely organised into bundles, and

occasionally filaments were seen to converge at electron dense plaques. Other cell types present were: superficial flattened cells, many having large glycogen deposits, nuclei and traces of organelles; supra basal cells; and basal cells both of which exhibited numerous mitochondria and rough endoplasmic reticulum. Some leukocytes were seen beneath the basement membrane and interposed between epidermal cells of the epidermis, however, these were relatively few in number. The predominant inflammatory cell was the lymphocyte with the occasional macrophage and plasma cell visible in the dermis (see Chapter 4).

The choice of electron microscopy for the study of VVS, has resulted in a detailed evaluation of the ultrastructural characteristics of the syndrome, contributing information not previously available using light microscopy. Scanning electron microscopy was an important addition to the investigation of normal, and pathological vestibular tissue. Normal vestibular tissue was found to be characterised by a pavement like surface of polygonal epithelial cells, with a complex network of microridges. Cell boundaries were prominent and raised, and microridges were rounded in appearance (see Chapter 4). These cells were similar in appearance when compared to surface cells of the vagina, cervix and bladder (Hackeman *et al.*, 1968; Odor *et al.*, 1989).

In addition to well defined microvilli, exfoliated cells of the cervix show cell boundaries, which have been described as 'crests' and also as 'furrows / grooves'(Ludwig & Metzger, 1976 as cited by Davina *et al.*, 1981). Furrows and grooves were seen on the surface of both control and VVS affected epithelia, where cell junctions from overlying cells had caused slight indentations. Davina *et al.* (1981) described changes in orientation of microridges into circles during pregnancy. In post-menopausal women the pattern of ridges changes again, the number decreases and the structures become less prominent, the cell borders are reported to become coarse instead of fine (Ludwig & Metzger, 1976 as cited by Davina *et al.*, 1981). This is

consistent with VVS samples, which when compared with normal (control) vestibular samples, appeared to be less densely populated with microridges, which are seen to be disorganised in overall structure, and shrunken in appearance. However, in the present study no change in appearance of the cell boundaries was observed. Davina *et al.*(1981) summarised past protocols for the processing of ecto-cervical cells for SEM, and concluded that these cells are particularly robust, and no alterations in surface characteristics were observed when processed by different methods. The lack of ultrastructural change using various methods was reported, to be due in part, to the partially keratinised state of the surface cells which are stable during processing. TEM of both vaginal and vestibular epithelium did not reveal keratinisation of the surface cells or the presence of an involucrin envelope. However, surface cells of vestibular and vaginal epithelium did show some thickening of the cytoplasmic membrane.

Using SEM, pores were visible distributed over the surface of perineal, vaginal and vestibular epithelia. These structures were more commonly seen in vestibular tissue and particularly in tissue samples from women with VVS. Samples from VVS patients were removed using a colposcope to ensure that the biopsy was taken from beside the Skenes' gland. It has been reported that in this area there may be numerous minor vestibular glands (Woodruff & Friedrich, 1985). In 1978, Robboy *et al.* described the origin and structure of these glands using light microscopy, and commented on their incidence at autopsy. It is possible that the pores visible in the vestibular samples are in fact minor vestibular glands, which have a similar appearance to mucus secreting glands and goblet cells evident in other epithelia. This would account for the increase in the incidence of these structures in VVS samples, as it is unlikely that an increase in these ducts / glandular structures would result in, or be a result of VVS.

Transmission electron microscopy (TEM), has indicated several factors of interest which may be important in future studies of VVS. These observations centre around the presence of intensely staining cells present in the epidermal cell layers of vestibular epithelium:

- Electron dense, shrunken cell profiles.
- Lobules of cytoplasm with no detectable origin.
- An inflammatory cell infiltrate below the basement membrane and visible in epidermal cell layers.

Intensely staining cells, which were not evident in large numbers in the control samples, have not previously been described in association with VVS using light microscopy. The fact that these cells have remained undetected is not surprising as they are often indistinguishable from other epidermal cells when examined using light microscopy. The use of methylene blue staining has resulted in the visualisation of these unusual cells at the light microscopy level. However, these cells would probably not have been detected unless ultrastructural examination of vestibular tissue had indicated their abundance in VVS samples. In some VVS samples approximately 10-20% of the basal and intermediate cell population appeared to be intensely staining and shrunken in appearance. Using light microscopy, these intensely staining cells have a similar staining intensity when compared with the nuclei of lymphocytes and normal basal cells, and are, therefore, hard to detect. Consequently, these cells appear more prominent in the supra-basal and superficial cell layers when using light microscopy. Ultrastructural investigation of normal vestibular tissue identified some similar intensely staining cells, but these cells were isolated, rarely occurring in groups.

When examined using TEM, several distinct changes were noted when comparing normal and intensely staining epidermal cells. As reported, the cytoplasm of these conspicuous cells appeared electron dense, possibly due to the condensation and clumping of cytokeratin filaments. In comparison, cytokeratin filaments in control samples and in normal areas of the

VVS samples, were pale staining, and not arranged in clearly defined bundles (see Chapter 4). In intensely staining cells, organelles were intact with little evidence of shrinkage, whereas the cell as a whole appears shrunken and the cytoplasm vacuolated. The nuclear membrane appeared undamaged. When grouped in clusters, these cells appear to be at various stages of change, denoted by the degree of condensation of cytokeratin filaments and vacuolation of the cytoplasm (Plate 19). The appearance of these cells initially suggested that the fixation schedule was not suitable for this tissue, however, when ultrastructural detail was compared with control samples fixed using the same method, the incidence of intensely staining cells was obviously greater. When the fixation protocol was varied there was no change in the appearance, distribution, or frequency of the intensely staining cells in VVS biopsies. The variation in electron density of these epidermal cells may be due to a natural phenomenon, such as metabolic differences between cells, however, it is suspected that these cells may be undergoing a form of cell death due to necrosis, apoptosis or a related process.

Necrosis, which is characterised by cell swelling and inflammation had for a long time been considered to be the only means of cell death. Necrotic cells are normally pale in appearance with disintegrating cytoplasm. (Kerr *et al.,* 1972; Wyllie, 1980; Wyllie *et al.*, 1984). Previous observations which did not fit the pattern of necrosis have been disregarded as anomalous. However, reported anomalies of necrosis are now believed to be due to apoptosis, a process which has been, and often still is, masked by the process of necrosis. In the last 20 years, a considerable amount has been learnt about this alternative form of cell death. The term apoptosis, proposed by Kerr, Wyllie and Curie in 1972, describes a process which results in cell deletion by shrinkage of the cell, fragmentation of the cellular DNA, and the formation of apoptotic bodies. Kerr *et al.* (1972), suggested a two-stage process involving the formation of apoptotic bodies and subsequently the phagocytosis and degradation of these bodies by other cells. Due to the continued confusion of the two processes a majority of reviews have focussed

on the similarities and differences of the two processes (Searle *et al.*, 1982; Bursch *et al.*, 1992; Gerschenson & Rotello, 1992; Buja *et al.,* 1993; Cohen, 1993a & b; Collins & Rivas, 1993; Budtz, 1994). Necrosis is determined by environmental, often traumatic factors, and not by factors intrinsic to the cell itself as in apoptosis. In addition, necrosis is not involved in developmental processes, and does not require the expression of new mRNA or proteins, both of which are characteristic of apoptosis (Arends & Wyllie, 1991; Buja *et al.,* 1993; Cohen, 1993a & b; Schwartz & Osbourne, 1993; Martin *et al.*, 1994). Apoptosis in skin has been reported on several occasions, however, its role and regulation are still obscure. Links with terminal differentiation have confused the issue of apoptosis in skin (Hashimoto, 1976; Lovas, 1986; Arends & Wyllie, 1991; Haake & Polakowska, 1993; Budtz, 1994). Experiments with granular cells showed endonuclease activity and DNA fragmentation, suggesting that apoptosis may occur in line with terminal differentiation of keratinocytes (Rosenbach *et al.,* 1993). In addition, Dispasquale & Youle (1992), have reported the transfer of apoptosis between the nuclei of heterokaryons. Terms such as dyskeratosis or colloid bodies, which are frequent observations in skin pathology, are often used to describe shrinkage of cells similar to that occurring during apoptosis. Conditions such as Bowenoid papulosis, steroid induced skin atrophy and lichen striatus may therefore all involve apoptosis (Hashimoto, 1976; Rosenbach *et al.,* 1993).

Whether the intensely stained cells commonly seen in VVS are affected by necrosis, or programmed cell death, was not determined during this investigation, however, these cells were patently being damaged by some mechanism. This appears be related to the symptoms of VVS as these cells were visible in significant numbers in the VVS samples. Image analysis of control and VVS affected epithelia was completed to determine the incidence of intensely staining cells in the epithelium (see Chapter 7).

Many of the characteristic cell developments associated with apoptosis, such as; cell shrinkage, intact organelles, membrane 'blebbing' and vacuolation of the cytoplasm, were apparent in samples from women with VVS. However, several of the diagnostic factors associated with apoptosis do not apply to VVS (Table 5.1). Condensation of nuclear chromatin was evident, however, margination and the formation of crescent shaped caps of chromatin, as well as apoptotic bodies containing nuclear fragments were not common in the VVS samples (Fig. 5.0). A similar appearance was evident however. Crescent shaped nuclei were seen commonly in epidermal cells, however, these were not due to margination of chromatin. The nuclei of these cells appeared arc shaped, and displaced in order to phagocytose cytoplasmic bodies. When present in epidermal cells, cytoplasmic bodies were visible almost surrounded by the nucleus.

Despite the fact that all of the characteristics of apoptosis were not present in VVS samples, an alternative diagnosis of necrosis is less convincing. Necrosis was not evident when examining patients with VVS, and biopsies exhibiting the characteristics of necrosis have not previously been reported in the literature. Necrosis is a degenerative phenomenon which results in exudative and often chronic inflammation. Although a mild to moderate inflammatory cell infiltrate was present in VVS samples, the degree of inflammation was not suggestive of necrosis. In comparison, during cell death by apoptosis, dying cells and cell debris are rapidly packaged into apoptotic bodies to prevent the initiation of a potentially damaging inflammatory reaction (Savil *et al.*, 1993). The presence of a moderate number of inflammatory cells in VVS samples may suggest a protracted form of apoptosis, where the dying cells are not cleared quickly, and therefore a degree of inflammation occurs. Research has indicated that there are possibly several subsets of apoptosis which may have less well defined characteristics (Rosenbach *et al*,.1993). The use of immunological markers is essential to determine the mechanism of cell death in VVS. Rosenbach *et al.* (1993), highlight two main types of apoptosis, one which is a receptor mediated and genetically controlled process, and another less

well defined form. The latter form of apoptosis, may occur due to a general, cellular response to injury, a similar process to that seen in cell mediated cytotoxicity, and which may be occurring during VVS.

In support of this, Schwartz and Osbourne (1993), report experiments which show various types of apoptosis. Apoptosis is only one aspect of programmed cell death (PCD), not all dying cells display the changes associated with apoptosis. During PCD in the intercostal muscles of the tobacco hawk moth, *Manduca sexta*, ultrastructural examination reveals that membrane blebbing, chromatin margination and DNA fragmentation do not occur. The classical nuclear changes associated with apoptosis are replaced by pyknosis and rounding of the nucleus. The appearance of these cells is similar to the intensely staining cells present in VVS biopsies. The nuclei in these samples retained their shape and nuclear membrane, however, the chromatin appeared more dense, possibly pyknotic (Chu-wang and Oppenheim, 1978, as cited by Schwartz and Osbourne, 1993).

Potten (1987) describes a form of cell death where the cells appear darkened. Enhanced staining of the cytoplasm and, or, the nucleus resulting in a 'dark' cell appearance, has been described in the tissues of sheep, rodents and man. A majority of reports using TEM have involved glutaraldehyde and osmium fixation, and embedding in a plastic resin for examination by TEM. It has been suggested that these processing steps are linked to the visualisation of 'dark' cells by TEM. In one study twice as many 'dark' cells were visible after fixation with glutaraldehyde and osmium and embedding in Epon, than tissue embedded in wax (Klein-Szanto *et al.*, 1982a & b). Murakami *et al.* (1985), described the appearance of similar dark keratinocytes following intracutaneous injection of Cholera toxin in mouse skin. Chiba *et al.*, (1984) described two types of 'dark' cells. The first had many features characteristic of actively synthesising cells, and resembled immature keratinocytes, having few filaments and mitochondria. The second

type had condensed nuclei and clumped chromatin, dilated vesicles, cytoplasmic vacuolation and swollen mitochondria. This type of 'dark' cell had numerous organelles and prominent cytokeratin filaments organized into bundles. Chiba *et al.,* (1984), suggests that these cells are degenerating or undergoing involution.

Parsons *et al.,* (1983) classified 'dark' cells into four groups, dependant on the degree cytoplasmic and nuclear condensation. This study suggested that these cells may be undergoing a type of apoptosis, although nuclear condensation was even, there was no marginal clumping of chromatin, and there was no evidence of phagocytosed material in surrounding cells and leukocytes. Nuclear changes and the overall appearance of cells are consistent with the present study of VVS affected tissue, where nuclear condensation was evident, but was not accompanied by margination, or the formation of arcs of chromatin. The visualisation of darkened cells using electron microscopy may in fact be linked to the use of aldehyde fixatives, as these cells have not previously been described in VVS biopsies processed for light microscopy.

Tumour promoters have been shown to induce the formation of 'dark' cells. The action of these chemicals is varied, however they are capable of causing acute tissue damage. Studies using rat respiratory epithelium have shown a direct relationship between the number of 'dark' cells and the degree of epithelial atypia. This has also been demonstrated in human oral leukoplakia (Klein-Szanto *et al.*, 1982a & b). Cytoplasmic condensation *per se* is not indicative of apoptosis. Cells with moderately condensed cytoplasm but normal ultrastructure in general can be found in most tissues (Potten, 1987). Condensed cells with swollen mitochondria are almost certainly dying, however, these cells have a different appearance from cells affected by apoptosis. This type of cell death is not characterised by the formation of blebs or the segregation of chromatin. Instead the dying cell is 'squeezed out' amongst other epithelial cells

Figure 5.0 Diagrammatic representation of the processes of apoptosis and necrosis

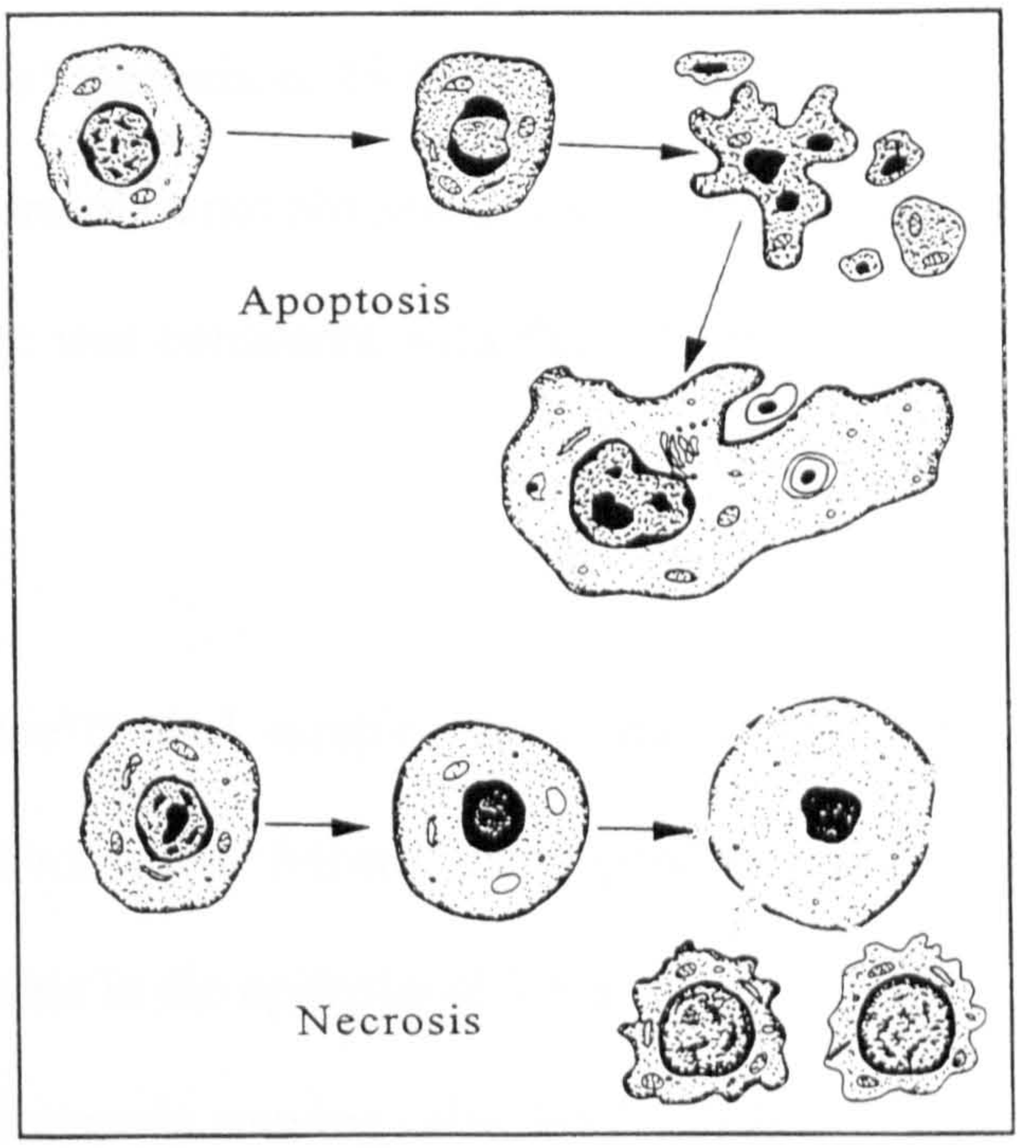


Table 5.1. Documented differences between the processes of apoptosis and necrosis

APOPTOSIS	NECROSIS
■ Individual cells or populations of cells are deleted	■ A non selective degenerative phenomenon
■ Significant cell shrinkage, mitochondria remain intact. Characteristic cytosol and chromatin condensation.	■ Cell swelling, mitochondria and other cytoplasmic compartments swell, resulting in rupture of membranes.
■ Apoptosis based on fragmentation of nuclear DNA by endonucleases - fragments of 200-300 bp, irreversible	■ Biochemical factors are linked to membrane breakdown and alterations in ion homeostasis.
■ No evidence of micro-environmental inflammatory reaction	■ Strong inflammatory reaction observed around dying cells
■ Membrane 'blebbing' or zeiosis, resulting in fragmentation of the cell into apoptotic bodies prevents release of noxious factors which trigger inflammation.	■ Rupture of cell membranes releases noxious factors resulting in macrophage activation.
■ Rapid phagocytosis of whole apoptotic cells or apoptotic bodies by neighbouring cells or macrophage	■ Inflammatory cells attracted to damaged cells phagocytose dying cells

This process, which is similar to apoptosis, has been described in pathological and non-pathological conditions. Abnormalities in mitochondria during this process suggest that it may be a variant of necrosis (Johannison, 1968, and Cooper *et al.,* 1975, as cited by Potten, 1987). Changes in mitochondria were not obvious in VVS, some mitochondria appeared damaged, but the degree of damage was consistent with that of control tissue and was probably due to processing.

Epidermal cells in the control samples were characterised by the presence of numerous cytoplasmic projections which formed interdigitating junctions between the cells. These projections were evident in the epithelia of VVS samples, but were less pronounced and less frequent around the intensely staining cells. Wyllie (1980) described a similar change which occurred concomitant with nuclear changes during apoptosis,'microvilli if present disappeared and blunt protuberances of the cell membrane form, desmosomal junctions if present may break down at this stage'(Wyllie, 1980; Searle *et al.,* 1982). In VVS samples there was no evidence of desmosomal breakdown. However, desmosomes appeared to be less frequent, and cytoplasmic projections were less prominent around intensely staining cells common in VVS samples, when compared with controls.

The most perplexing observation was the presence of membrane bound lobules of cytoplasm, visible interposed between epidermal cells, which appear to have no origin. On serial sectioning no nucleus was evident in the cytoplasmic lobules. As these bodies were devoid of cytoplasmic junctions and projections, it may be concluded that they may be either projections of migratory cells, or alternatively, membrane bound by-products of cells. These structures may be interpreted as projections of leukocytes, or possibly the arms of Langerhans cells. Although Langerhans granules were observed on several occasions in the cytoplasm of non-epidermal cells, neither complete granules, nor components of Langerhans granules were evident in the

cytoplasmic lobules. There was an increased number of non-epidermal cells present in VVS samples compared with the control samples. Many of these cells had large lobed nuclei and granular cytoplasm. The nature of these cells is unknown as immunological marking was not possible on the glutaraldehyde fixed tissue. However, it is possible that a percentage of these cells were Langerhans cells, which may account for the presence of such lobules interposed between epidermal cells in the VVS samples. Marshall & Steven (1969), described membrane bound structures in rumen epithelium using TEM. These structures had a similar appearance to cytoplasmic lobules described in this investigation. These authors attributed these structures to cytoplasmic processes of dendritic cells. Langerhans cells are an important component of the skin immune system, and are particularly vital antigen presenting cells, involved in the development of the immune response (Stingl *et al.*, 1980; Edwards & Morris, 1985). The presence of an extensive network of dendritic process of Langerhans cells, may account for the increase in the inflammatory cell infiltrate evident in cases of VVS. However, this does not account for the presence of large numbers of apoptotic-like cells, or the presence of cytoplasmic lobules contained within epidermal cells.

An alternative origin of these membrane bound lobules may be that they are the bi-products of apoptosis, termed 'blebs' or 'apoptotic bodies'. Apoptotic bodies have previously been confused with autophagic vacuoles, as identification rests on the recognition of nuclear fragments or the presence of organelles not usually found in the ingesting cell. Terms such as zeiosis, popcorn-like cytolysis, necrobiosis and extrusion subdivision are all terms which have been used to describe the phenomena now termed apoptosis (Wyllie, 1980; Wyllie *et al.*, 1984; Cohen, 1993a & b). Lobules of this type were visible in the epidermis of control samples, however, they were discrete and few in number. The lobules were seen in similar situations in the control and VVS affected vestibular tissues, usually associated with leukocytes and intensely staining apoptotic-like cells. During the process of apoptosis membrane bound blebs

of cytoplasm and nuclear contents are formed as the dying cells are removed. These extracellular bodies never show evidence of degradation and may be capable of metabolic activity even though they are programmed to die (Zychilinsky, 1993). This is consistent with the findings of the present study which indicated that membrane bound lobules of cytoplasm showed no evidence of cell death.

The lobules of cytoplasm seen in the VVS samples are consistent with the descriptions of apoptotic bodies (Olson, 1975; Wyllie, 1980; 1984; White *et al.,* 1993), being of varying sizes and shapes, and containing organelles and nuclear fragments. On several occasions nuclear remnants were visible contained within the lobules, however this was not a common observation. Other authors have described phagocytosis of apoptotic bodies by macrophages and by neighbouring epidermal cells. Although lobules of cytoplasm were seen in close contact with macrophage-like cells in the epithelium, there was no evidence of phagocytosed apoptotic bodies in these cells. Frequently, neighbouring epidermal cells were seen to extend cytoplasmic projections, which were seen to make close contact with lobules of cytoplasm. On many occasions similar cytoplasmic bodies were seen contained within epidermal cells, and may have been phagocytosed. Often the nucleus of the cell containing the cytoplasmic body was displaced, and arc shaped. In classical apoptosis it is essential that macrophages and phagocytes quickly clear cells undergoing apoptosis, and apoptotic bodies. If these structures rupture they will evoke a potentially harmful immune response. In order to attract phagocytes and macrophages to the apoptotic cells, it has been proposed that several changes in the cytoplasmic membrane occur to increase the 'eat me' status (Savill *et al.,* 1993).

There is evidence that lymphocyte attachment to epithelial cells is often followed by apoptosis, which may rule out increased membrane permeability as the primary event in targeted cell death. In cases where cell death is believed to be induced by cell mediated immunity *in vivo*.

numerous apoptotic bodies have been found to occur in close proximity to the infiltrating lymphoid cells (Potten, 1987). This is consistent with observations of membrane bound lobules in this investigation, where leukocytes were seen to make close contact with darkened epidermal cells, and with surrounding lobules of cytoplasm. This type of cell mediated apoptosis is not restricted to lymphocytes, macrophage have also been shown to trigger apoptosis. Cell mediated apoptosis has also been identified in chronic active hepatitis, lichen planus, liver allograft rejections and graft *vs*. host disease (Hashimoto, 1976; Potten, 1987).

The death of cells by apoptosis has become particularly important in pathology, as the process may be identified as a significant factor in many diseases. This process of cell deletion has already been shown to occur in areas of embryogenesis, T cell development, tissue remodelling, homeostasis, and tissue involution (Wyllie, 1980; Wyllie *et al*., 1984; Jenkinson *et al*., 1989). If the ability to harness and use the power of programmed cell death becomes reality this may be used in the treatment of cancers. Anti-apoptotic drugs would also be useful, particularly in areas such as dermatology where hyper-proliferative diseases such as psoriasis are common. It has been proposed that there are a large number of receptors for apoptosis, as well as many noxious agents which may induce this process. It is probable that apoptosis is an extremely complex process, and that many subsets of the process occur. It has been shown that cell death can occur without DNA fragmentation, however, the mechanism of DNA destruction which is usually present is not clear (White *et al.,* 1993). Two main theories have been suggested; firstly, that DNA endonuclease activity is induced or, secondly, the cellular DNA is made available for fragmentation by an endogenous endonuclease. Other factors such as calcium-dependant unfolding of the chromatin, and the effect of low pH may also be important in the process of apoptosis.

The link between apoptosis and VVS needs to be pursued further as this investigation has only uncovered part of a complicated chain of events, which may be attributed to apoptosis. Immunocytochemistry to detect proteins such as Bcl2 or Bax linked to the regulation of apoptosis should form the next stage of the investigation. It is essential to determine whether cells are affected by a type of apoptosis, by which a degree of cell mediated immunity occurs, and the classical nuclear changes are absent, which would account for the mixed, mild to moderate inflammatory cell infiltrate reported by many authors (Pyka *et al.,* 1988; Prayson *et al.,* 1995). It has been reported that apoptosis may occur in response to an autoimmune reaction (Tan, 1994). It is conceivable, that VVS may be due to a cell signalling defect, which results in the damage of epidermal cells in the apparent absence of an etiological agent. The presence of auto-antibodies on some epidermal cells in VVS samples could be investigated in future studies if tissue samples were processed in wax, or by LTFS for TEM. In addition, the presence of dendritic process of Langerhans cells, may be examined using light and electron microscopy, and associated immunological marking techniques to identify the numerous cytoplasmic bodies present in VVS samples (Hanau *et al.*,1986).

Figure 5.1 Schematic presentation of the ultrastructural characteristics of vestibular tissue from VVS patients. Illustrating the presence of glycogenated (G) superficial (Su) and intermediate cells (Ic), darkened, apoptotic-like cells (D), lobules of cytoplasm (Lb), and the infiltration of leukocytes (Le), characteristics which are not so prominent in normal vestibular tissue. Note also the presence of a small lymphocyte (L) in the basal cell layer (Bc), and a plasma cell (Pl) and mast cell (Mc) in the dermis.

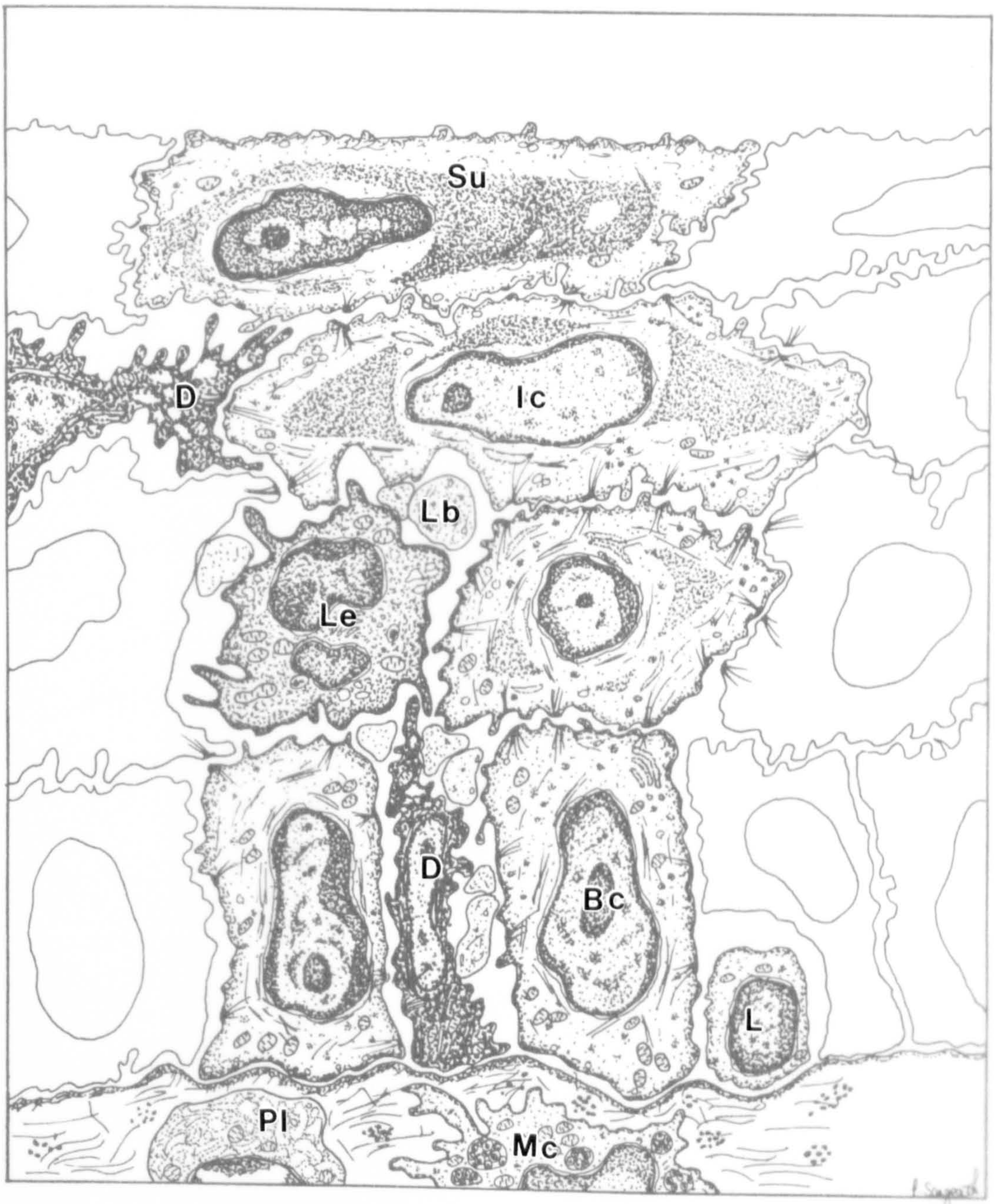
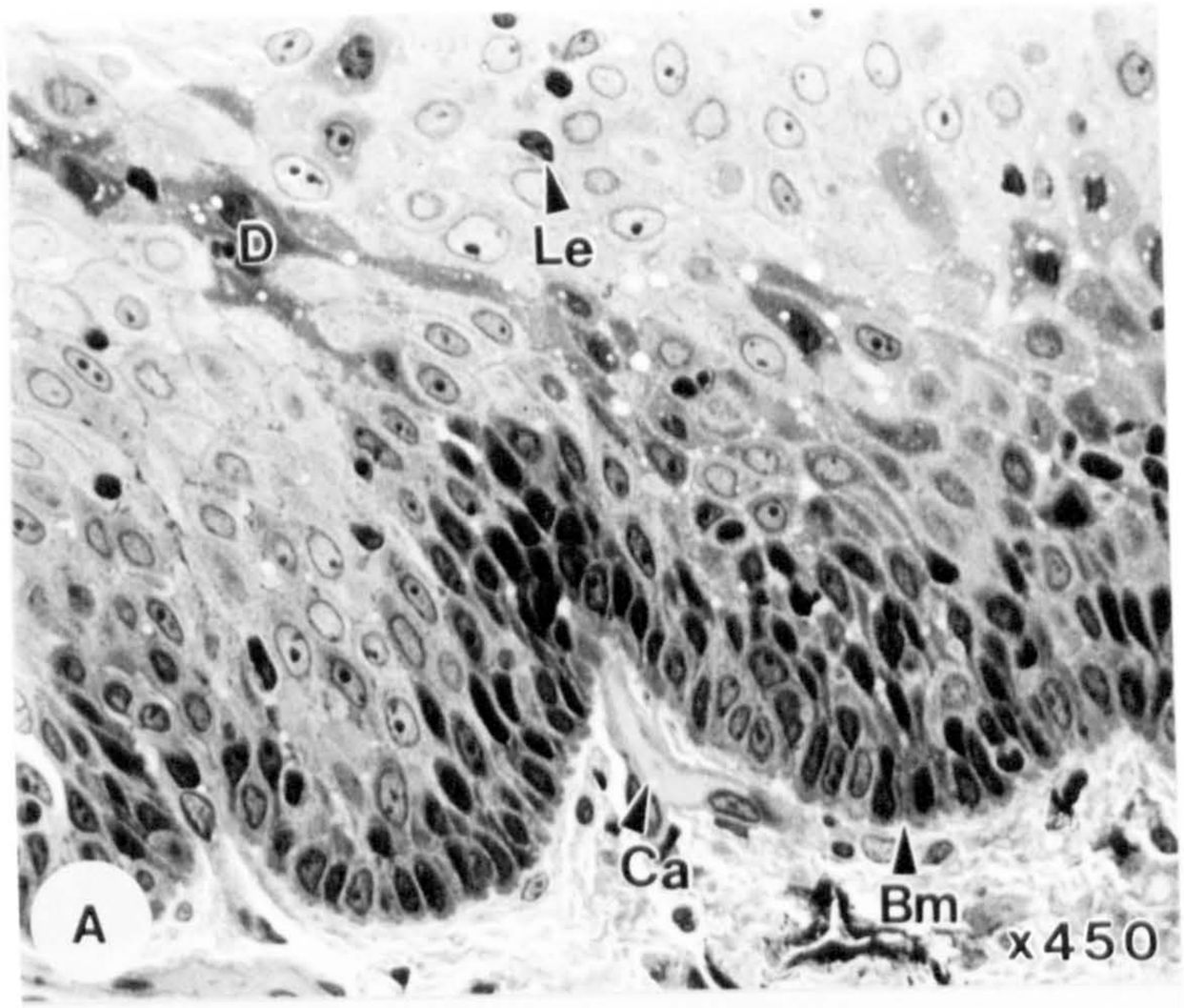
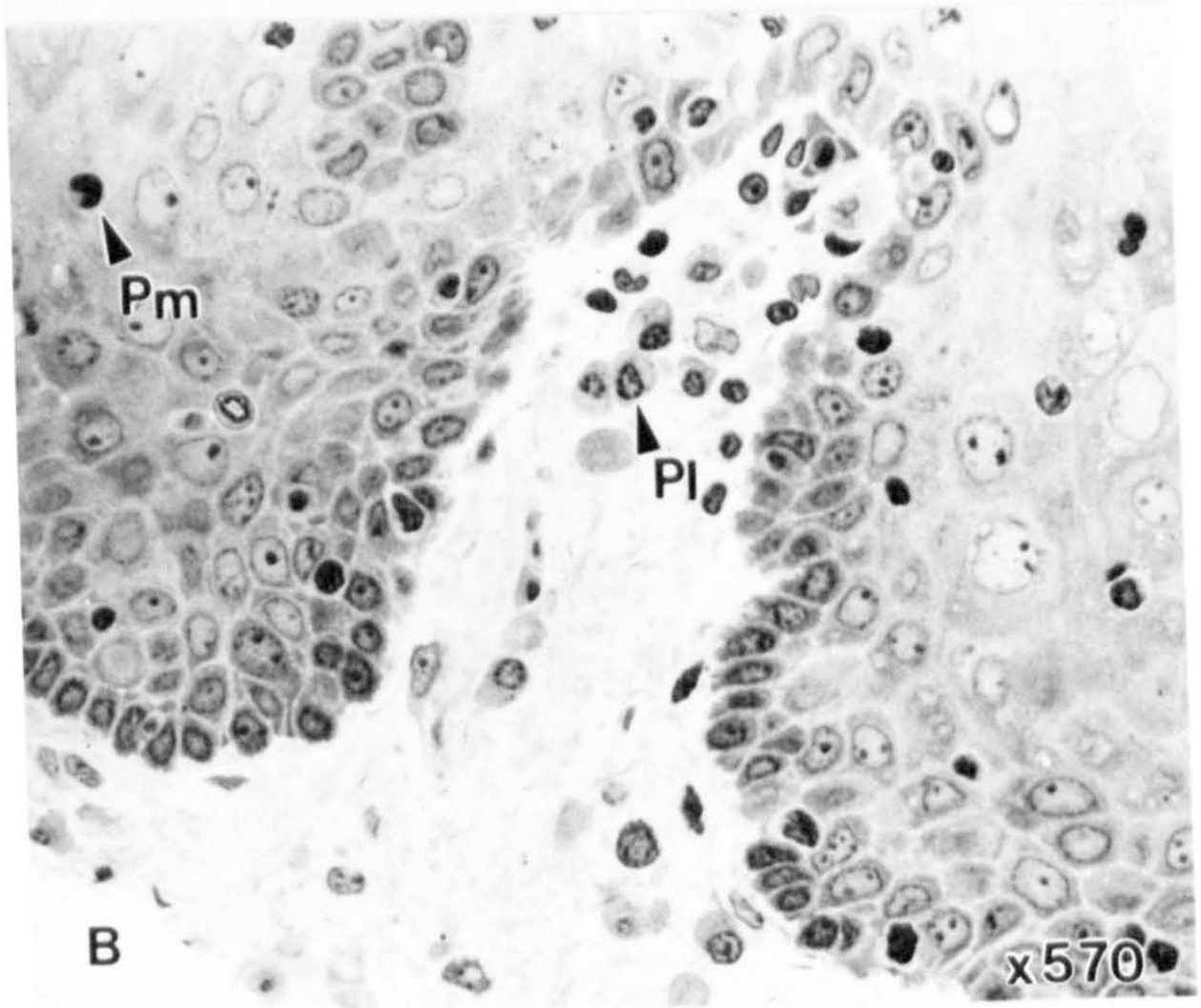

Su
D
Ic
Lb
Le
D
Bc
L
Pl
Mc

Plate 14 a LM of VVS affected epithelium. Resin sections were stained with 1% methylene blue. Dark staining cells are visible at this magnification (D). Leukocytes are seen penetrating the epidermis (Le), and a capillary (Ca) is visible in close contact with the basement membrane (Bm). x 450

Plate 14b Plasma cells (Pl) and other leukocytes in the dermis of a VVS sample, immediately below the basement membrane. A polymorphonuclear leukocyte (Pmn) is visible in the epidermis. x 570

Plate 14c The dermis of a VVS sample, illustrating a moderate inflammatory cell infiltrate, composed of mainly lymphocytes, and plasma cells. x 450


D
Le
Ca
Bm
A
x450


Pm
Pl
B
x570

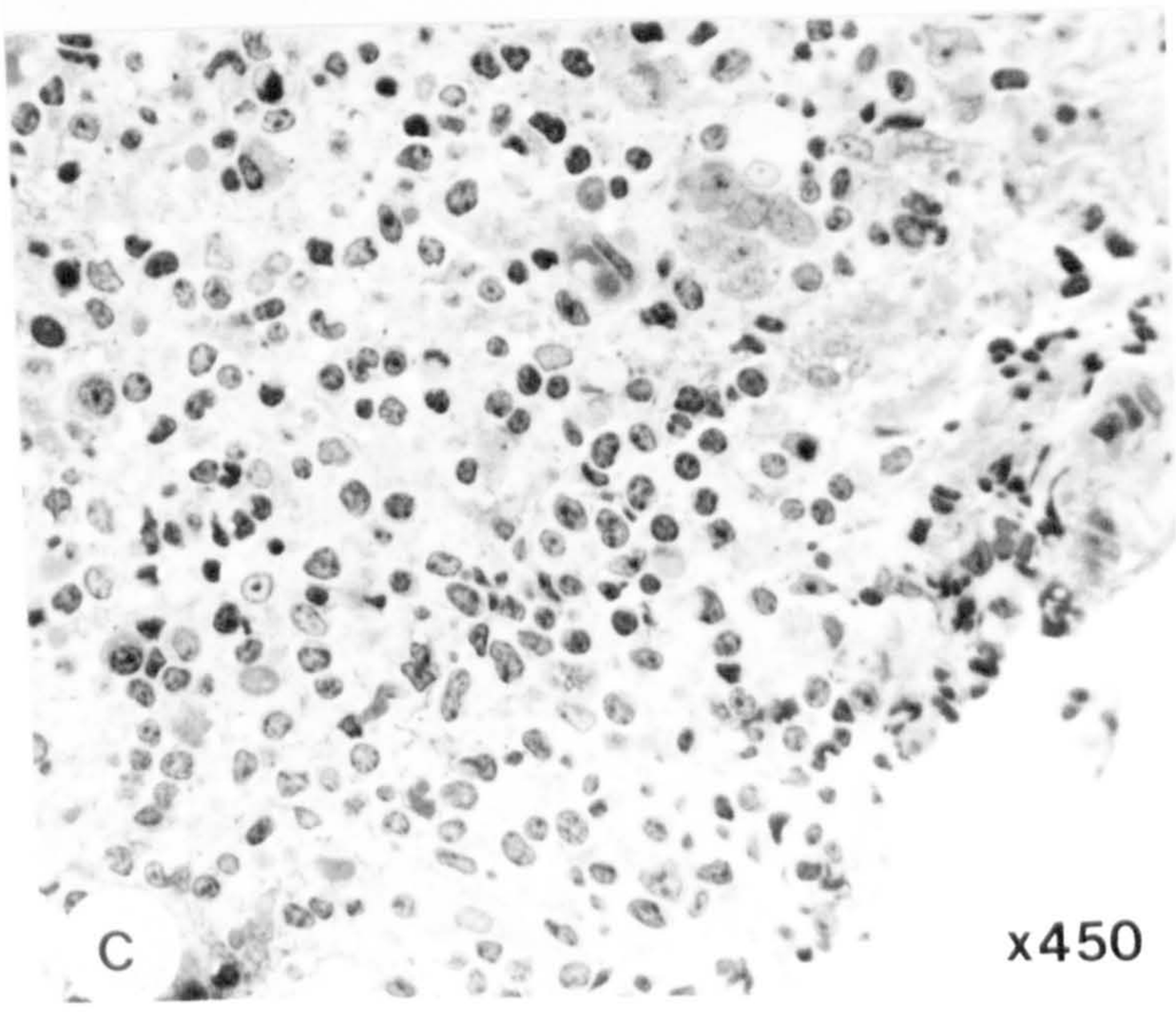
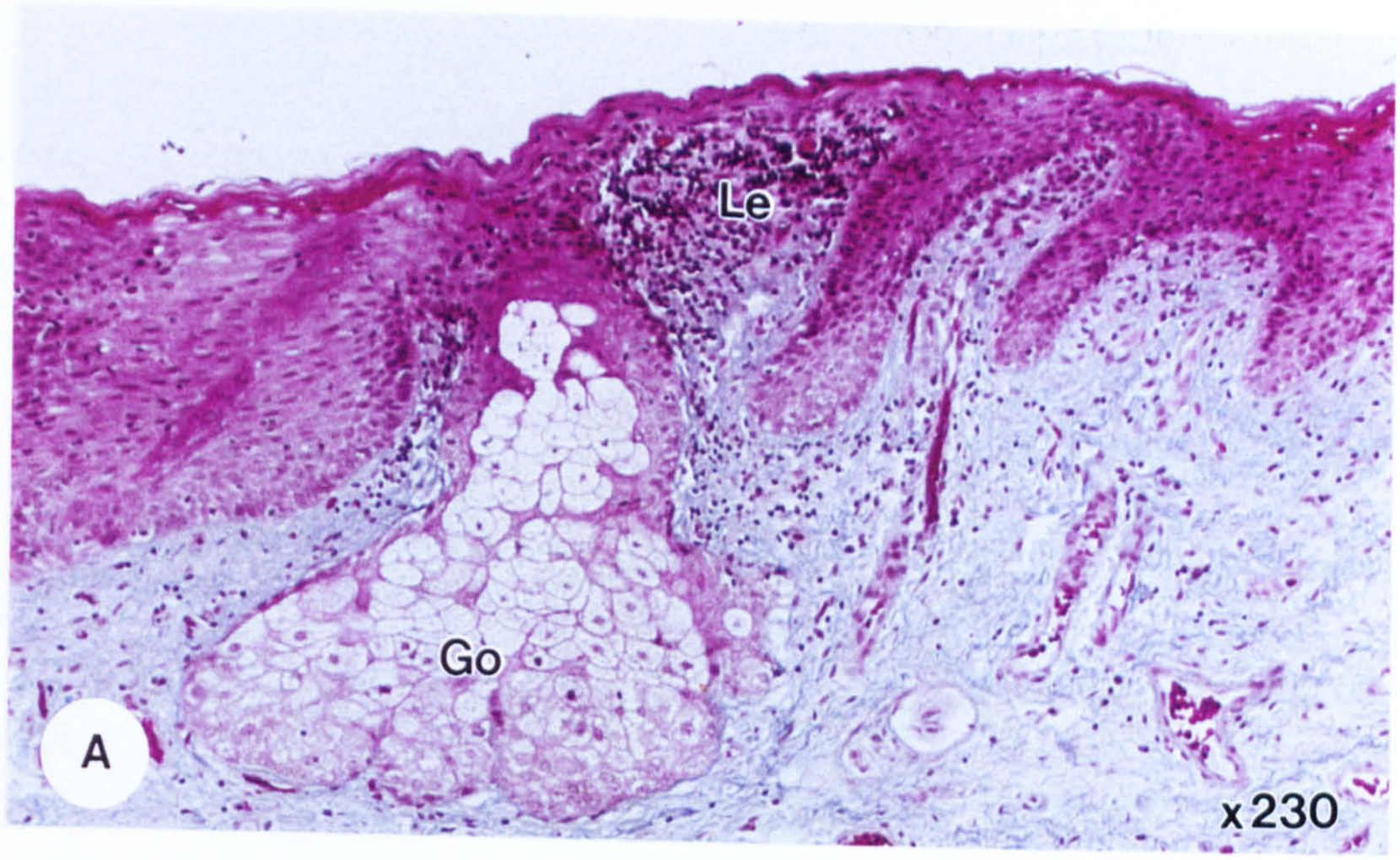

C
x450

Plate 15a

LM photograph of vestibular epithelium from a patient with VVS. Wax sections were stained with Mallorys. Illustrating the presence of a glandular structure which is similar in appearance to a sebaceous gland (Sg). Leukocytes are seen to be concentrated around this structure, but are not seen in the lumen of this gland. x 230

Plate 15b

Vestibular epithelium from a VVS patient, illustrating a mild inflammatory cell infiltrate in the dermis (Le). Surface cells are nucleated and slightly flattened (Su). x 600


Le
Go
A
x230

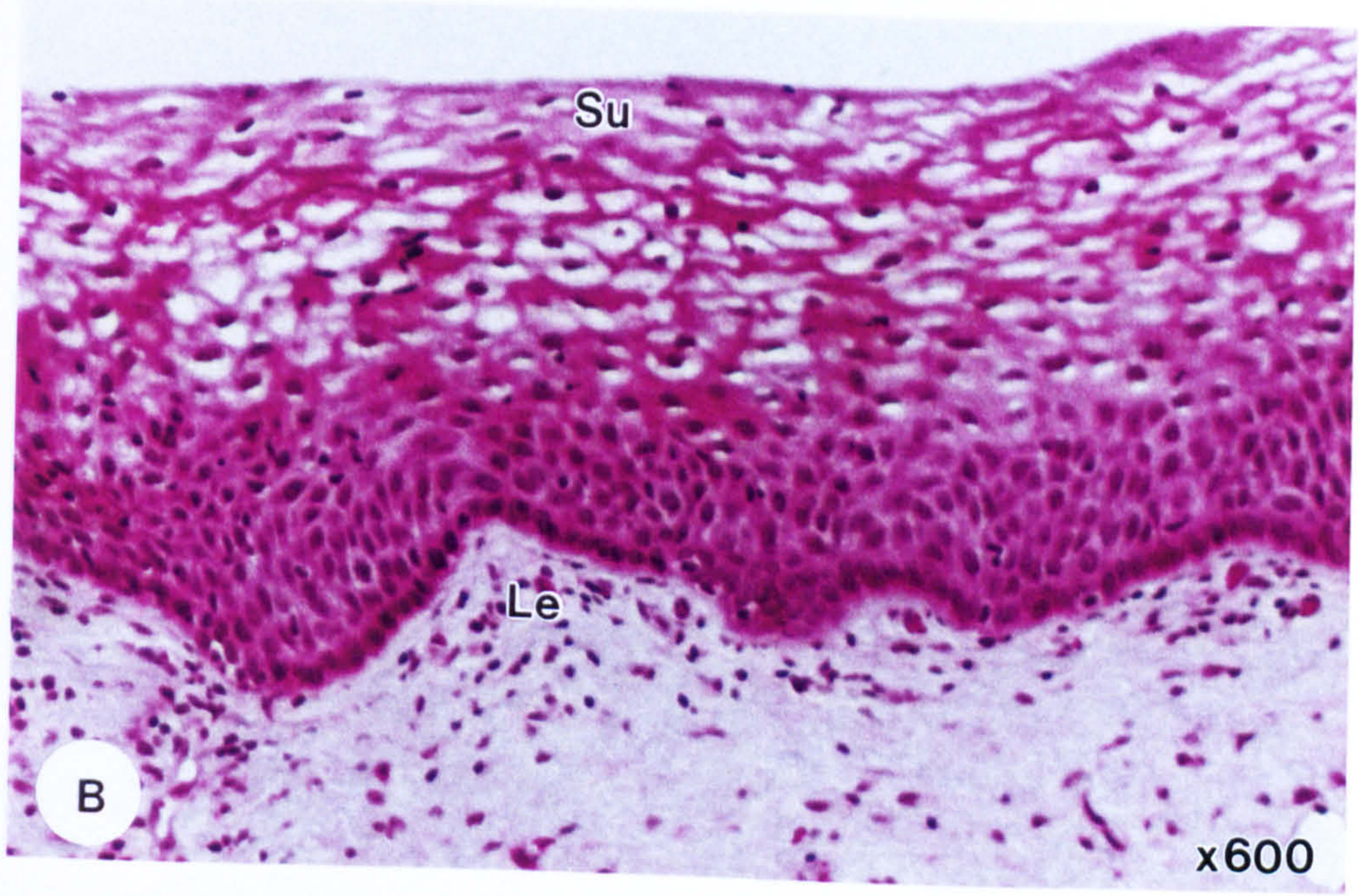
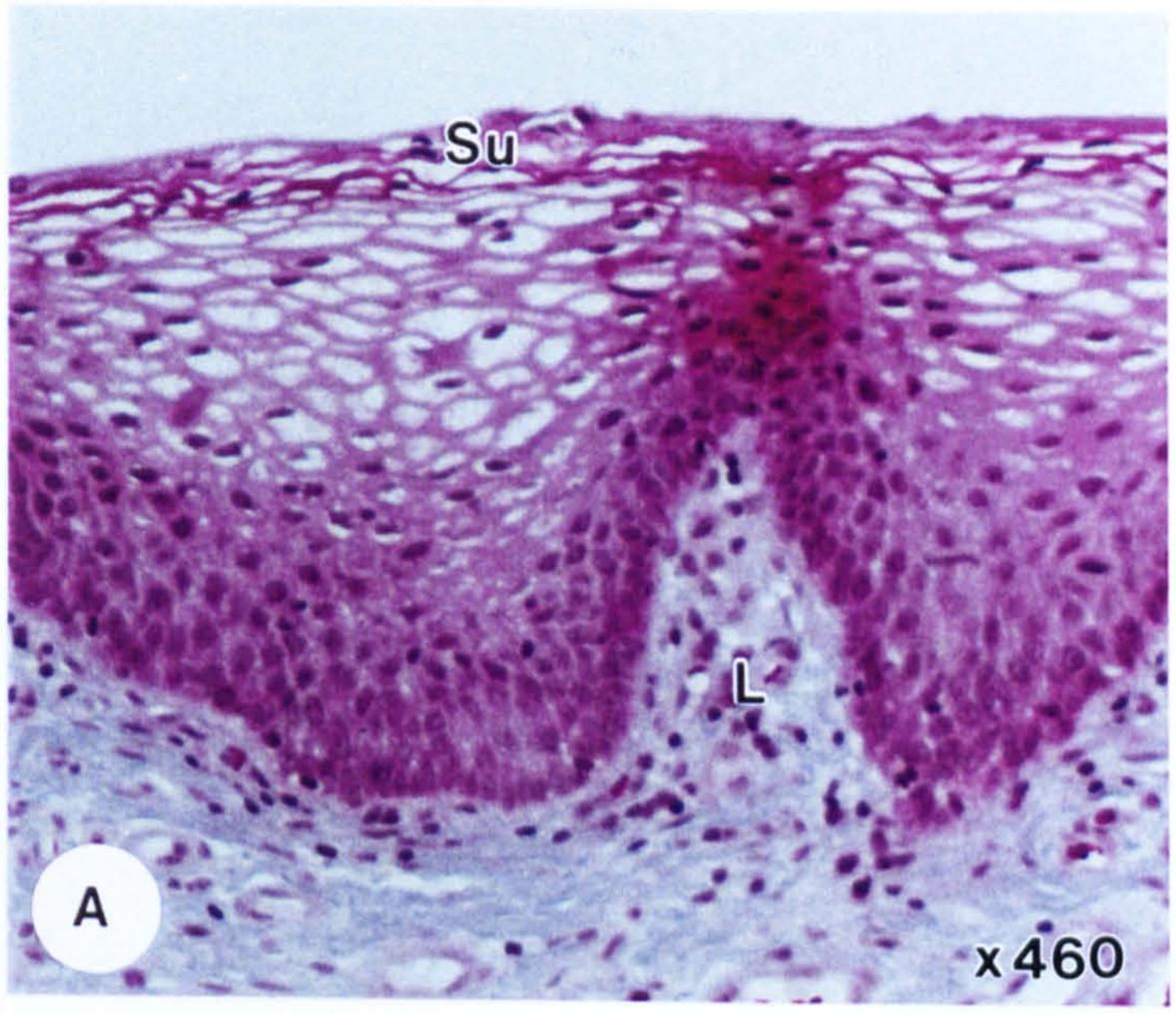
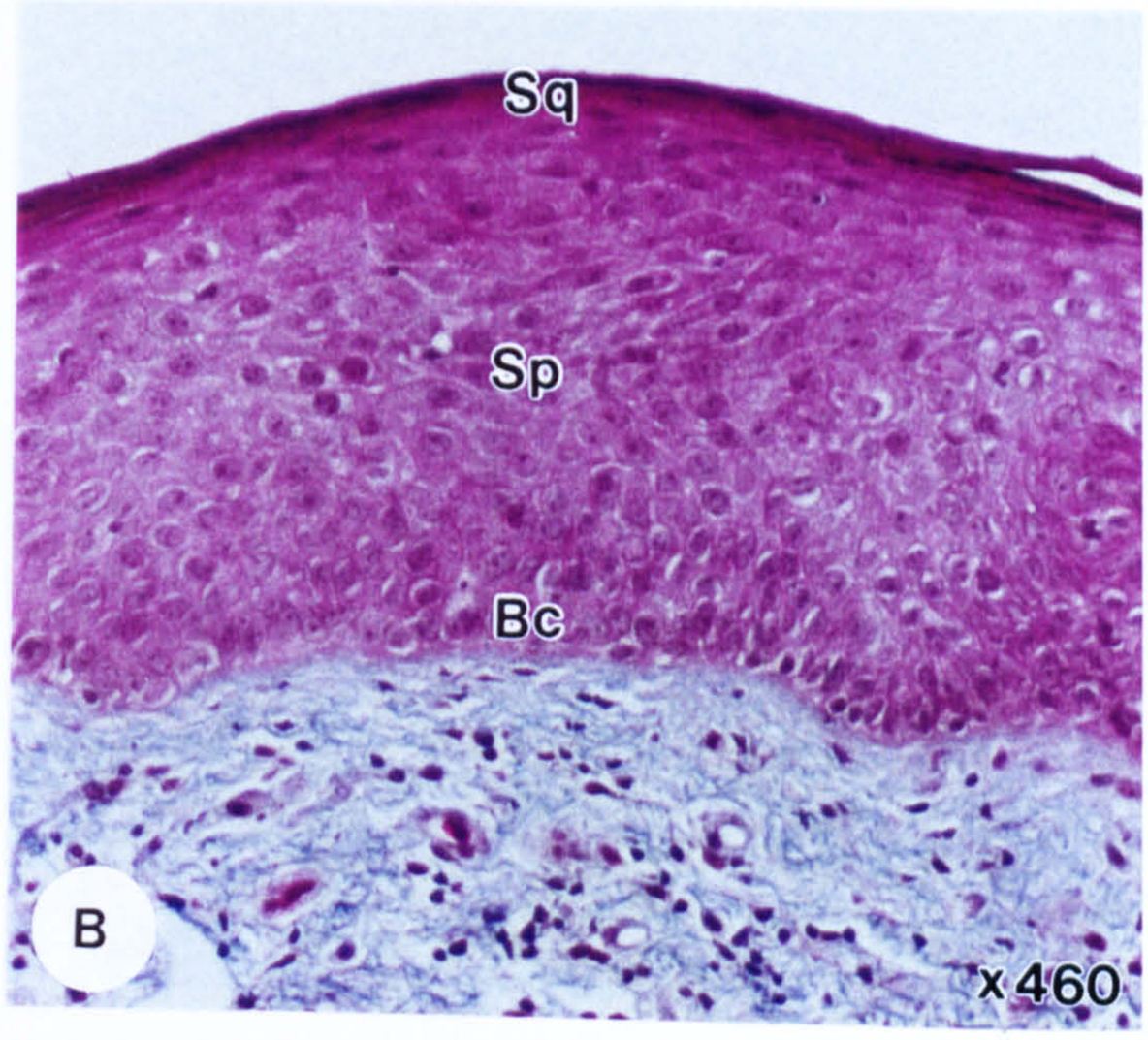

Su
Le
B
x600

Plate 16 a LM photograph of vestibular epithelium from a patient with VVS, stained with Mallorys. Illustrating a non-keratinised area of vestibular epithelium, removed during vestibulectomy as treatment of VVS. Surface cells are flattened (Su), and a leukocytes are seen in the dermis below the basement membrane (Le). x 460

Plate 16b LM of a partially keratinised area of vestibular epithelium stained with Mallorys. Surface cells are thickened and do not have nuclei (Sq), spiny cells (Sp) and basal cells (Bc) are also illustrated. x 460

Plate 16c LM area of keratinised vestibular epithelium, which appears to have been removed from an area close to Harts' line, which indicates the transition from non-keratinised to keratinised epithelium. Surface squames are seen to be desquamating (Sq), some clear cells are seen along the basement membrane. x 460


Su
L
A
x460


Sq
Sp
Bc
B
x460

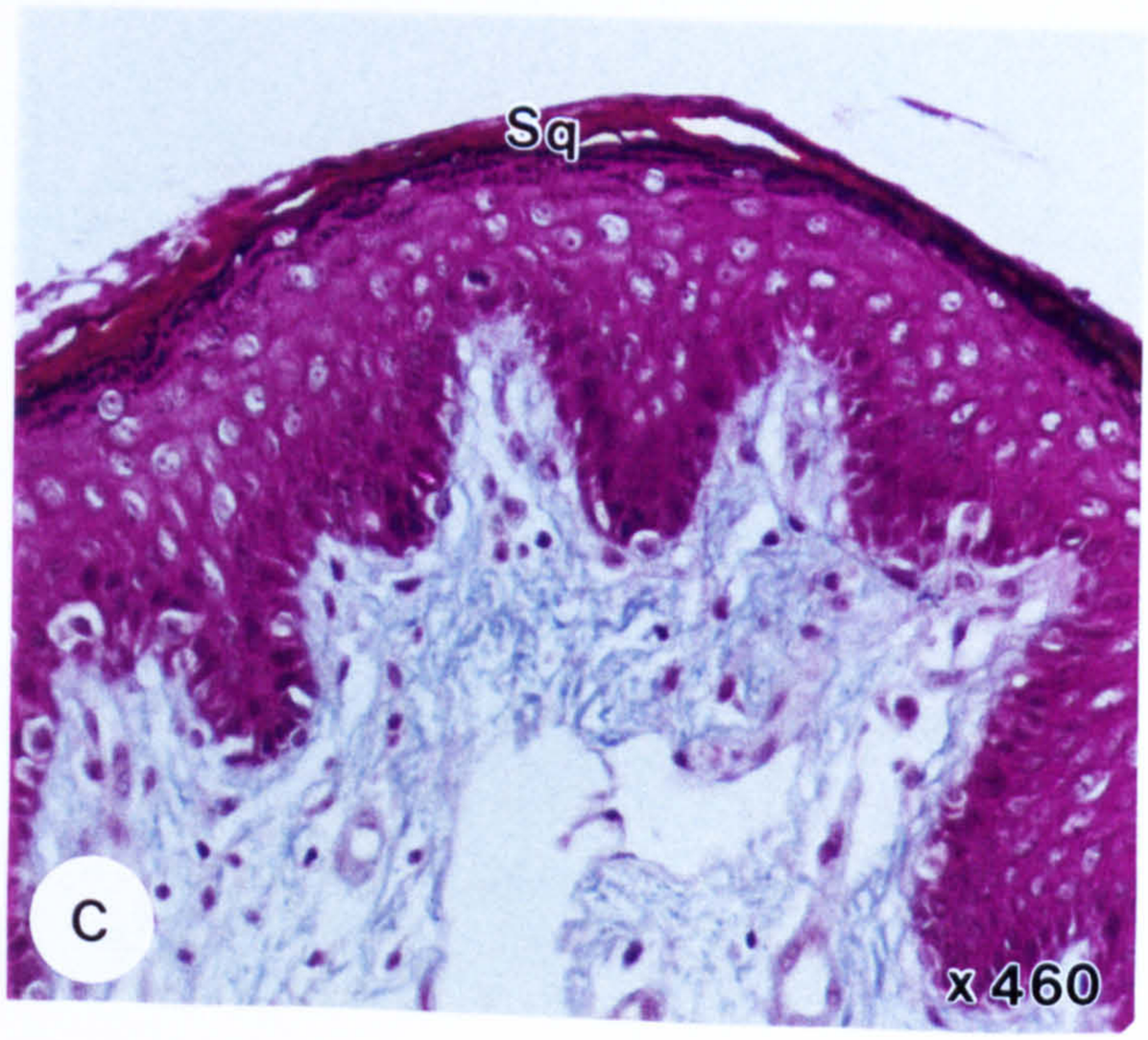
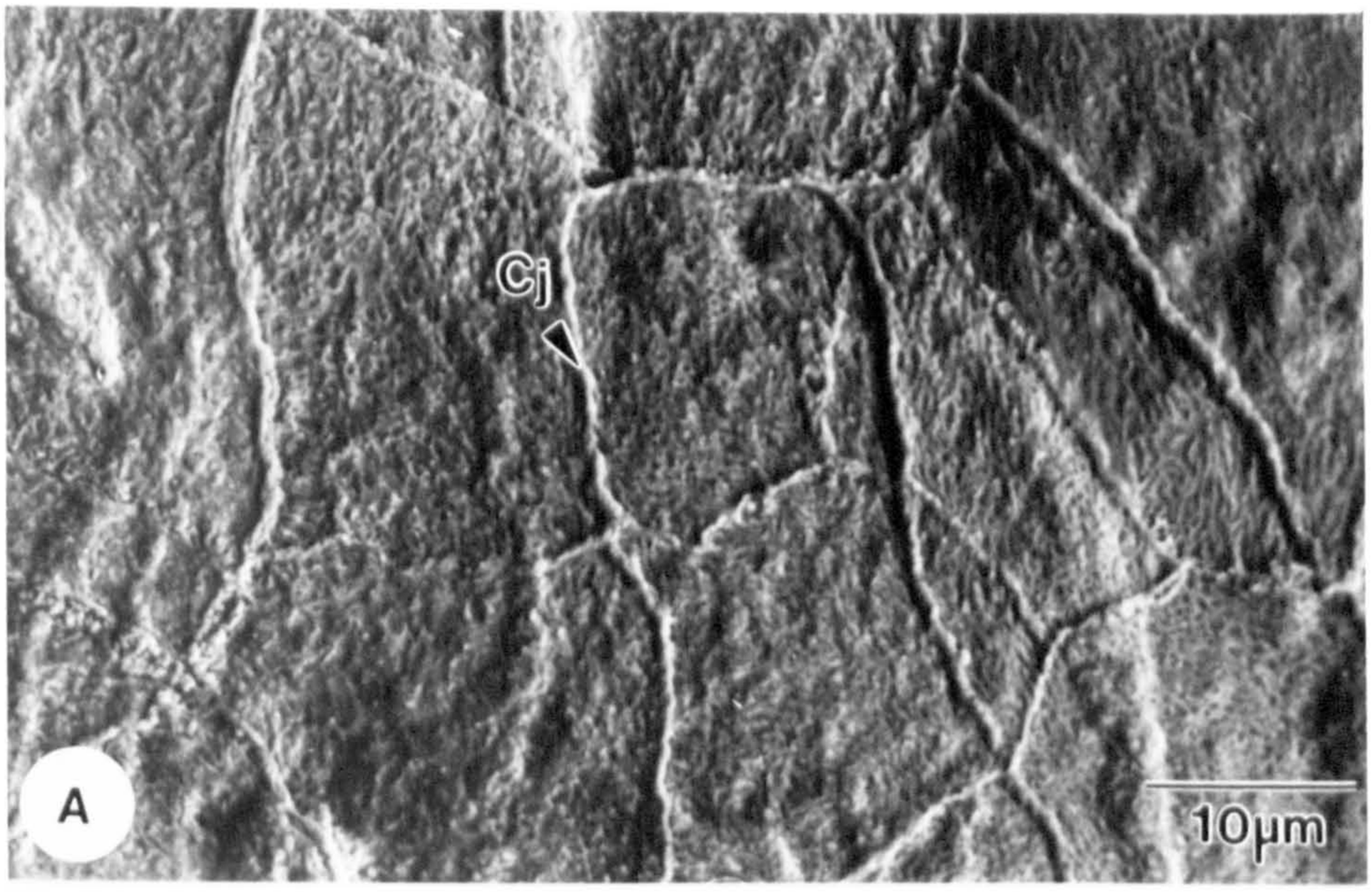
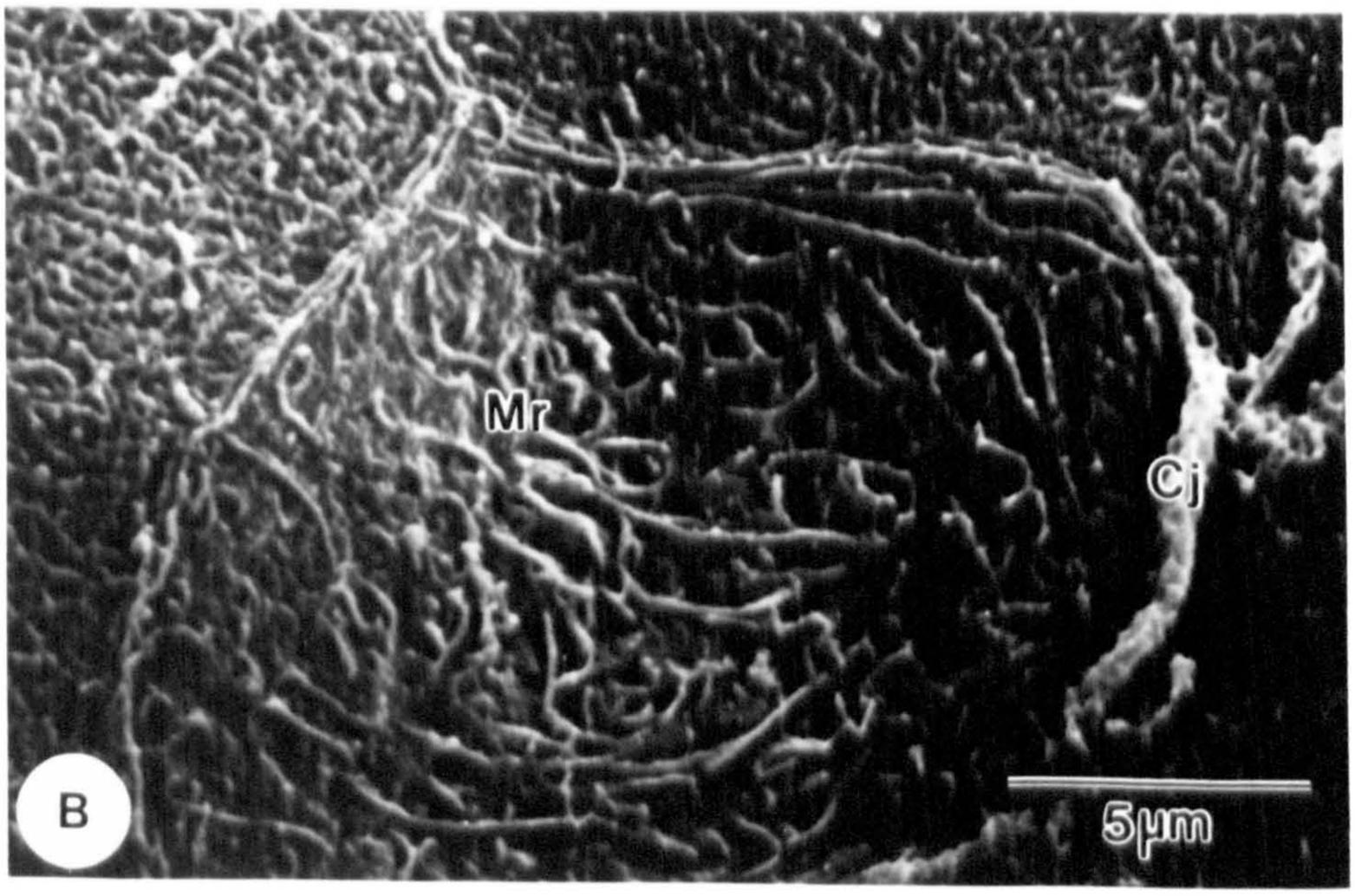

Sq
C
x460

Plate 17a Low power scanning electron micrograph illustrating the surface topography of vestibular epithelium affected by VVS. Surface cells are similar in appearance to normal epithelium at this magnification. Cell junctions are prominent and raised (Cj). These surface cells are polygonal in shape. Bar =10μm

Plate 17b At a higher magnification, microridges (Mr) on the surface cells of VVS samples appear to be shrunken. Microridges are straight in appearance and not raised as in control samples. Cytoplasmic junctions (Cj) are intact and are fine in appearance. Bar = 5μm

Plate 17c Illustrating a surface cell with shrunken microridges (Mr). Cell marked * has a different surface topography, being almost flat in appearance with no microridges. Bar = 5μm


Cj
A
10μm


Mr
Cj
B
5μm

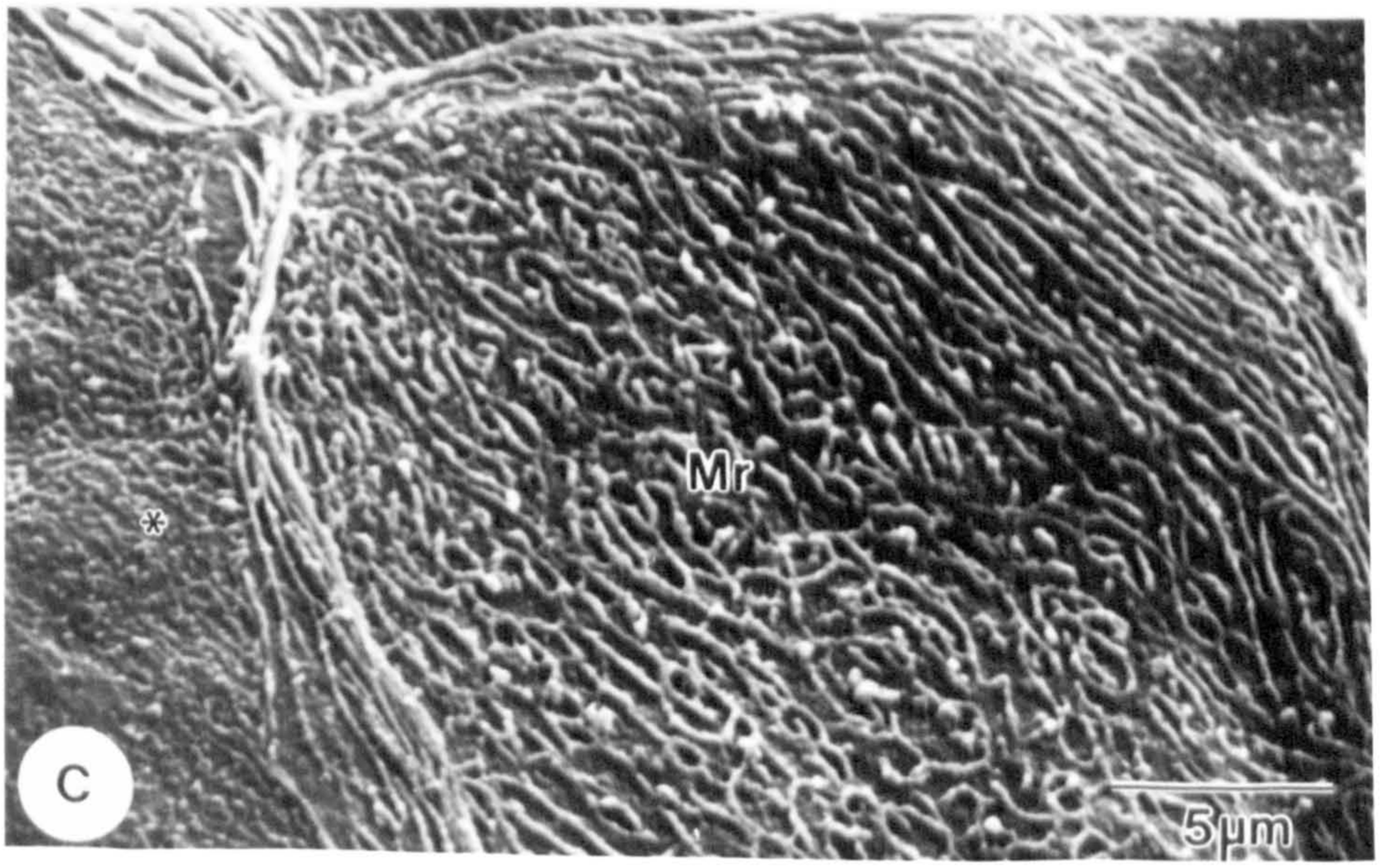
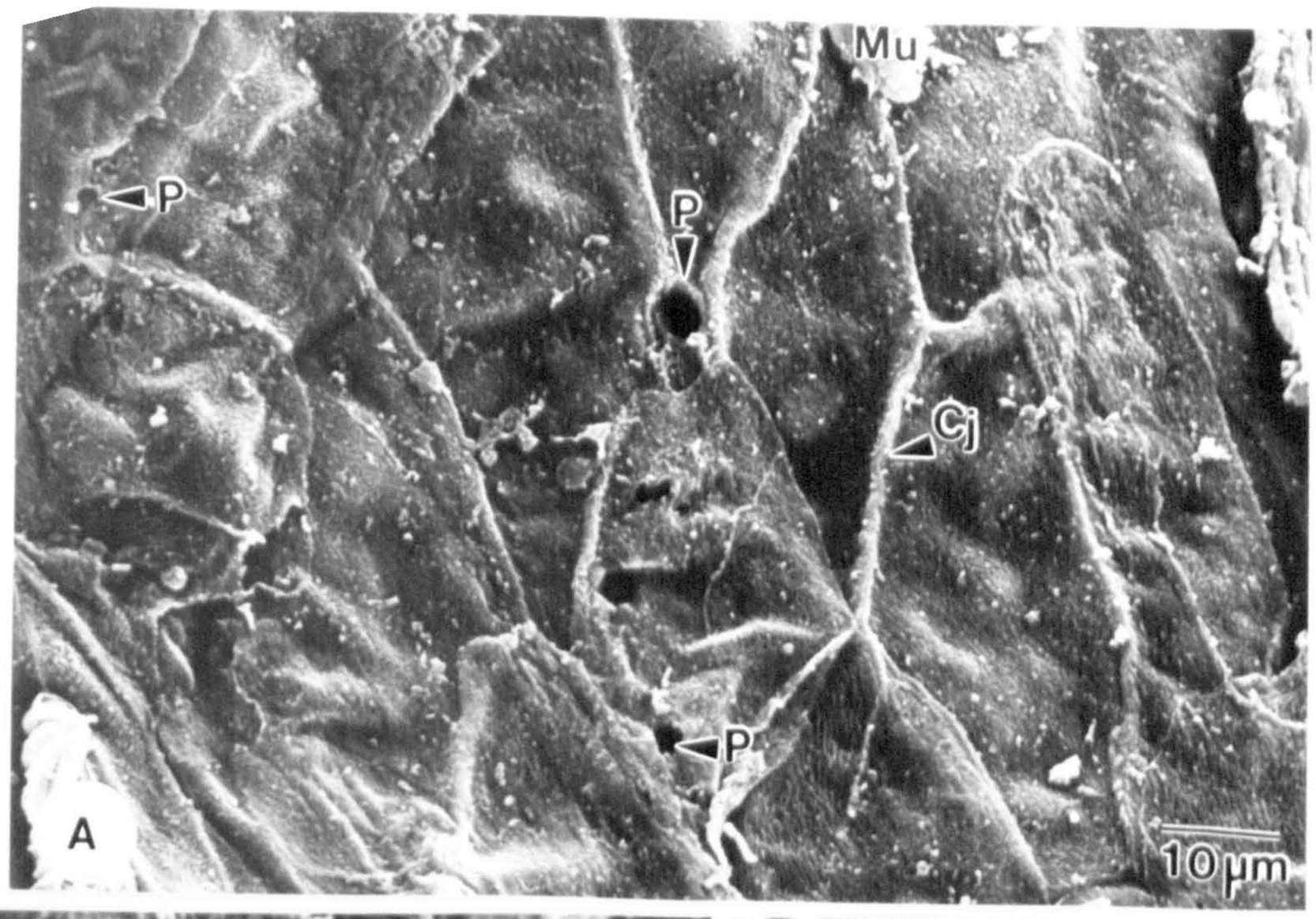

Mr
C
5μm

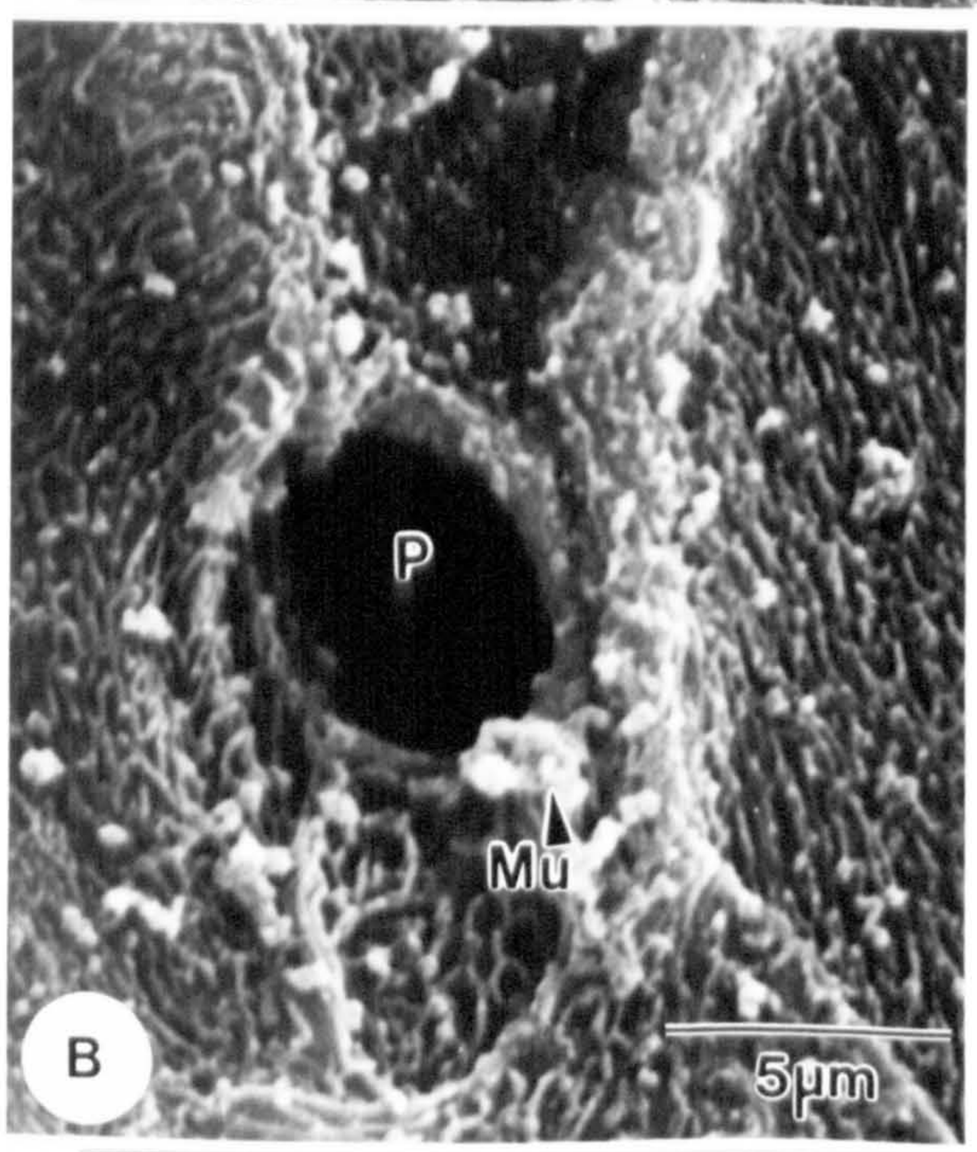
Plate 18a VVS samples were characterised by the presence of pores (P) in small groups. These openings were more common in VVS samples than in controls. Situated at the junctions (Cj) between surface cells, these pores may be minor vestibular glands. Mucus is common, often masking the surface ultrastructure (Mu). Bar = 10μm

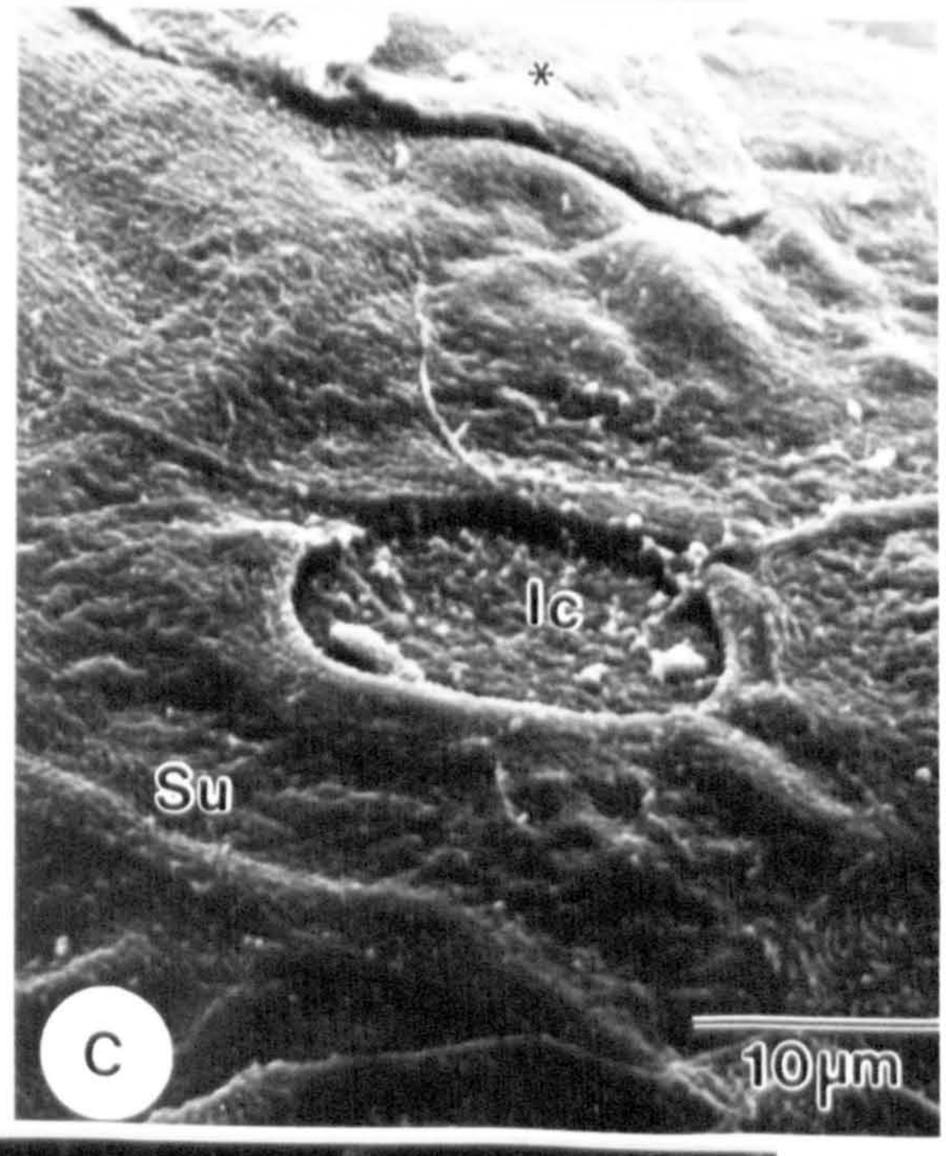
Plate 18b Higher power micrograph of a typical surface pore (P). Debris similar to mucus (Mu) is visible around the pore opening. Bar = 5μm

Plate 18c Surface cells (Su) of VVS affected epithelium. Illustrating an area where a surface cell appears to have been exfoliated. An intermediate cell (Ic) is visible, with more prominent surface microridges. Another desquamating cell is visible marked *. Bar = 10μm

Plate 18d Illustrating the depth of surface pores (P) seen between superficial cells (Su), and the microvillus appearance inside a typical pore (Mv). Mucus (Mu) is visible within the pore opening. Bar = 2μm


Mu
P
P
Cj
P
A
10µm


P
Mu
B
5µm


*
Ic
Su
C
10µm

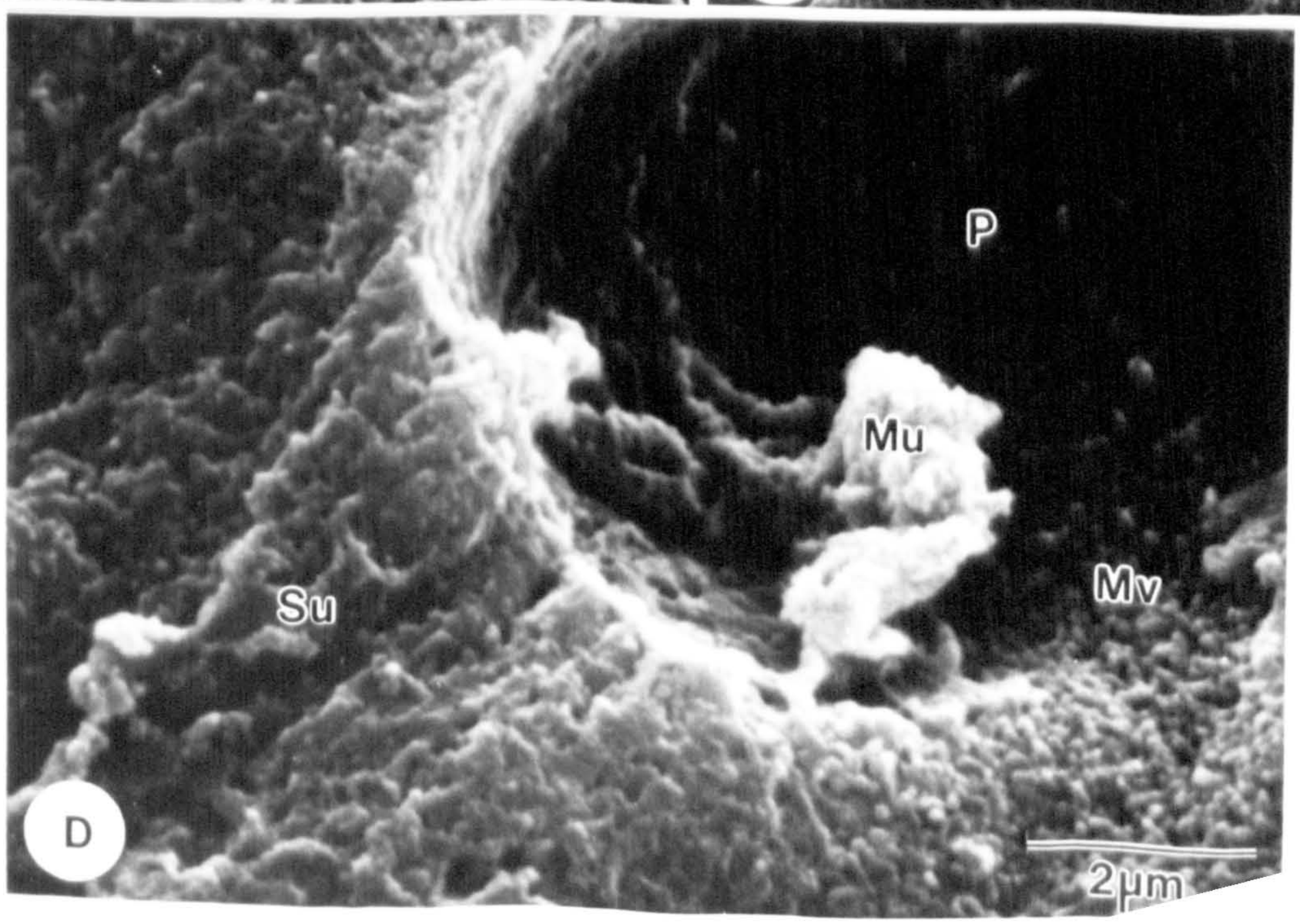

P
Mu
Mv
Su
D
2µm

Plate 19 Low power transmission electron micrograph of vestibular epithelium, illustrating the presence of dark staining cells (D) in VVS samples. The darkened cell appears shrunken, and the cytoplasm vacuolated (V). Several mitochondria (M) are visible, situated close to the basement membrane (Bm). Note the lobules of cytoplasm (Lb) and the presence of a small lymphocyte (L).The basal cells (Bc) surrounding the darkened cell also appear to have condensed or shrunken nuclei. In these cells the cytokeratin filaments (F) appear to be moderately staining, however in normal intermediate cells (Ic) the cytokeratin filaments were pale staining in appearance. x 8,850

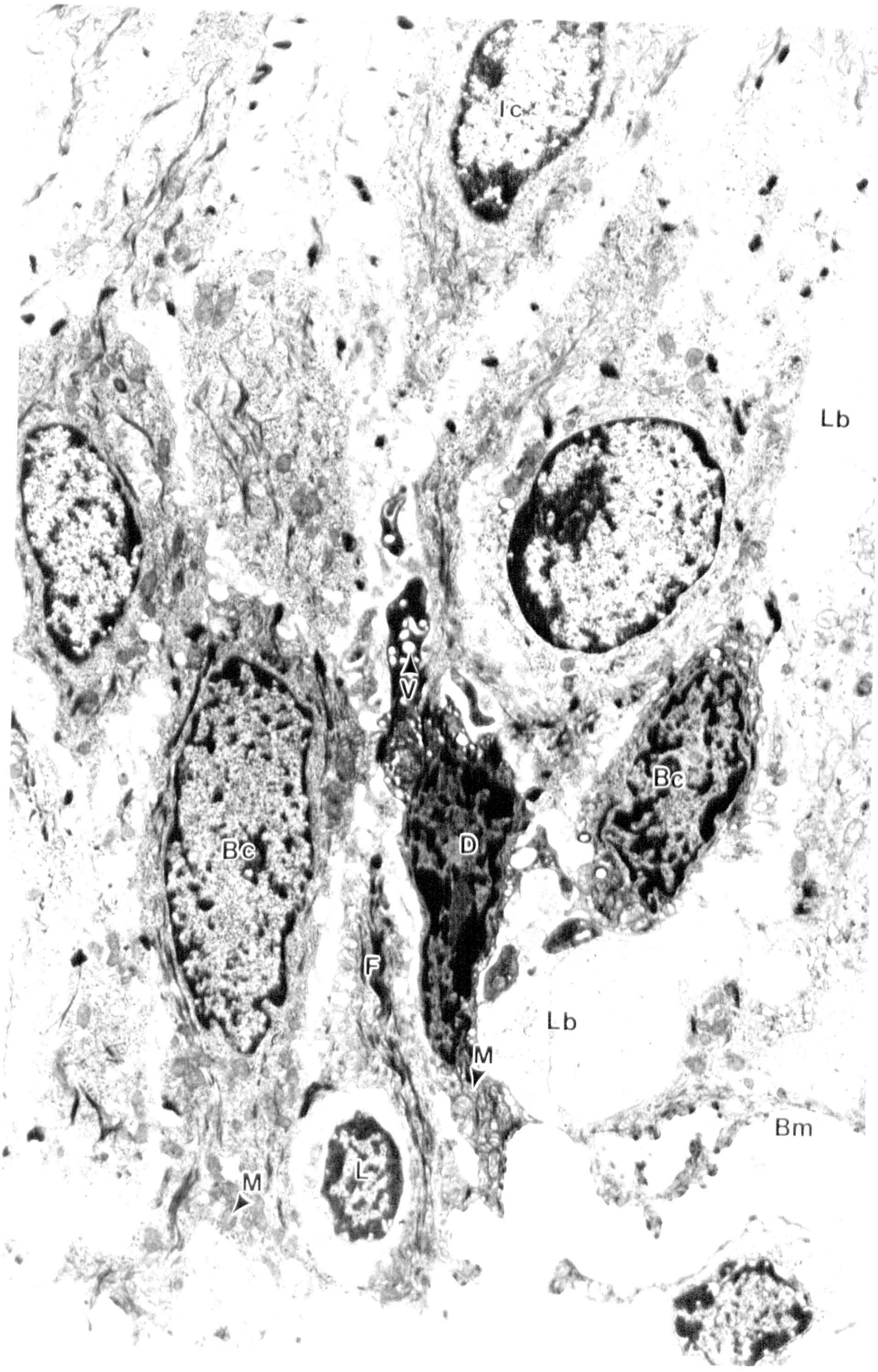

Ic
Lb
V
Bc
D
Bc
F
Lb
M
Bm
M
L

Plate 20a & b Basal cells of VVS affected epithelium. The basal cell marked (Bc) is apparently normal. However, the darkened cell profile (D), is shrunken in appearance with vacuolated cytoplasm. Desmosomal junction are maintained (De), mitochondria (M) appear to be slightly damaged, the cytoplasm is dark in appearance, and cytokeratin filaments are condensed. Cytoplasmic folds (Cp) which were common on adjacent basal cells are absent.

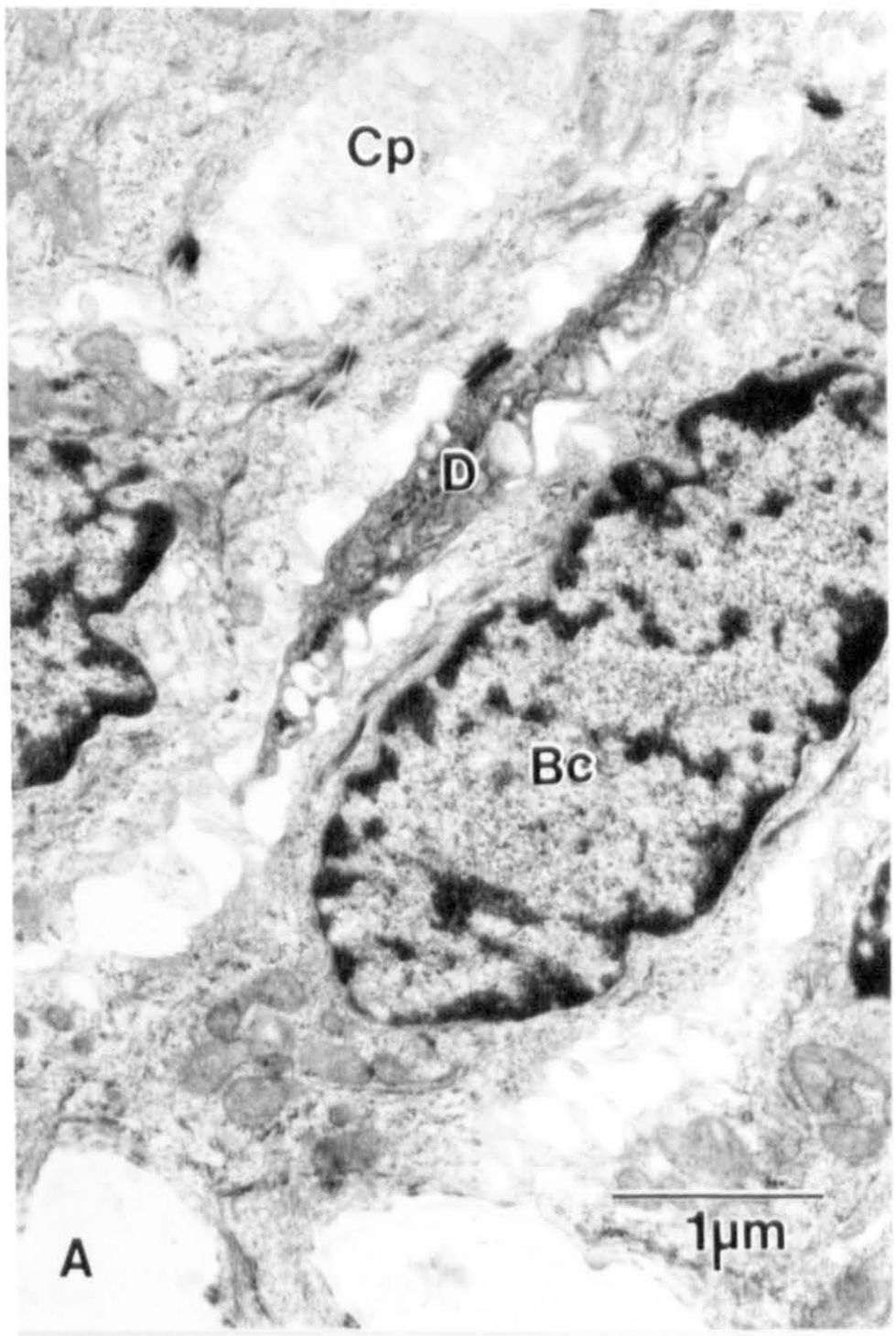
Plate 12a x 17,000

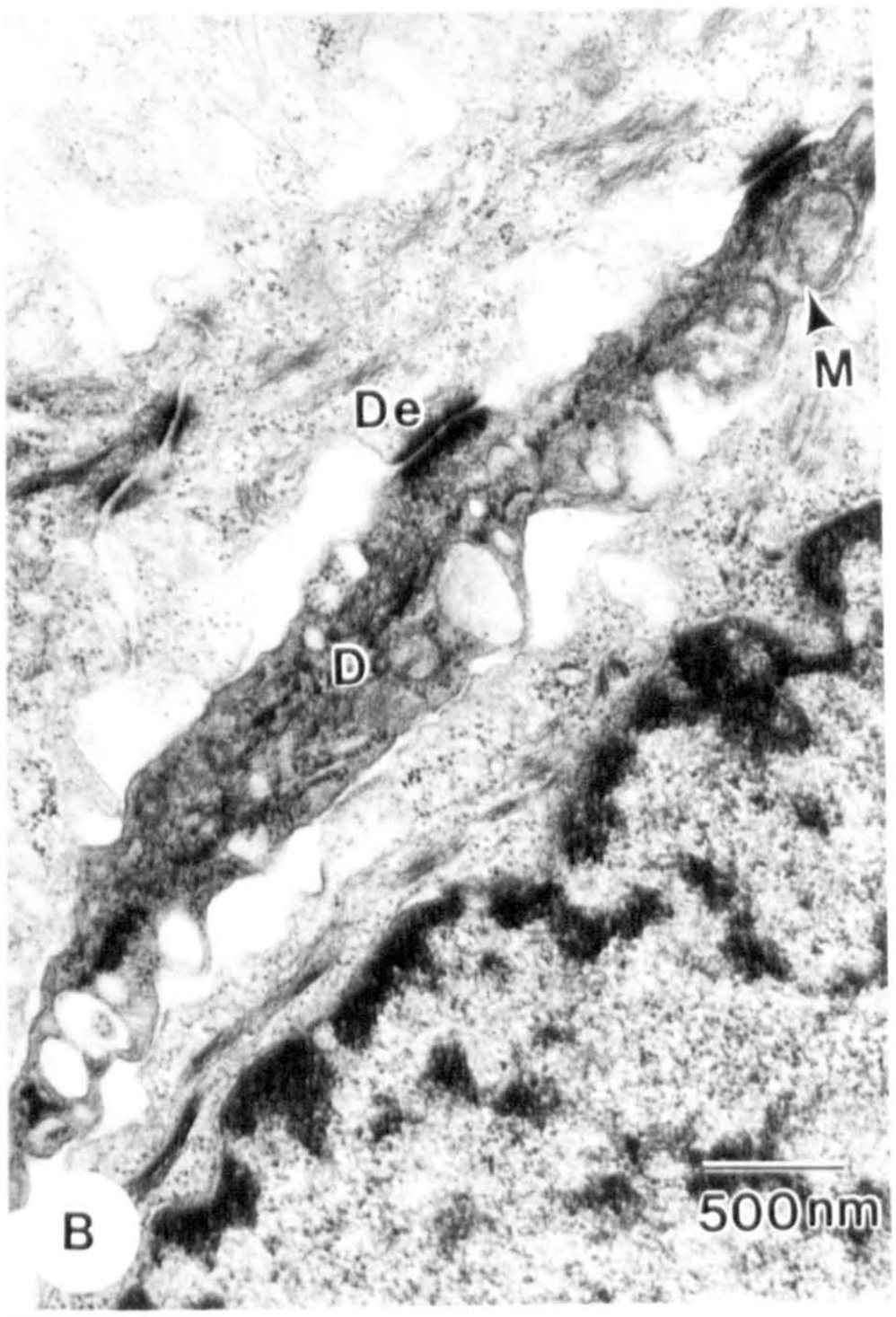
Plate 12b x 21,500

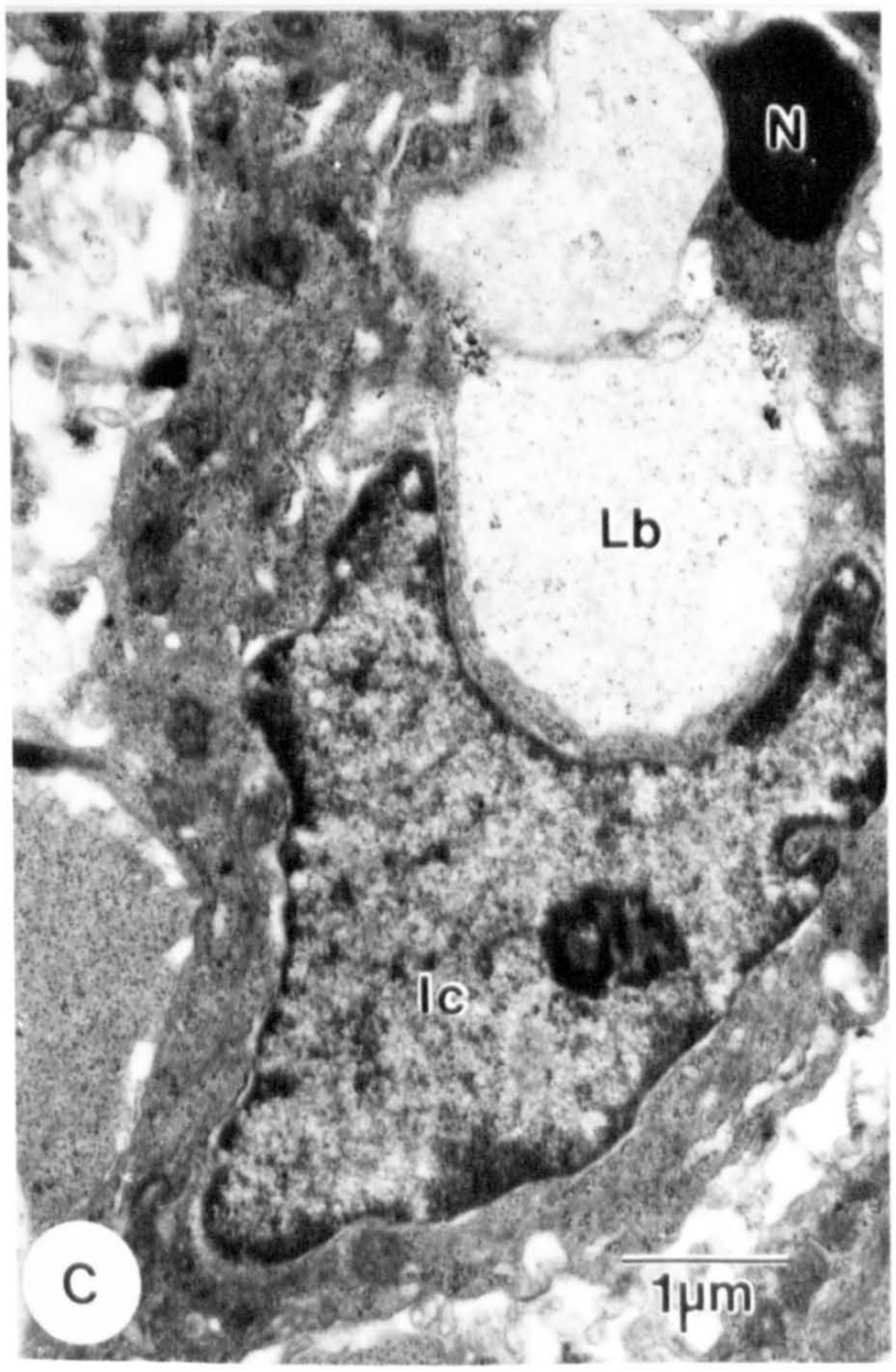
Plate 20c & d Intermediate cells (Ic) of vestibular epithelium. These cells appear to be engulfing lobules of cytoplasm found in intercellular spaces in VVS samples. Several membrane (Me) bound cytoplasmic lobules (Lb), of varying appearance are seen in this intermediate cell. One electron dense body (N), has a similar staining intensity as chromatin, however, a nuclear membrane was not detected. Another membrane bound cytoplasmic lobule is characterised by having vacuoles (V).

Plate 12c, x 22,500

Plate 12d x 11,700


Cp
D
Bc
1µm
A


De
M
D
500nm
B


N
Lb
Ic
1µm
C

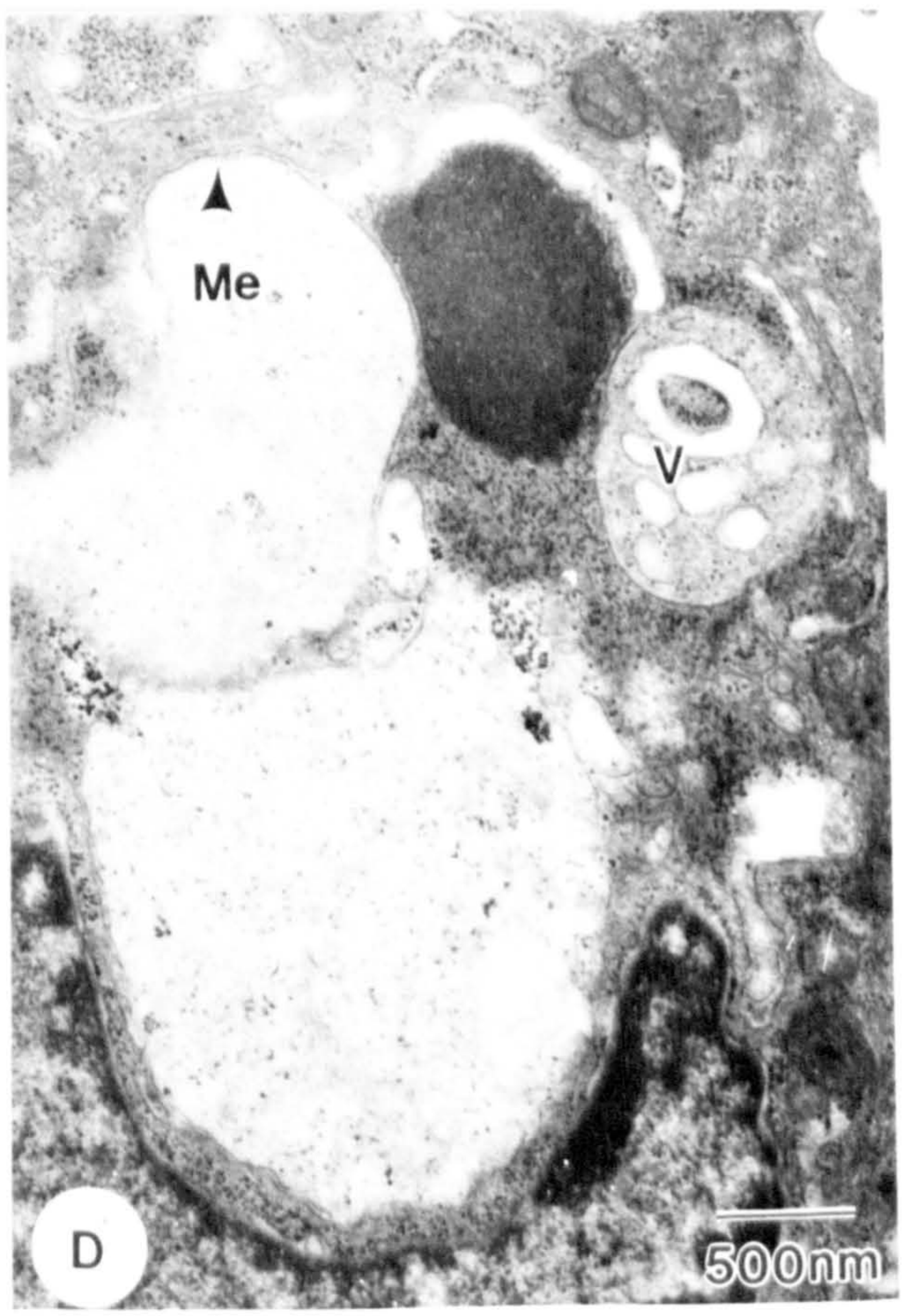
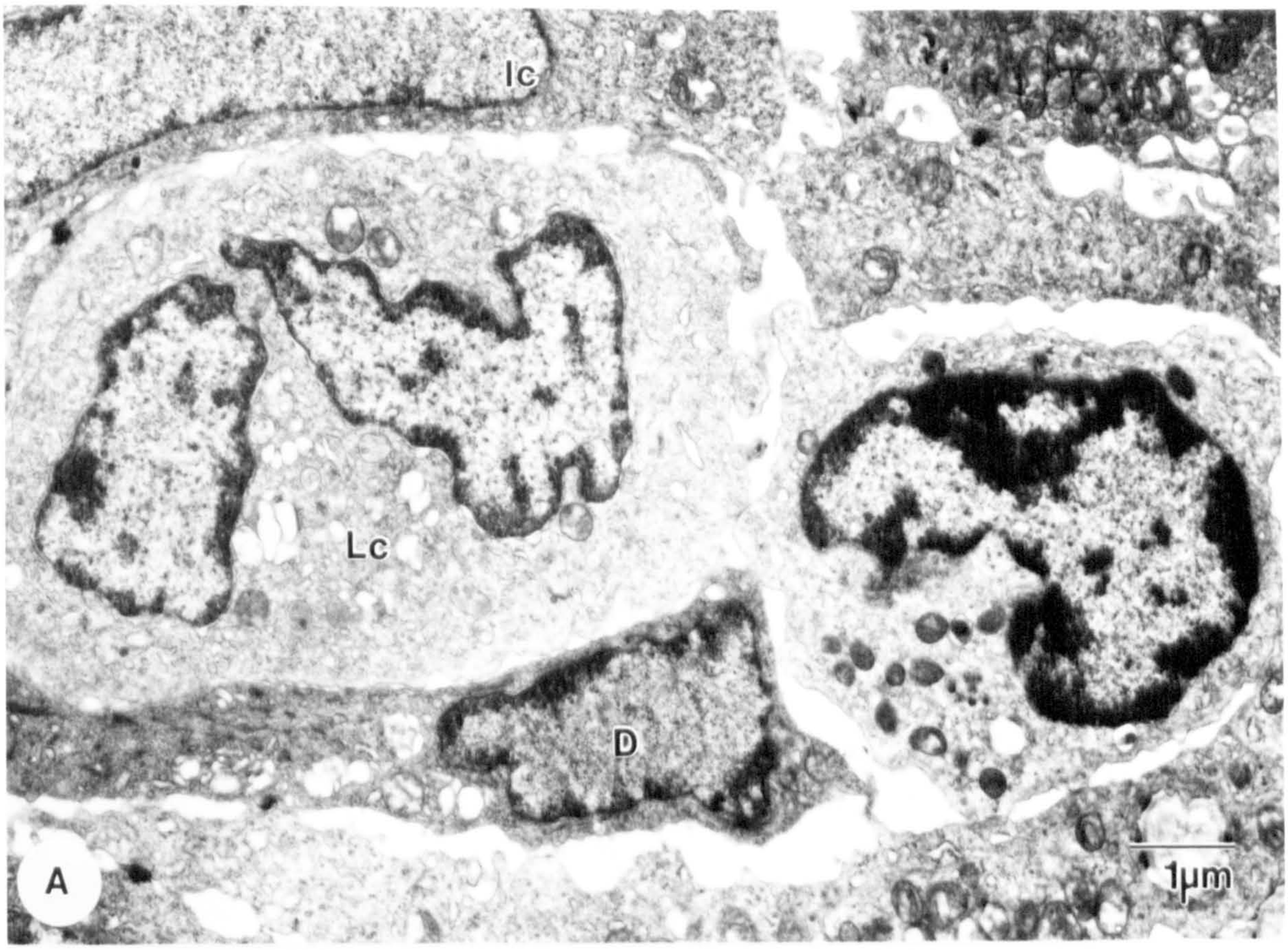

Me
V
500nm
D

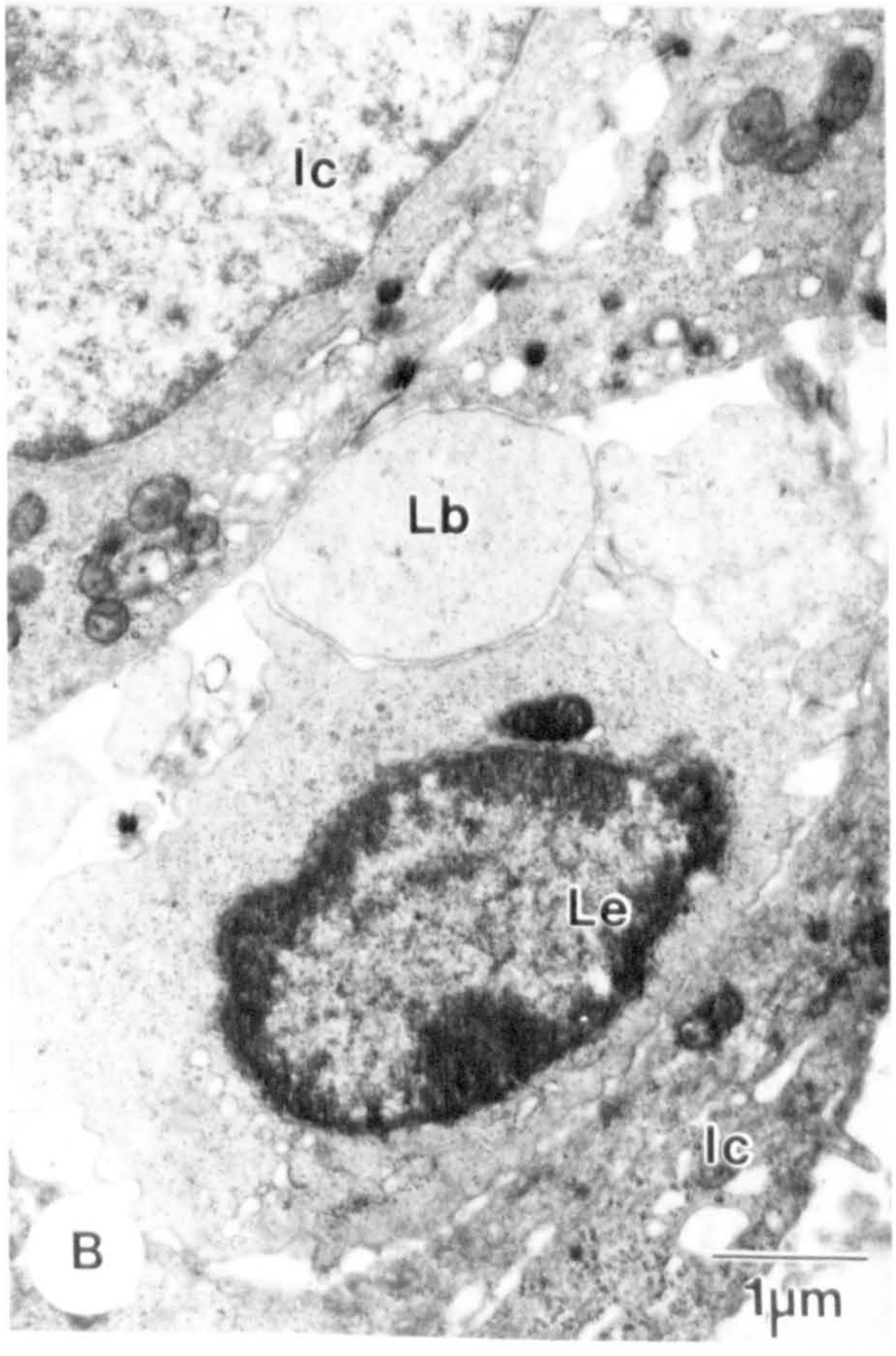
Plate 21a Leukocytes (Le) in the intermediate cell (Ic) layer of VVS affected vestibular epithelium. Leukocytes are surrounded by several intensely staining intermediate cells (D) which are slightly vacuolated. Leukocytes are seen to make close cytoplasmic contacts with the epidermal cells. One of the leukocytes appears to be a Langerhans cell (Lc), however, complete Birbeck granules, characteristic of Langerhans cells, were not identified. The other leukocyte has a slightly lobed nucleus and may be a NK cell, neutrophil or a large granular lymphocyte. x 11,000

Plate 21b & c Leukocytes (Le) in the lower (Plate 21b) superficial cells (Su) and upper intermediate (Plate 21c) cell layers (Ic). Note the presence of cytoplasm lobules (Lb) close to the leukocytes and near a lymphocyte (L). Leukocytes have cytoplasmic projections suggesting that they are activated. The leukocyte in Plate 21b. appears to be phagocytosing a membrane bound lobule of cytoplasm (Lb). The two leukocytes in Plate 21c have dark granules.

Plate 21b x 9,200

Plate 21c x 12,000.


lc
Lc
D
A
1μm


lc
Lb
Le
lc
B
1μm

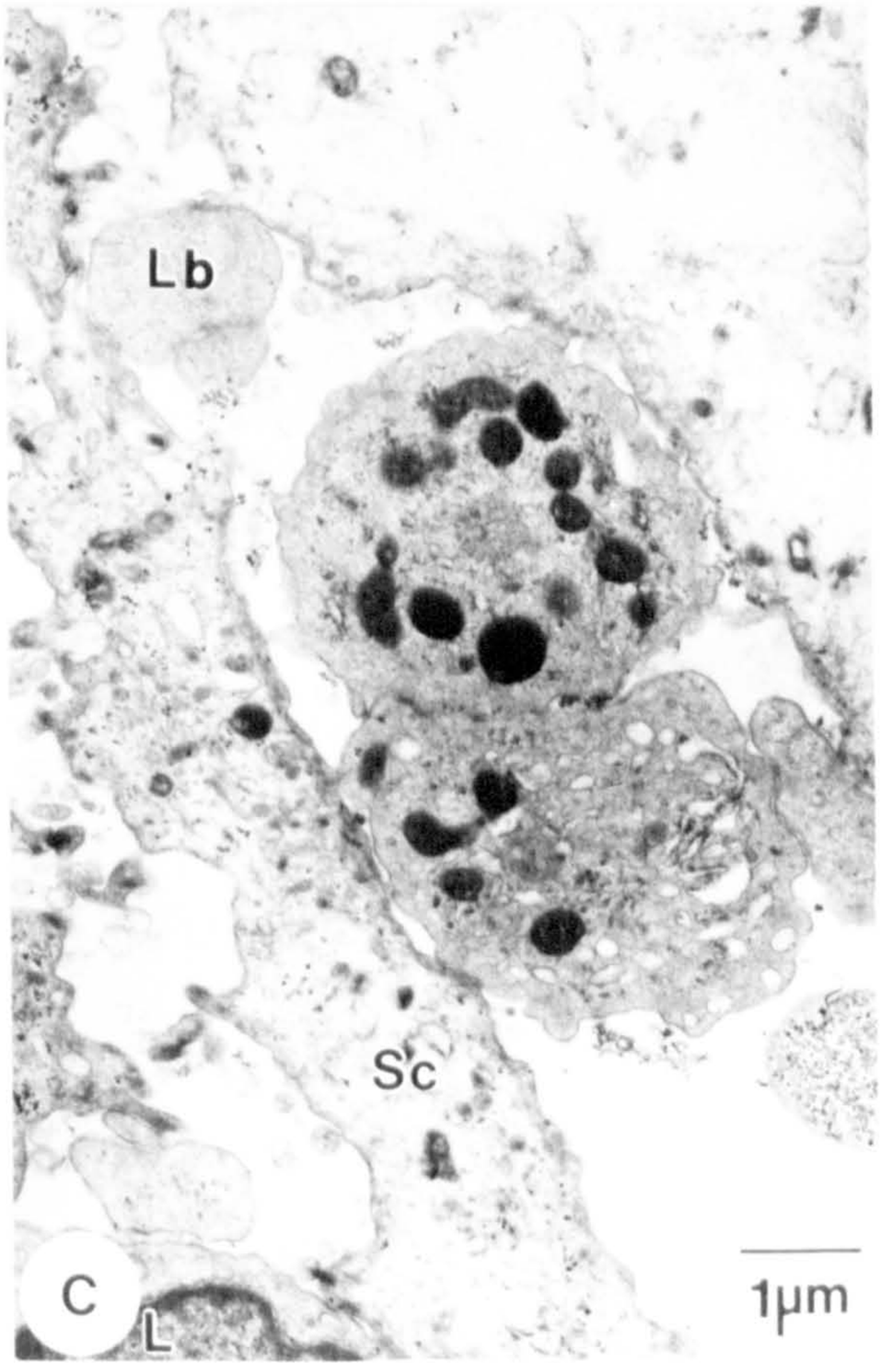
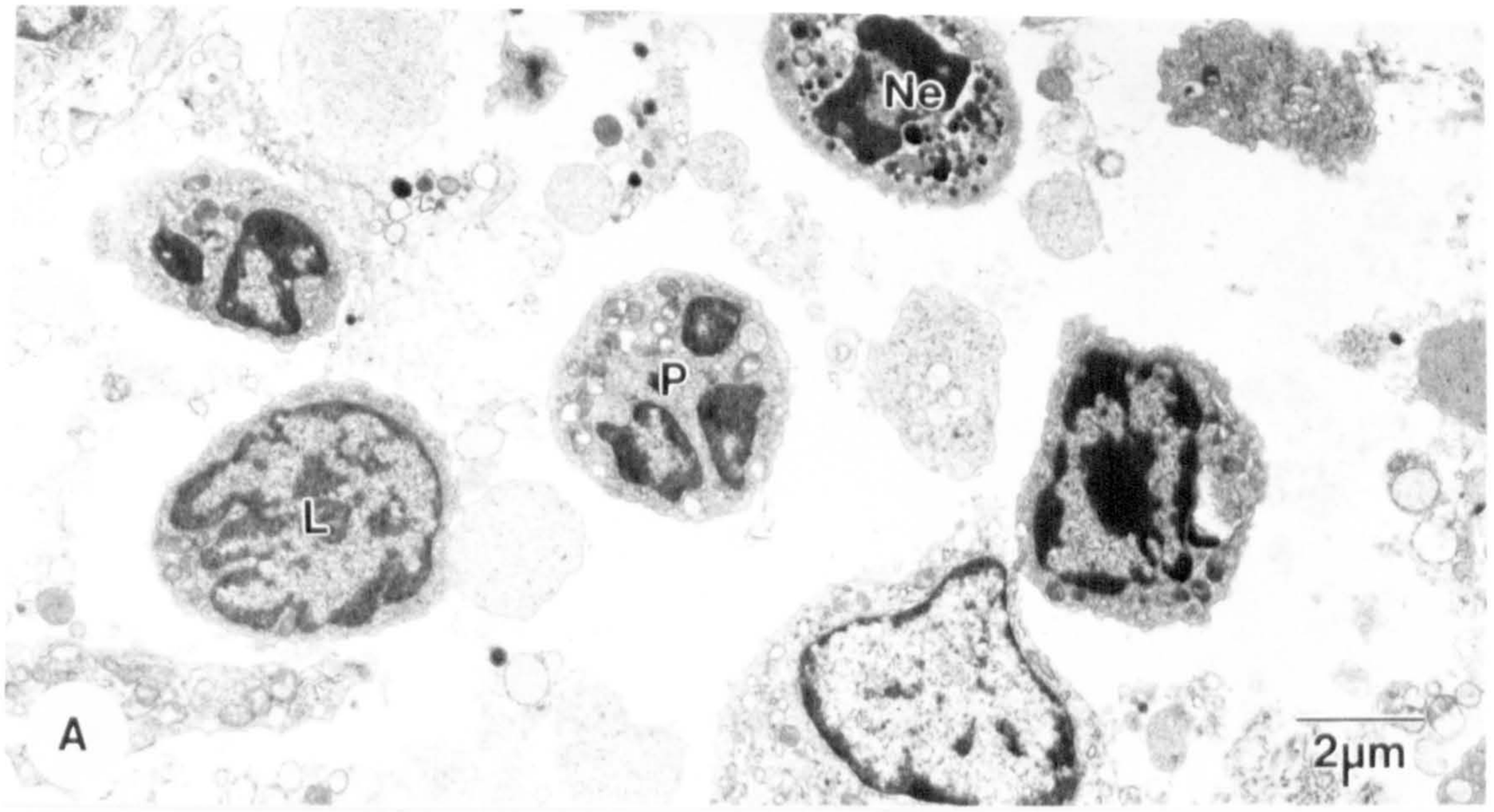

Lb
Sc
C
L
1μm

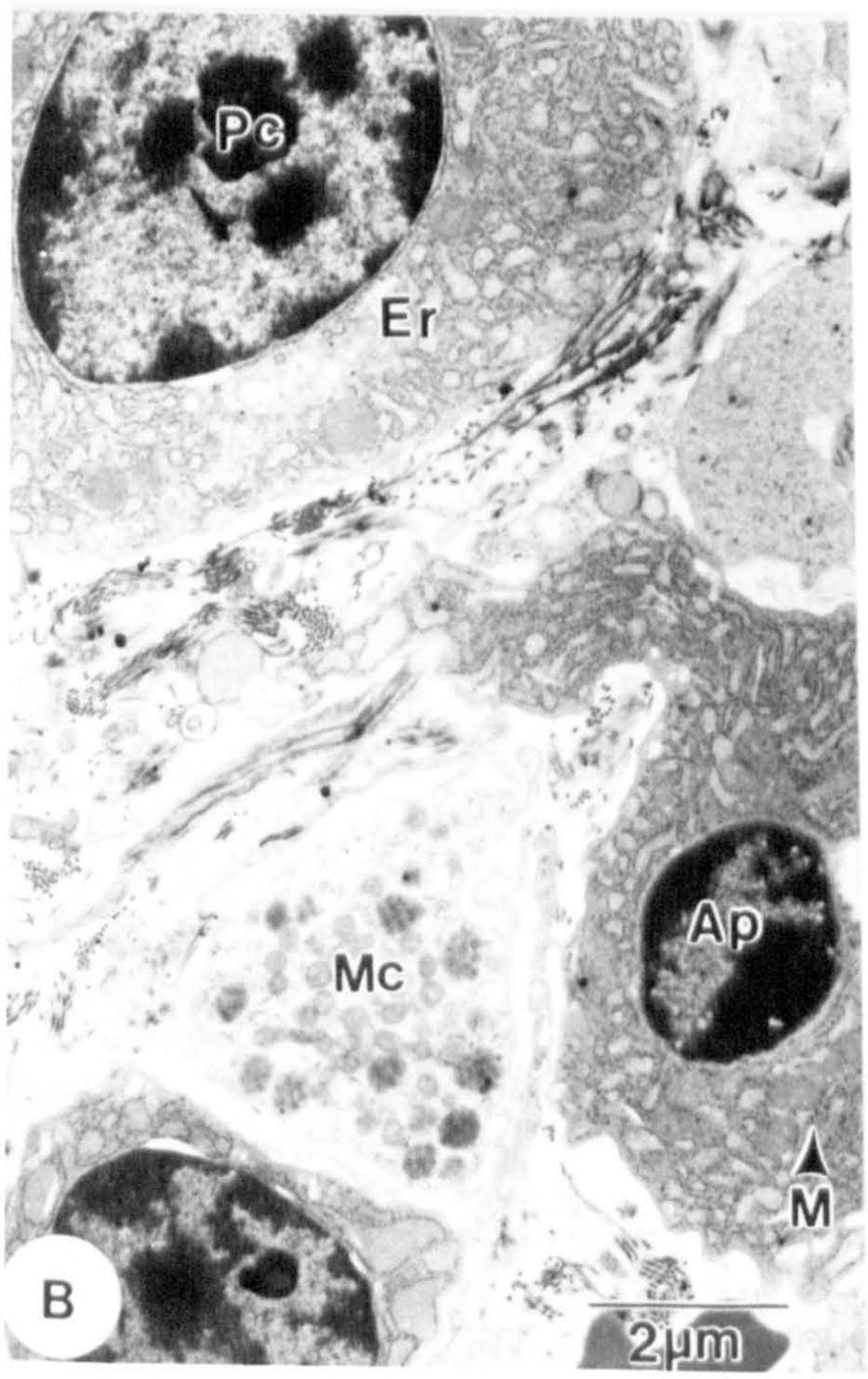
Plate 22a Leukocytes in the dermis of a VVS sample, close to the basement membrane. Illustrating an inflammatory infiltrate characterised by a possible neutrophil (Ne), polymorphonuclear cells (Pmn), and lymphocytes (L). x 5,700

Plate 22b & c Illustrating a mature plasma cell (Pc) and a mast cell (Mc) close to a capillary (Ca) (Plate 22b & c). Plasma cells characteristically have a clock face clumping of chromatin, however, two plasma cells in 22b and 22c appear to be apoptotic (Ap). The chromatin of these two plasma cells is marginated characteristic of cells undergoing apoptosis. All plasma cells had extensive endoplasmic reticulum (Er) and numerous mitochondria (M).

Plate 21a x 7,500

Plate 21c x 6,500


Ne
P
L
A
2μm


Pc
Er
Mc
Ap
M
B
2μm

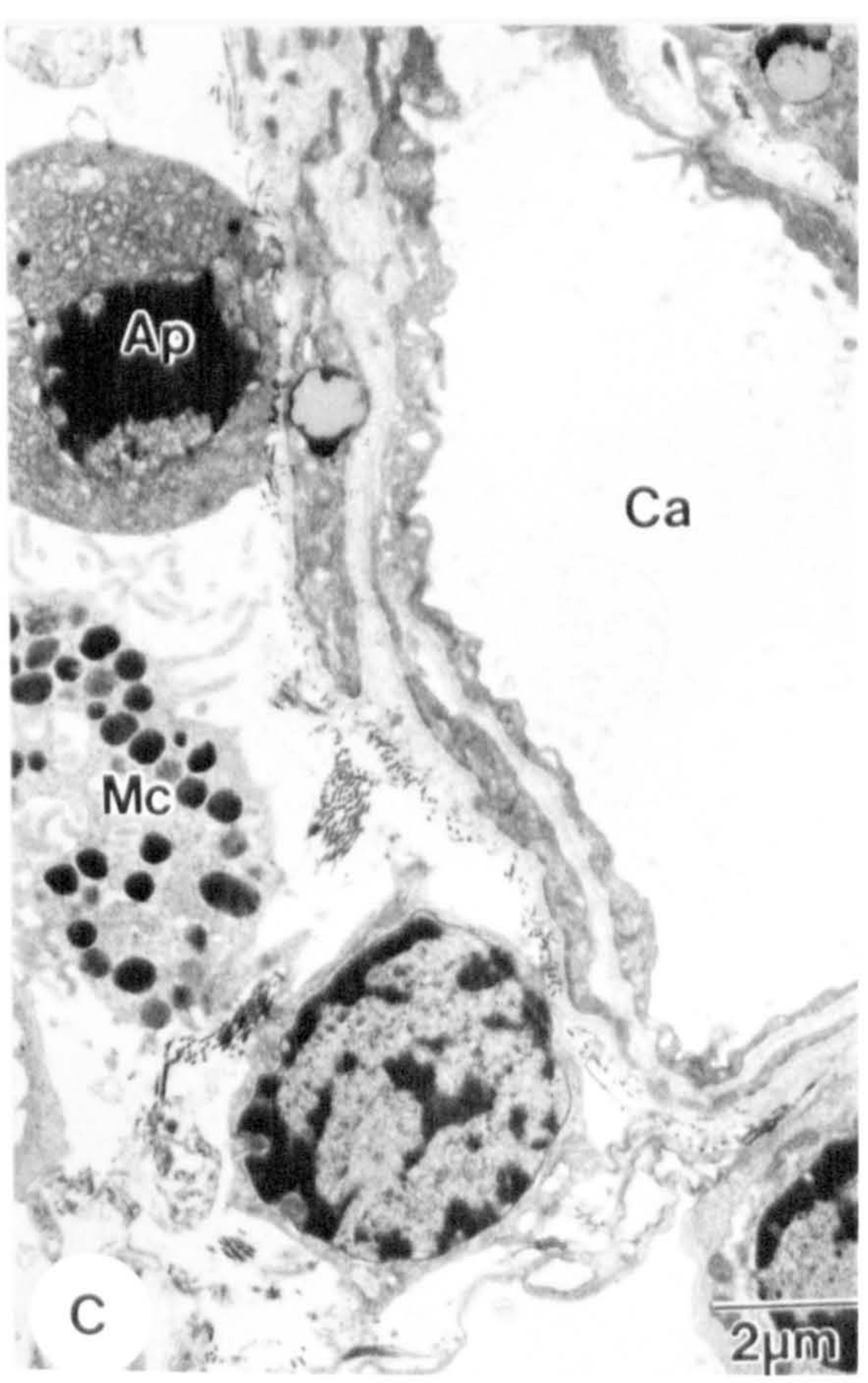
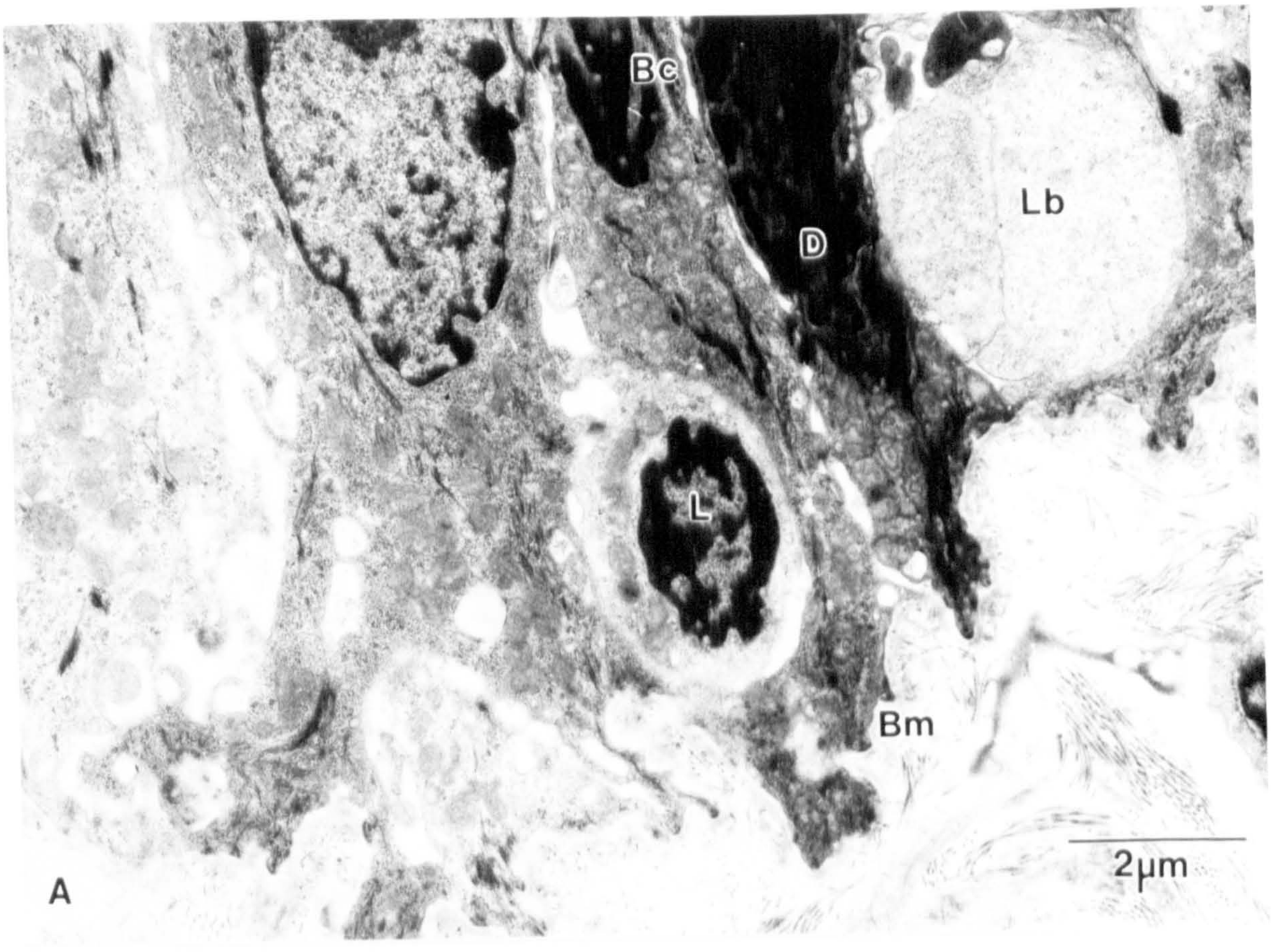

Ap
Ca
Mc
C
2μm

Plate 23a & b Illustrating the presence of membrane bound lobules (Lb), a lymphocyte (L) and a darkened cell (D) in the close proximity of basal cells (Bc). Lobules of cytoplasm (Lb) are making close contacts with the darkened basal cell (D). The darkened cell (D) has very little cytoplasm and a condensed, intensely staining nucleus. Several mitochondria (M) are visible close to the basement membrane (Bm).

Plate 23a x 13,500

Plate 23b x 9,850


Bc
D
Lb
L
Bm
2μm
A

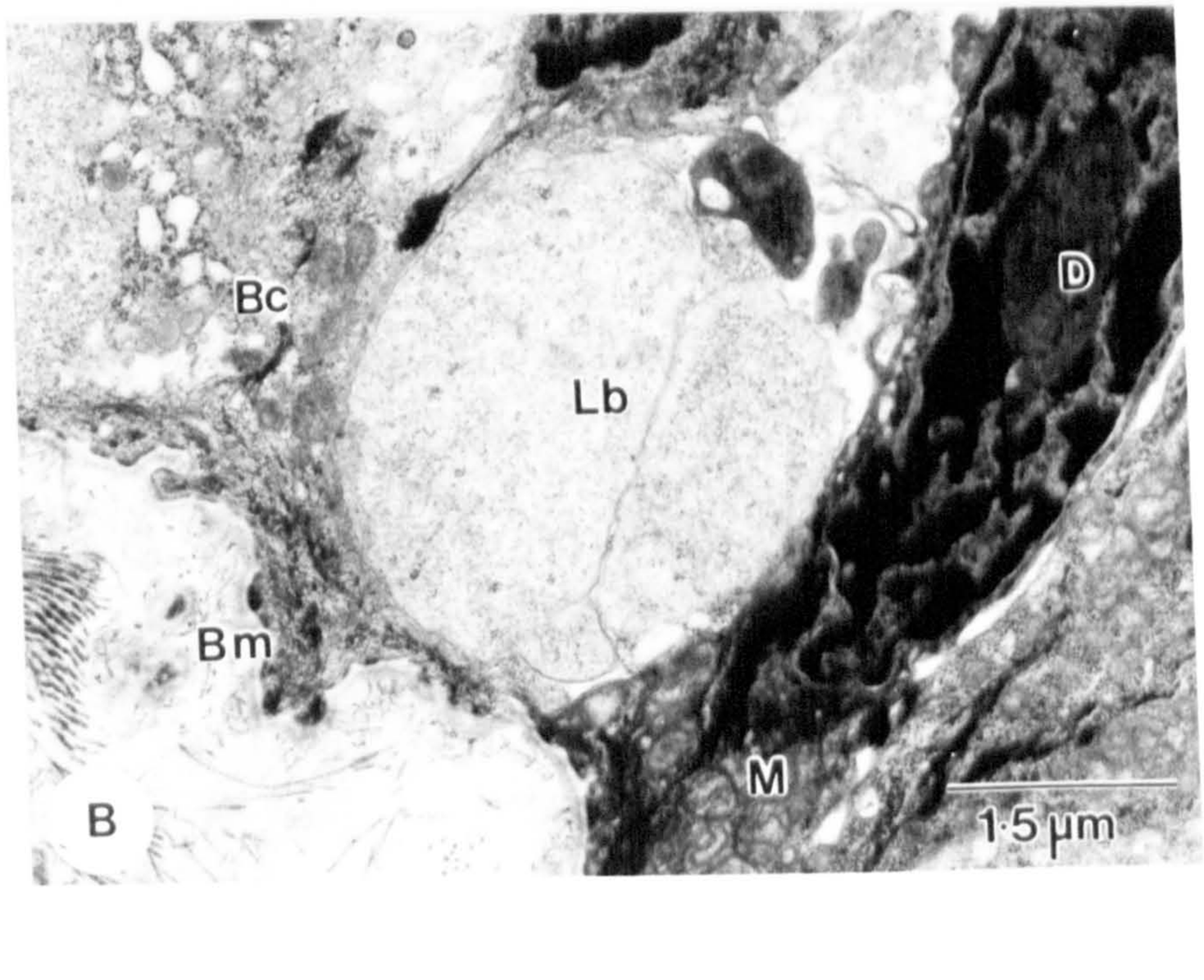

Bc
Lb
D
Bm
M
B
1·5 μm

Plate 24a-c Illustrating cytoplasmic lobules which were frequently seen interposed between epidermal cells of all cell layers in VVS samples. Lobules (Lb) are seen to make close cytoplasmic contacts with neighbouring epidermal cells (Ic), which in some cases appear intensely staining. Commonly membrane bound lobules are seen to be phagocytosed by epidermal cells (Plate 24c) or in the process of being engulfed (Plate 24a). Note the presence of a leukocyte process (Le) close to cytoplasmic lobules. No nucleus is evident on serial sectioning. Lobules of cytoplasm were usually characterised by the presence of ribosomes (R) and fine cytoplasmic filaments (F). Occasionally vesicles (Ve) were evident in these cytoplasmic lobules.

Plate 24a x 15,000

Plate 24b x 45,000

Plate 24c x 13,500

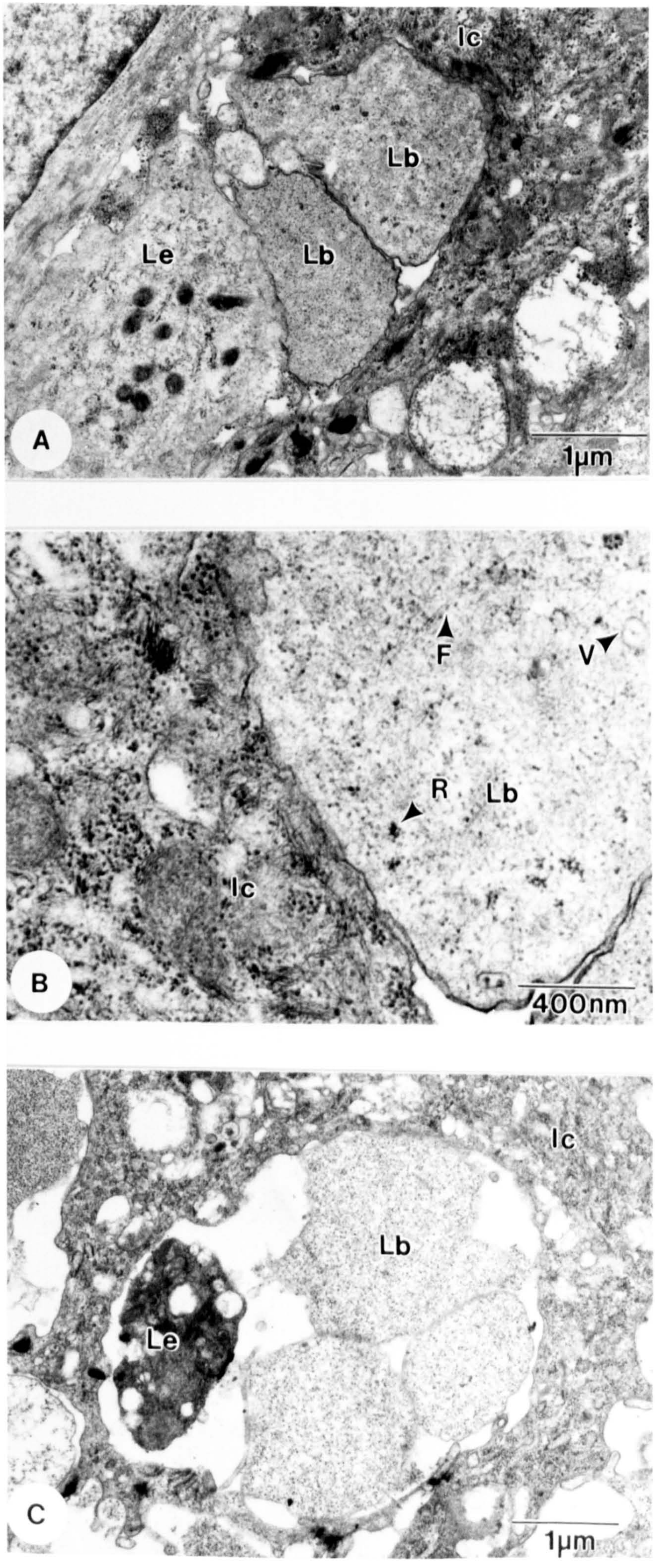

Ic
Lb
Le
Lb
A
1µm
F
V
R
Lb
Ic
B
400nm
Ic
Lb
Le
C
1µm

Plate 25 Lower intermediate cells of VVS affected vestibular epithelium. Intermediate cells (Ic) are visible engulfing lobules of cytoplasm (Lb). The nuclei of several intermediate cells appears arc shaped. The lobules of cytoplasm appear to be broken down within the epidermal cells. This area of the epithelium appears disorganised, and many of the epithelial cells are electron dense and some have developed vacuoles (V). Mitochondria (M) are intact, as are desmosomal junctions (De). x 12,500


De
V
M
Lb
Lb
Ic
Lb
1μm

Plate 26a Superficial cell (Su) of VVS affected epithelium. Cytoplasm of this cell is deteriorating as the cell is exfoliated. The nucleus is intact but slightly flattened in appearance. Vesicles (Ve) are visible along the cytoplasmic membrane of the surface cell, commonly fusing with the membrane. Short desmosomal junctions are evident (De). x 9,000

Plate 26b Superficial cells (Su) are characterised by microvillous-like projections (Mv) of the cytoplasmic membrane. These projections vary in shape, length and distribution along the surface of these cells. The plasma membrane of the surface cells is slightly thickened but not keratinised. There are no keratohyalin granules present. Large deposits of glycogen (G) are common in these cells. x 30,000

Plate 26c Superficial cells indicating the presence of large aggregates of glycogen (G), and vesicles (Ve) in close contact with the cytoplasmic membrane. Folding of the cytoplasmic (Cf) membrane is not as pronounced when compared with the lower cell layers. Desmosomes are short (De). x 7,000

Plate 26d High power micrograph of vesicles (Ve) commonly seen in the surface cells of VVS affected epithelium. This cell has retained its nucleus (Nu). Vesicles (Ve) were generally electron lucent, but occasionally vesicles with electron dense cores were evident, which were similar in appearance to membrane coating granules found in keratinised skin. x 25,000

CHAPTER SIX

The Effect of Ketoconazole on the Ultrastructural Characteristics of VVS

6.0 Abstract

Ketoconazole is a topical antifungal preparation, which had been anticipated to be of use in the treatment of VVS, due to the mild inhibitory effects of this drug on the production of leukotriene B_4.The etiology of VVS remains elusive, however, pathologically VVS presents as a chronic inflammatory condition affecting the vestibule of the vulva. Investigation of the inflammatory response has revealed very little information pertaining to the cause of the syndrome (Pyka *et al.*, 1988, Prayson *et al.*, 1995). Post-treatment biopsies, revealed an ultrastructural appearance similar to that seen in pre-treatment samples. Apoptotic-like cells were present in similar numbers when compared with the pre-treatment samples, however, these dark cells were more heavily vacuolised and shrunken in appearance. Despite the number of leukocytes being similar in the majority of cases (see Chapter 7), the nature of the inflammatory cells present appeared different. Lobed nuclei, characteristic of macrophage-like cells were common in post-treatment samples, particularly in the dermis. As in the pre-treatment samples, plasma cells and lymphocytes were also present. Lobules of cytoplasm were numerous, and as in pre-treatment samples, these bodies were found contained within macrophages, and also within neighbouring epidermal cells. A majority of the post-treatment samples showed increased numbers of cytoplasmic lobules in the dermis, often close to capillaries. Despite a documented decrease in vulvar sensitivity after treatment with ketoconazole (Chapter 3), this was not accompanied by significant ultrastructural changes. In conclusion, ketoconazole may be manipulating the inflammatory response by reducing the production of LTB_4; however, the cause of the inflammation is probably not being affected. Therefore, when treatment with ketoconazole is discontinued, increased sensitivity of the vestibule often recurs.

6.1 Introduction

Treatment of patients with inexplicable vulvar pain is particularly difficult. Many clinicians have attempted to treat the vulvar pain associated with VVS, but the majority have had little success. Patients with VVS are particularly complex to treat, as there is often no obvious etiological agent present. Pathological clues to the cause of the syndrome are scarce, and patients are often distressed, and commonly difficult to examine. Treatment regimes for VVS have included: calcium citrate (Solomons *et al.*, 1991); carbon dioxide laser treatment (Davis, 1989); alcohol injection; topical steroids (Friedrich, 1988); pelvic floor assessment and treatment (Glazer *et al.*, 1995); and surgical removal of the vestibule (Woodruff & Parmley, 1983; Bornstein *et al.*, 1989, 1995). The pathology of the condition is not distinctive , however, there have been repeated reports of koilocytic cells in the superficial cell layer of the vestibular epithelium, which is considered indicative of HPV. The association of HPV and VVS, has resulted in a large number of investigations utilising the anti-viral properties of interferon (see Chapter 2). Interferon is currently the most widely used treatment regime for VVS, however, the results pertaining from these investigations are not wholly conclusive (Wilkinson *et al.*, 1993; Bergeron *et al.*, 1994). More recently, studies have indicated that the methodology used for detecting HPV in tissue samples, has influenced the number of 'positive' cases obtained. The use of basic histological observation for the detection of HPV has been heavily scrutinised, and this means of detecting HPV has frequently been found to be inaccurate (Bergeron *et al.*, 1994). Generally, it is accepted that interferon therapy is successful in a small number of carefully selected VVS cases, where HPV has been equivocally shown to be present using PCR.

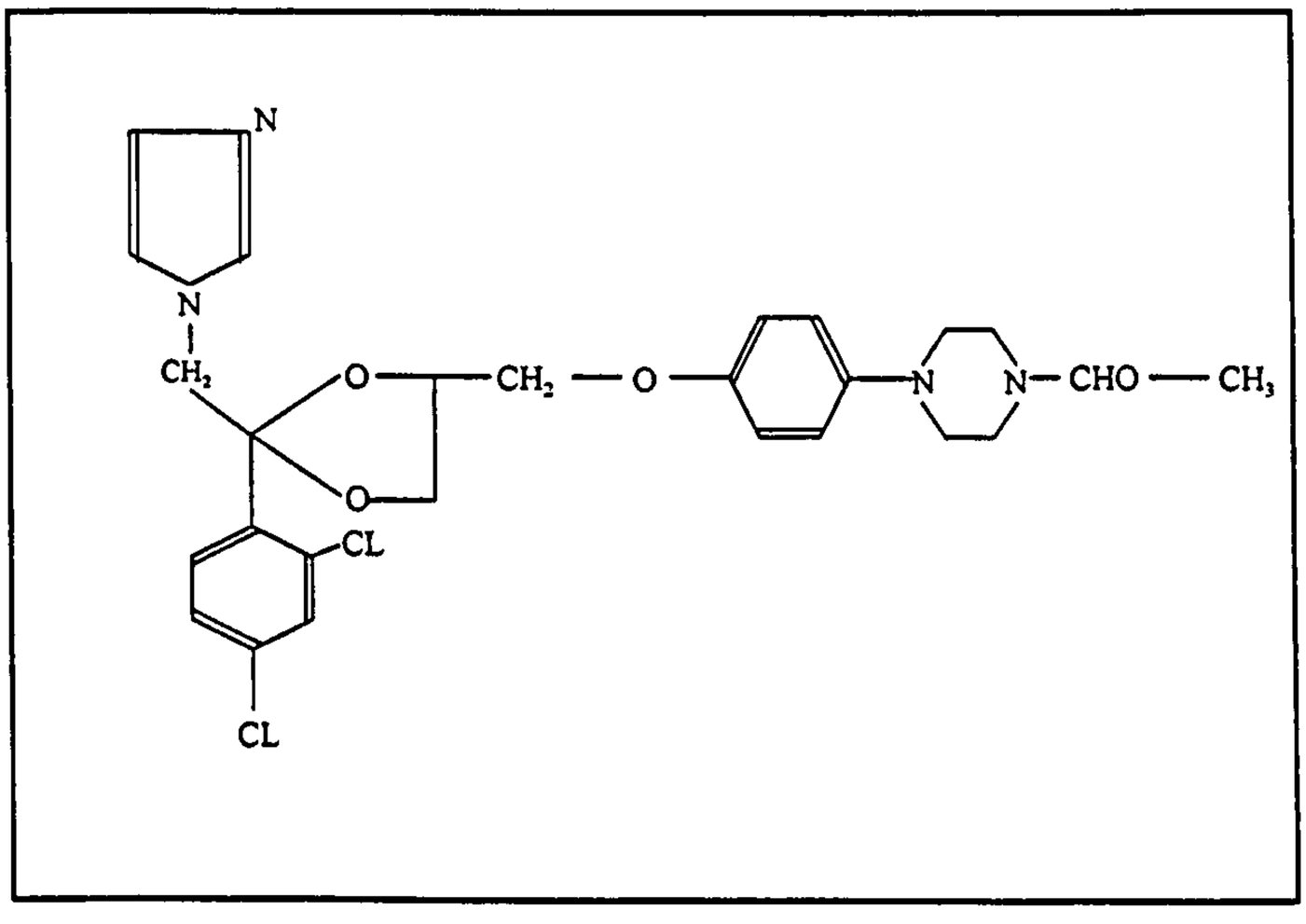
Vulvar Vestibulitis Syndrome is characterised by a non specific inflammatory cell infiltrate in the tissue surrounding the vestibular glands; the inflammation does not affect the gland walls or lumens (Pyka *et al.*,1988). The inflammatory response has been investigated on several occasions, however, all studies report mild to moderate, mixed inflammatory response, which

is not characteristic of a particular disease process (Pyka *et al.*, 1988) (see Chapter 2). In the present investigation, periductal vestibular tissue from VVS patients, was found to have several distinct characteristics which were not apparent in tissue from control patients. Prior to treatment with ketoconazole, the vestibular epithelium was infiltrated by a moderate number of leukocytes which were present in all cell layers, consistent with the findings of Pyka *et al.* (1988), Wilkinson *et al.* (1993) and Prayson *et al.* (1995). However, the most interesting observation was the presence of dark-staining, possibly apoptotic cells seen in small groups throughout the epithelium. Dark cells were characterised by the presence of shrunken, often vacuolated cytoplasm, and the cytoplasmic membranes of the dark cells were characteristically intact. Membrane bound, cytoplasmic lobules, were seen in close contact with dark cells, and were also found engulfed by neighbouring epidermal cells and leukocytes (see Chapter 5).These unusual and distinctive findings have not previously been described in association with VVS. It was interesting that these features were seen in biopsies taken both at Cambridge and Plymouth G.U.M. clinics, and that the overall ultrastructural appearance of these samples was similar.

Ketoconazole, a topical antifungal preparation, was considered by the manufacturer to have beneficial effects which may be useful in the treatment of Vulvar Vestibulitis Syndrome. Ketoconazole is a member of the imidazole family of therapeutic chemicals, used mainly for the treatment of fungal infections (Fig. 6.0). Since the introduction of these drugs in 1969, imidazoles have become one of the most widely used group of antifungals in human and vetinary medicine, and are also used for the protection of fruit and vegetables from fungal infections (Dooms-Goosens *et al.*, 1995). Other members of this widely used group of drugs include: clotrimazole, bifonazole, miconazole, triconazole, fluconazole and croconazole. These drugs can be administered orally as well as topically, and have an effect on both superficial and deep mycoses (Feldman, 1986). Dooms-Goosens *et al.* (1995), reviewed the past literature with

reference to the incidence of contact sensitivity to this group of drugs. Their results indicated that reactions to imidazoles are quite common, however, often the reaction is in response to the base cream, and not to the drug *per se*. It has also been reported that reactions to ketoconazole were uncommon, compared with reactions to other imidazoles.

Fig.6.0 The chemical structure of ketoconazole



(Adapted from Di Pietra *et al.*, 1992)

Imidazoles act by inhibiting ergersterol production which is an important component of fungal cell membranes, an effect which may result in direct cellular damage to the fungus (Feldman, 1986). The antifungal properties of this drug originate from its ability to bind to yeast and fungal cytochrome P450. In response to the binding of this enzyme, the fungal cells become depleted of ergersterol, and contain increased levels of 14α methysterols which disturb membrane properties and the activity of membrane bound enzymes, therefore affecting growth (Furr & Wakeling, 1986). The affinity of ketoconazole for fungal, as opposed to mammalian sterol synthesis enzymes, is consistent with few reports of adverse side effects. However, side effects do become apparent when the maximum dose is exceeded. In man, hepatic dysfunction seems to be a unique, non-dose dependent reaction (Willard *et al.*, 1986). Other side effects of

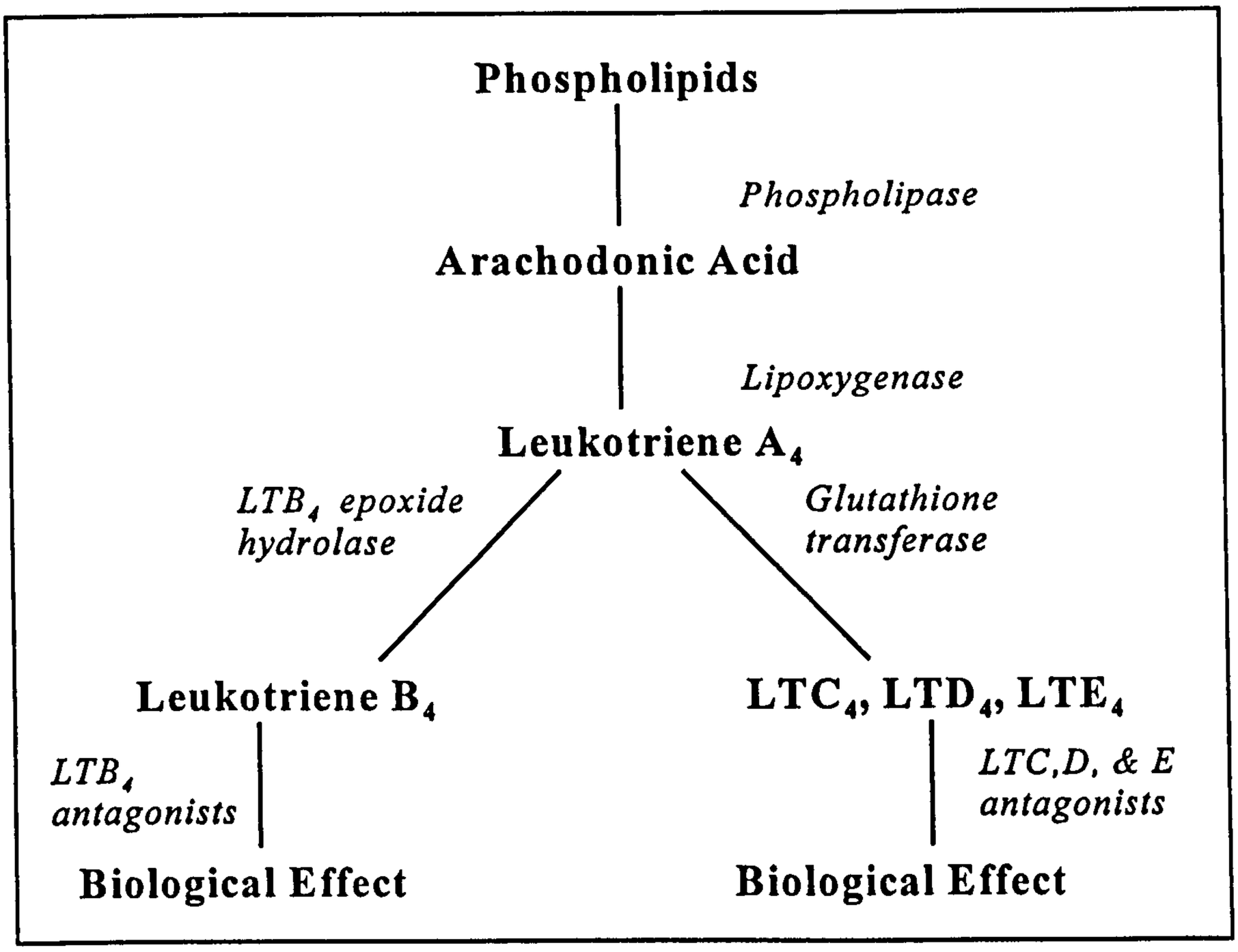
ketoconazole include reports of hypoadrenocorticism (Addisons disease), decreased libido, gynecomastia, impotence, oligospermia, and is particularly problematic in patients with previous liver complaints (Willard, 1986; Eil, 1992).

Ketoconazole and other imidazole derivatives are known to inhibit steroidogenesis. Other drugs such as metronidazole and orthiobendazone, which contain imidazole or azole structures may also have some effect on hormone synthesis. The effects of oral ketoconazole administration include a reduction in the levels of plasma testosterone, androstenedone, dehydroepiandosterol, cortisol and corticosterone. The inhibition of steroidogenesis by ketoconazole in both the adrenal gland and the testis, may become important in the treatment of conditions when a reduction in the steroidogenesis would be a benefit, such as in prostatic cancers or Cushings' syndrome (Furr & Wakeling, 1986; Willard *et al.*, 1986; Amado *et al.*, 1990; Forgue-Latiffe, 1992). Beetens *et al.* (1986), examined the activity of ketoconazole compared with other known lipoxygenase inhibitors, and found the action of this drug to involve the selective inhibition of leukotriene biosynthesis. Ketoconazole has an inhibitory effect on the production of 5-hydroxy-eicosatetraenoic acid (HETE) and LTB_4 in rat peritoneal polymorphonuclear leukocytes, but has no effect on the cyclooxygenase pathway, or on the 15 and 12 lipoxygenase system (Beetens *et al.*,1986; Hillber, 1990). For all LTB_4 inhibitors, inhibition takes place at the level of the 5-lipoxygenase enzyme (Figure 6.1). Hillber (1990) has demonstrated that the most selective and strongest 5-lipoxygenase inhibitor was itraconazole.

Although a fungal etiology has not been considered a major candidate of the cause of VVS, one proposed action of ketoconazole includes the inhibition of leukotriene synthesis. The use of a LTB_4 inhibitor for the treatment of VVS, may reduce the degree of inflammation, resulting in a decrease in the sensitivity of the vestibule. The process of inflammation involves the production of leukotrienes (LTs), so called, because they were first isolated from leukocytes

(Borgeat & Naccache, 1990). Leukotrienes are a group of biochemically active molecules, which are products of the 5-lipoxygenation of arachadonic acid.

Figure 6.1 The production of leukotrienes via the arachadonic acid pathway



The formation of LTs is completed by arachadonic 5-lipoxygenase, an enzyme which is present in phagocytes, mast cells and basophils. Two groups of these chemicals are produced during an inflammatory response; LTA_4 and LTB_4, and LTC_4, LTD_4, LTE_4 and LTF_4 (Fig. 6.1). Inflammatory stimuli such as chemotactic peptides, phagocytosed particles and immunological stimuli which activate phagocytes and mast cells, are all capable of stimulating LT synthesis (Soter *et al.*, 1983; Borgeat & Naccache, 1990). Work involving these chemicals, has concentrated mainly on their role in asthma and inflammation, due to the discovery that peptido-leukotrienes form part of the slow reacting substance of anaphylaxis, and are also involved in smooth muscle contraction during bronchio-constriction (Bray, 1986).

Leukotriene B_4 has a relatively weak smooth muscle contracting ability, but is a strong mediator of leukocyte chemotaxis (Plambland *et al.*, 1981; Bray, 1986; Borgeat & Naccache, 1990; Zimmerman *et al.*, 1990; Fretland *et al.*, 1993; Iwamoto *et al.*, 1993) . The presence of an inflammatory mediator is often signified by vasodilation, enhanced vascular permeability, pain and the symptoms of tissue destruction; for example increased numbers of phagocytes and the release of degradative enzymes. During an acute and, or chronic inflammatory reaction, the cells which are found to generate the most leukotrienes, are macrophages, neutrophils and eosinophils. Several inflammatory mediators are able to stimulate LT formation by inflammatory cells such as macrophages and neutrophils. Leukotriene B_4 is capable of enhancing the random movement of neutrophils, eosinophils, lymphocytes and macrophages from both experimental animals and from man. In addition, resident tissue cells are also able to respond to an inflammatory stimulus by releasing LT, and therefore enhancing the local inflammatory response. Keratinocytes, which are similar structurally to vestibular epithelial cells, have the ability to produce cytokines in response to antigen (Bray, 1986).

The local effects of an inflammatory response include redness and heat, due to vasodilation and increased blood flow, accompanied by pain, due to the stimulation of local thermal, mechanical and chemo-receptors. A process which is accompanied by swelling, due to increased vascular permeability and extravascular plasma leakage and cell migration. All of these symptoms are common in cases of VVS, where patients complain of sensitivity to pressure on the vestibule, a burning pain, and physical findings limited to erythema and slight oedema. Generally LTB_4 is a weak inducer of plasma leakage, however, when injected with prostaglandin E2 (PGE_2) the LTB_4 response is markedly enhanced. Soter *et al.*, (1983), found that local injection of LTB_4 elicited a wheal and flare reaction, which was followed by an inflammatory reaction and a cell infiltrate predominated by neutrophils. The combination of PGD_2 and LTB_4, elicited focal tenderness and a more intense infiltration of neutrophils. Leukotriene B_4 has been shown

to affect the secretory function of leukocytes and may affect the tissue degradative phase of the inflammatory response. It has been suggested that LTB_4 may play a role in the development and maintenance of chronic inflammatory lesions, a situation analogous with that of VVS (Bray, 1986).

The main aim of this part of the investigation was to investigate the effect of the topical, anti-inflammatory cream, 2% ketoconazole (Nizoral, Janssen Pharmaceuticals, Belgium) on VVS. It was proposed that this treatment would assist in the improvement of VVS, by inhibiting the production of LTB_4 via the arachadonic acid pathway, and thus reducing the degree of inflammation. Existing VVS patients attending Plymouth G.U.M. clinic, had been treated with ketoconazole for between one and six months duration, with encouraging results (Chapter 3). A majority of patients showed some improvement over several months, suggesting that changes in the epithelium may be occurring due to treatment. Originally it was anticipated that a placebo controlled investigation of a stronger leukotriene inhibitor would be completed. However, problems with the formulation of this topical preparation prevented its use in the present investigation. The affect of ketoconazole, on the sensitivity of the vestibule in VVS patients was assessed over a four month period. Two biopsies of vestibular tissue were taken, one prior to treatment with ketoconazole, and another after four months of treatment. It was anticipated that any beneficial affect of the drug may become evident in the ultrastructural characteristics of the tissue when compared to pre-treatment biopsies.

6.2 Methods

Patients presenting at the G.U.M. clinic at Plymouth (Freedom Fields), with idiopathic vulvar pain were assessed using Friedrichs' (1987), diagnostic criteria (see Chapter 2), and subsequently were assessed for the degree of vulvar sensitivity using the 'Vulvar Algesiometer' (see Chapter 3). Patients with typically low scores on all four ducts, who also met Friedrichs' diagnostic criteria, were asked to participate in the investigation. Ethical committee approval was required for the inclusion of patients in the trial, and for the removal of vulvar biopsies; this was obtained in February 1994 (Appendix 1). The exclusion criteria for the investigation were:

(a) Post- menopausal women, or women under 16 years of age

(b) Patients who presented with any gross pathology, e.g., warts, or VIN.

(c) Patients with acute vulvitis due to the presence of an etiological agent, e.g., *Candida.*

The pharmacological information provided by Janssen Pharmaceuticals (Belgium) is included in Appendix 3. The cream used for the treatment of VVS was 2% ketoconazole (Nizoral, Janssen). Informed consent was obtained from each patient before the initial biopsy was taken. Patients were informed that they could withdraw from the investigation at any time, and their treatment would not be affected. A biopsy of periductal vestibular epithelium was removed from the lateral border of the right Skenes' gland, prior to treatment, under local anaesthetic (see Chapter 4, Fig. 4.0). Patients were instructed to apply the cream liberally, twice a day for one month, after which they were required to attend the G.U.M. clinic to be assessed. After four months of treatment with ketoconazole, a second biopsy was removed under local anaesthetic from the lateral border of the left Skenes' gland. Biopsies of vestibular epithelium were processed for TEM.

A 3mm Keyes punch biopsy sample was removed from the lateral border of the left Skene's gland and immediately transferred to a vial containing the primary fixative, 4% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.2) at 4°C, for two hours. After 30 minutes the biopsies were subdivided into tissue blocks of approximately 1mm x 1mm whilst immersed in fixative (4°C). The tissue blocks were returned to fresh primary fixative for a further 90 minutes. Blocks were subsequently washed twice in 0.1M sodium cacodylate buffer (pH 7.2), post fixed in 1% osmium tetroxide for 60 minutes (4°C) and finally washed twice in 0.1 M sodium cacodylate buffer (pH 7.2). Tissue blocks were block stained in 2% uranyl acetate for 20 minutes in the dark, washed in distilled water and dehydrated in a series of graded ethanol, 30%, 50%, 70%, 90% and absolute alcohol, for 10 minutes in each mixture. Dehydration was completed by two 10 minute washes in propylene oxide. Infiltration of resin was carried out over several days to ensure full penetration of the resin into the tissue. A series of graded mixtures of propylene oxide and Spurr resin (Spurr, 1969) were used (3:1, 1:1, 1:3, Pure x 2). Blocks were polymerised at 70°C for 8 hours. Ultrathin sections, cut on a Reichert Ultracut ultramicrotome, were collected on copper grids, stained for 10 minutes in uranyl acetate and 20 minutes in lead citrate, and subsequently viewed using a Jeol 1200 transmission electron microscope. Semi-thin sections, of 0.5µm were cut and stained with 1% methylene blue for routine light microscopy.

In total, fifteen patients participated in the trial of ketoconazole and its effect on the ultrastructural characteristics of VVS. These patients had not received any other treatment for VVS prior to the initial biopsy being taken.

6.3 Results

6.31 Light microscopical observations of post-treatment vestibular epithelium.

Semi-thin sections stained with methylene blue showed an epithelium approximately 20 cells thick. Basal cells were dark staining in appearance; the basement membrane was undulant, forming wide, shallow projections into the dermis (Plate 27a). Inter-cellular junctions were visible surrounding cells of the intermediate cell layer (Plate 27b). Surface cells often retained their nucleus, which was frequently laterally displaced. These cells were pale staining in comparison with intermediate and basal cells. Some intensely staining cells were visible often situated in groups, distributed throughout the epithelium. Some large intermediate cells appeared vacuolated, with pyknotic nuclei (Plate 27b). Leukocytes were common in all cell layers of all post-treatment vestibular samples; plasma cells and macrophage-like cells were present in the dermis, positioned immediately below the basement membrane (Plate 27). Capillaries were situated close to the basement membrane, and were often packed with leukocytes, particularly lymphocytes. Eosinophils and mast cells were uncommon in the dermis of post-treatment samples.

6.32 Transmission electron microscopy of post-treatment vestibular epithelium

A schematic representation of the general ultrastructural characteristics of post-treatment samples from VVS patients is shown in Figure 6.2. The overall ultrastructural appearance, was similar to that of the pre-treatment vestibular biopsies from VVS patients. Surface cells were flattened in appearance, and commonly contained large deposits of glycogen. Microvillous projections were numerous on the surface of the superficial cells. Vesicles were visible in close contact with the cytoplasmic membrane of upper intermediate and superficial cells. The epithelium was loosely organised into different cell layers, and was dominated by the presence of a large population of intermediate cells. These cells were not spiny in appearance, but were characterised by the presence of interdigitating cytoplasmic processes. Dark staining cells were

common in the post-treatment samples, and were found to correspond with the intensely staining cells visualised in semi-thin sections stained with methylene blue. It was estimated that these cells probably formed 20-30% of the basal and intermediate cell population. However, an accurate quantification of the number of dark staining cells was not possible using TEM. The cytoplasm of these cells was generally extensively vacuolated. Commonly, dark cells were seen in close contact with cytoplasmic lobules and lymphocytes (Plates 28 & 29). Intensely staining cells maintained intercellular junctions with neighbouring epidermal cells despite being shrunken in appearance. Desmosomes were structurally intact, however, these structures were less common surrounding dark staining cells. The nuclear, and organelle membranes of these cells remained intact. Intensely stained cells were seen as foci throughout the epithelium, but were particularly common in the basal and intermediate cell layers. Occasionally an intensely staining cell profile was visible in the superficial cell layer. Membrane bound lobules of cytoplasm, similar in appearance to apoptotic bodies, were seen in all layers of the epithelium, but were more common in the basal and intermediate cell layers (Plate 29a & b). These structures were frequently seen in groups in close contact with intensely staining cells. Cytoplasmic lobules frequently contained mitochondria, polyribosomes and fine cytoplasmic filaments, and appeared to be of epidermal origin (Plate 30a & b). Occasionally nuclear material was evident, contained within membrane bound cytoplasmic body (Plate 30c). Of various dimensions, these lobules of cytoplasm, were often found engulfed by epidermal cells, and commonly seen surrounded by cytoplasmic projections of macrophage-like cells or NK-like cells (Plate 31). These cytoplasmic bodies were not, however, seen contained within macrophages. In the dermis, identical membrane bound lobules of cytoplasm were seen close to capillaries (Plate 31).

In several post-treatment biopsies, electron dense, angular shaped inclusions were found in macrophage-like cells (Plate 32). Macrophages were common in the dermis of a majority of the

post-treatment biopsies, these leukocytes were characterised by the presence of a large lobed nucleus, numerous vacuoles and many mitochondria. Natural killer-type cells were also visible in the epidermis and dermis, these cells were characterised by a lobed nucleus, developed Golgi apparatus and numerous mitochondria (Plate 31). Lymphocytes of varying sizes were the predominant cell type in the epidermis. Some large leukocytes were visible which had abundant cytoplasm and numerous organelles, these were possibly large granular lymphocytes or NK cells. Lymphocytes were common in all cell layers, frequently these cells were seen in close contact with normal and intensely staining epidermal cells (Plate 30). At the point of contact between the lymphocytes and the epidermal cells, vesicles were frequently visible (Plate 33b). These vesicles often appeared to dispel their contents into the inter-cellular space.

6.4 Discussion

The ultrastructural features of post-treatment vestibular epithelium were very similar to that of the pre-treatment biopsies. Using light microscopy, the epithelium was found to be infiltrated with numerous leukocytes, which were seen in all cell layers, but appeared to be more common interposed between basal and intermediate cells. The characteristic nuclei of plasma cells and macrophages were evident in the dermis, particularly below the basement membrane, and were found to be distributed in a similar pattern to that observed in the pre-treatment biopsies. Plasma cells were generally mature, with well developed endoplasmic reticulum; in pre-treatment samples plasma cells were more common, but were at various stages of maturity.

Examination of post-treatment biopsies using TEM, revealed ultrastructural characteristics which were similar to those apparent in pre-treatment samples. As in the pre-treatment samples the ultrastructural observations were found to centre around the presence of dark staining, possibly apoptotic cells. In post-treatment samples the frequency of apoptotic-like cells in the epithelium appeared to be greater than in the pre-treatment samples. Quantification of the ultrastructural differences between the two groups was impossible using TEM. The number of apoptotic-like cells, was one of the factors which was investigated using image analysis, in order to quantify any differences between the two groups of biopsies (Chapter 7). Apoptotic-like cells were found at different stages of change, ranging from being darkened in appearance to heavily vacuolated, and shrunken. In pre-treatment samples, the apoptotic-like cells did appear vacuolated, but not to such a degree as in the post-treatment samples.

The presence of membrane bound cytoplasmic bodies was a common finding in the pre and post-treatment biopsies. These unusual structures, were again found in close contact with the dark staining cells, but were even more prolific in the post-treatment samples. Variable in size and appearance, these cytoplasmic bodies, were of the same appearance as those found in the

pre-treatment biopsies, and were believed to be apoptotic bodies. The increase in the number of these structures in the post-treatment samples was perplexing, as it was expected that the majority of these cytoplasmic bodies would be cleared by surrounding epidermal cells and infiltrating macrophages. Despite the presence of macrophages in both the pre- and post-treatment samples, engulfed apoptotic bodies were not frequently visible.

In the post-treatment samples, large lymphocytes and macrophages were seen making cytoplasmic contacts with dark staining cells, and with cytoplasmic bodies. As in pre-treatment samples, lymphocytes were the predominant cell type seen in contact with apoptotic-like cells, however, macrophages were seen in the epithelium in a small number of cases. Generally in the pre-treatment samples lobed nuclei of macrophages were not seen infiltrating the epidermis. The increase in the presence of macrophages in the epidermis of post-treatment samples may be due to the natural progress of the condition, and is unlikely to be due only to the effect of ketoconazole. It could be postulated that macrophages are present in the epidermis and dermis to clear the apoptotic cell debris and to remove any cytoplasmic bodies. In pre-treatment samples cytoplasmic bodies were seen in neighbouring epidermal cells, and contained within macrophage-like cells. In the post-treatment samples, very few macrophages were seen to contain the remains of epidermal cells. It was difficult to comment on the significance of this finding, as the plane of the sections examined may have prevented the visualisation of phagocytosed cytoplasmic bodies.

The presence of a significant number of inflammatory cells, is not consistent with the classical process of apoptosis, where an immune response is avoided by the processing of cellular components into apoptotic bodies (Wyllie, 1980; Arends & Wyllie, 1991; White, 1993). However, there were numerous cytoplasmic bodies in the post-treatment samples which have not been phagocytosed. It may be the case that these cytoplasmic bodies, are in fact due to the

process of apoptosis, as anticipated, and that the inflammatory cells present are due to another factor linked to the syndrome. Streilein (1978, as cited by Bos & Kapsenberg, 1986), coined the term skin associated lymphoid tissues (SALT), to describe the cells present in the skin which have immunological properties, and are important in the initiation and development of an immune reaction (Table 6.0).

Reactive oxygen species (ROS) mediated skin damage has been implicated in many inflammatory skin conditions (Trenman *et al.*,1992). The production of ROS in the skin may be due to the infiltration of PMNs, UV radiation under aerobic conditions, drug toxicity, ageing and autoimmune disease. These processes essentially produce O_2; the reaction and subsequently the toxicity of $O_2^{\cdot}$ therefore depends on its conversion to more cytotoxic species such as H_2O_2 and $.OH.H_2O_2$. In immune mediated skin diseases such as allergic and contact dermatitis, it has been postulated that monocytes from patients with skin diseases produce more $O_2^{\cdot}$ than controls, creating an attenuated immune reaction. There is also evidence that ROS may contribute to other skin disorders. In patients with psoriasis, the number of phagocytes, and the locomotion and adherence of these cells are increased. Psoriatic neutrophils have also been shown to release other mediators of inflammation, such as myeloperoxidase (MPO) and catalase (Trenam *et al.*, 1992).

Table 6.0 Skin associated lymphoid tissue (SALT)

Skin Immune System (SIS)
Keratinocytes
Langerhans cells
Intermediate cells
Veiled cells
Neutrophillic granulocytes
Mast cells - connective tissue type
Mast cells- mucosal type
Vascular endothelial cells
Lymphatic endothelial cells
'Homing' T cells

(Adapted from Bos & Kapsenberg, 1986)

Despite ketoconazole having an effect on the degree of sensitivity of the vestibule (see Chapter 3), the ultrastructure of the vestibular epithelium did not appear to have altered considerably, and was not showing any evidence of returning to normal. Although dark staining cells, and leukocytes were present in the control samples, these cells were few in number and generally isolated. Perhaps, if treatment was continued for a further four months, the ultrastructural characteristics of the tissue may begin to resemble that of the control samples. Alternatively, women with VVS may have a greater proportion of apoptotic-like cells in the vestibular epithelia. This may be an inherent factor in the development of the vulvar discomfort, or a result of this syndrome.

Patients treated with ketoconazole generally responded very well to this method of treatment, however, this may be in part, due to a form of the placebo effect. The trial of ketoconazole did not involve the inclusion of a placebo, as this was a pilot investigation and sample numbers were not sufficient to permit the inclusion of a placebo. The influence of the placebo effect, may give rise to similar results as those obtained in this investigation. Patients with VVS have frequently consulted several GP's and, or gynaecologists, who rarely diagnose VVS. Subsequently, when women are told that they have a recognisable condition, they immediately feel more positive, even though the treatment of VVS is difficult and often unsuccessful. A diagnosis of VVS, may be sufficient to increase the well being of the patient, and consequently, reduce the amount of pain the woman reports on her next visit. However, this may also be due in part, to the bodies reaction to stress, which at increased levels is reported to result in an enhanced inflammatory response. Therefore, it must be considered that the effect of ketoconazole on the reported degree of sensitivity, may be due, in part, to a placebo effect. This is supported further, by the findings of the present investigation, that after completing a course of treatment, which may take up to 6 months, some women report that their symptoms return. These findings are also consistent with the results of this study, which did not show any

evidence of the tissue ultrastructure returning to normal.

An alternative theory, is that the inhibitory effect of ketoconazole on the production of LTB_4, may reduce the inflammatory response sufficiently to reduce the degree of vulvar sensitivity, however, when treatment is ceased, LTB_4 synthesis is resumed, and the pain returns. Ketoconazole is probably not targeting the cause of the problem in a majority of patients, however, a significant group of patients do show a complete recovery and remain in remission indefinitely. Successful treatment of this small group of patients, suggests that there may be yet another subgroup of patients with idiopathic vulvar pain who respond well to LTB_4 inhibitors. Alternatively, this may indicate that a subgroup of patients with VVS do in fact have a fungal infection which responds to treatment with ketoconazole. This is not however, consistent with the findings of other authors, who have reported, that very few VVS patients have *Candida* infections when they present with the symptoms of VVS (Goetsch, 1991; Marinoff &Turner, 1991, 1992; Bazin *et al.*, 1994). If a stronger LTB_4 inhibitor was used, this may be sufficient to successfully treat all VVS patients, alternatively a longer course of treatment could be encouraged if side effects do not become apparent.

A small percentage of VVS patients who did not respond favourably to treatment with ketoconazole, were subsequently treated with topical metronidazole gel. Several of the patients who received this treatment responded well, suggesting that this azole derivative, may also be of use for the treatment of VVS in certain cases. The number of patients receiving this treatment were small and the results not consistent, therefore, a pilot study would be required to investigate the effects of metronidazole on VVS before a full trial could be supported. However, the fact that both metronidazole and ketoconazole have a similar effect on the symptoms of VVS, may suggest an analogous action of these drugs. It may be the case, that the anti-inflammatory property of these compounds, is not as important in the treatment of VVS

as the effect on androgen synthesis, which may be a complicating factor in the development of VVS.

It has been reported by Stanisz *et al.* (1994), that women are more susceptible to autoimmune disorders such as rheumatoid arthritis, systemic lupus erythematous, Sjogren' syndrome, and other diseases involving mucosal surfaces. These authors also reported that during pregnancy many aspects, including the symptoms of autoimmune disease, are frequently found to show improvement. Estrogen has been shown to have a dose dependent, dual effect on the immune system, and may influence the functional activity of T cells. Estrogen has been demonstrated to enhance (10^{-10}M) or inhibit (10^{-7} M) IL6 induced IgM production by B cells. Estrogen receptors have been located in T suppressor cells , but not on T helper cells or on B cells. Receptors for estrogen are also found on monocytes, which produce IL1 and IL6 in response to this hormone, and therefore may indirectly affect both T helper and B cells. Estrogen has been reported to elevate antibody (IgM) production in response to T dependent antigens, enhance T dependent B cell proliferation, and the expression of surface Ig on plasma cells (Stanisz *et al,.* 1994). Macrophages function in terms of phagocytosis, lysosomal activity and IL1/IL6 production, has been shown to be increased in the presence of a physiological dose of estrogen, as is neutrophil chemotaxis and migration (Stanisz *et al.*, 1994).

Chemotaxis is a major mechanism by which polymorphonuclear leukocytes (PMNL), and other phagocytic cells move to the site of infection. The pain associated with VVS may be due indirectly, to chemotaxis, which does not necessitate the presence of an etiological agent or disease process. NK cells are able to degrade and phagocytose dead cells and antigens. However, these cells differ from other phagocytic cells, as they may target cells in the absence of antibody or antigenic stimulation, and may be non-specifically activated by agents such as mitogens and interferon. Therefore NK cells may be recruited by any antigen activated T cell

which releases interferon. Recognition of antigen is a highly specific process which is mediated by T lymphocytes, which produce and release effector molecules resulting in an amplification of the inflammatory response. Disruption of this complicated chain of events could take place at any step, resulting in an abnormal, and possibly deleterious reaction. The way in which cytotoxic T lymphocytes (CTL) recognise and kill target cells is still undetermined to a certain degree. Several proposals have been suggested, however, a majority of authors agree that the mechanism of cell lysis by NK and CTL probably requires the presence of perforin and granzyme and the formation of pores in target cells. Attack by CTL and NK cells, commonly results in apoptosis of the target cells, which involves fragmentation of the cellular DNA. This process may occur by cross linking of apoptosis-inducing cell surface molecules on the target cells. These molecules may include Fas which is part of the tumour necrosis factor (TNF) family and is a lytic effector molecule of CTLs (Berke, 1995).

Polymorphonuclear (PMN) neutrophil granulocytes exhibit increased adherence, chemotaxis, degranulation and bacterial killing after stimulation with substances formed within an inflamed site (Rossi *et al.*, 1993). These substances are essential for host defence but also result in tissue damage due to the extrusion of PMNL lysosomal enzymes and the production of toxic oxygen radicals (Plamblad *et al.*, 1981). These authors showed that LTB_4 stimulates neutrophil migration, aggregation and degranulation. In comparison LTC_4 and 5 HETE were not found to stimulate PMN functions; suggesting a definite role of LTB_4 in the inflammatory response. It was suggested by Plamblad *et al.* (1981), that LTB_4 was formed in order to attract more neutrophils to an inflamed site. Fretland *et al.* (1993) illustrated that LTB_4 elicits a dose dependent migration of PMNs when injected intradermally into guinea pig, and rabbit skin. These findings were also demonstrated in human skin, in addition, LTB_4 was also shown to stimulate the proliferation of keratinocytes in culture.

The degree of chemotaxis and therefore the production of LTB_4 may be assessed by the production of myeloperoxidase, a neutrophil marker enzyme. This means of measuring LTB_4 has been used as a qualitative index for tissue inflammation in the gastrointestinal tract (Fretland *et al.*, 1993). The production of LTB_4 could also be assessed in VVS samples with the use of a competitive radioimmunoassay, performed on tissue sections. This technique was described by Zimmerman *et al.* (1990), who demonstrated variations in the levels of LTB_4 using two lipoxygenase inhibitors. These authors found that LTB_4 accumulation was a cause, rather than an effect of granulocyte infiltration. Another possible means of assessing the amount of LTB_4 present in tissues from women with VVS would be to use an immunological marker, and quantify the intensity of the reaction obtained.

In summary, the effect of ketoconazole on the vestibular epithelium from VVS patients is unclear. Although there is a decrease in vulvar sensitivity, as assessed using the 'Vulvar Algesiometer' (Chapter 3), the ultrastructural characteristics of the tissue are unlike the control tissue, and more similar to the pre-treatment biopsies. The presence of a significant number of macrophage and NK-like cells in a majority of the biopsies, suggests that apoptotic-like cells and associated cytoplasmic bodies may be being cleared. However, there were numerous membrane bound, cytoplasmic bodies in both the epidermis and dermis in the post treatment samples, indicating that apoptosis may still be prevalent.

Figure 6.2 Schematic representation of vestibular epithelium from a patient with VVS, post-treatment with ketoconazole.Surface cells are flattened (Su), with large depostis of glycogen (G). Note particularly, the presence of large numbers of membrane bound cytoplasmic lobules (Lb), dark staining, shrunken, and vacuolated (V) epidermal cells (D) in the intermediate cell layer (Ic), and the presence of leukocytes (Le) in close contact with the dark cells. A polymorphonuclear leukocyte (Pmn) is illustrated near to a plasma cell (Pl) in the dermis beneath the basement membrane (Bm).

Su

D

Ic

G

Lb

Lb

Ic

D

L

V

Bc

D

Pl

Pmn

Ca

L

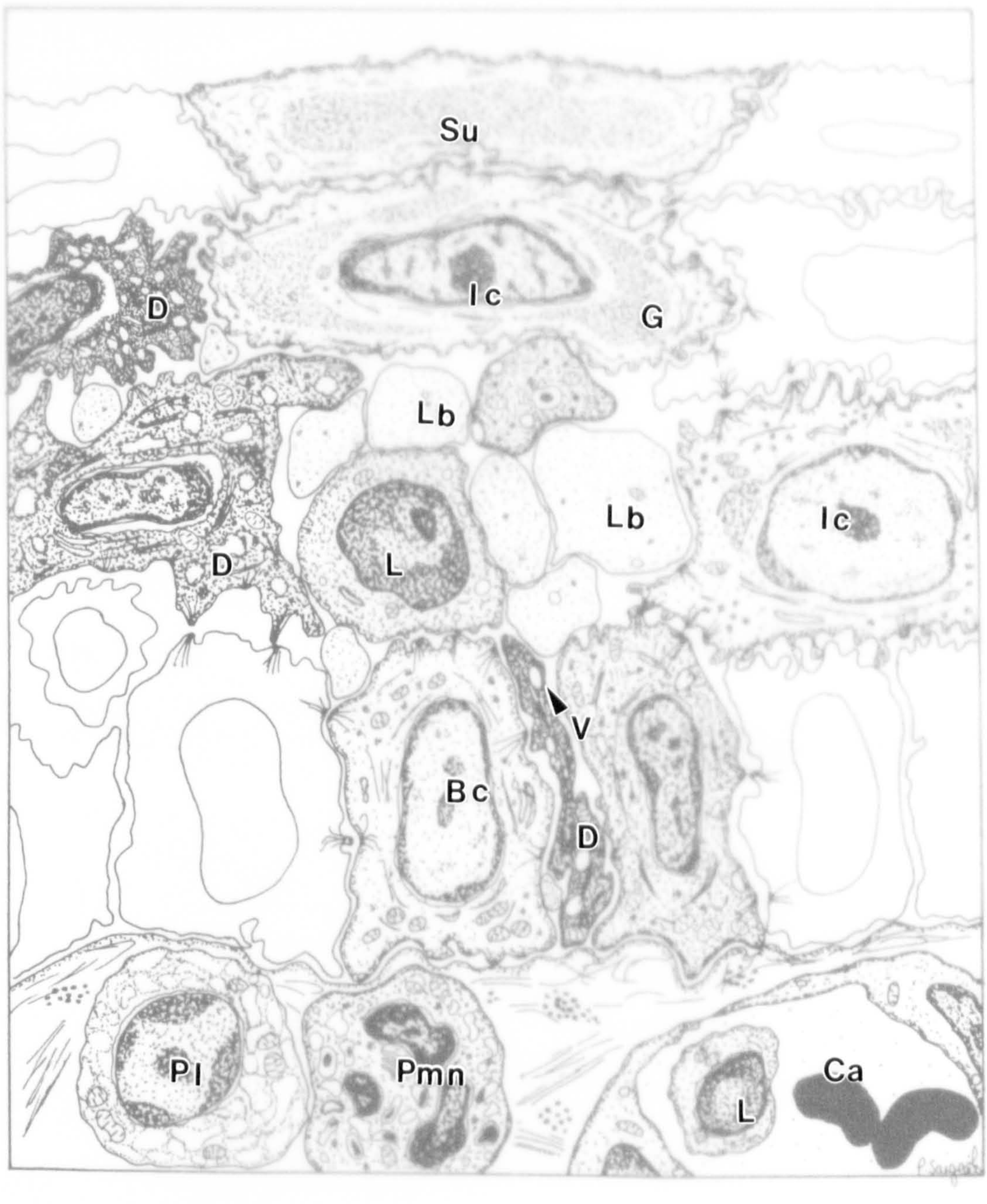
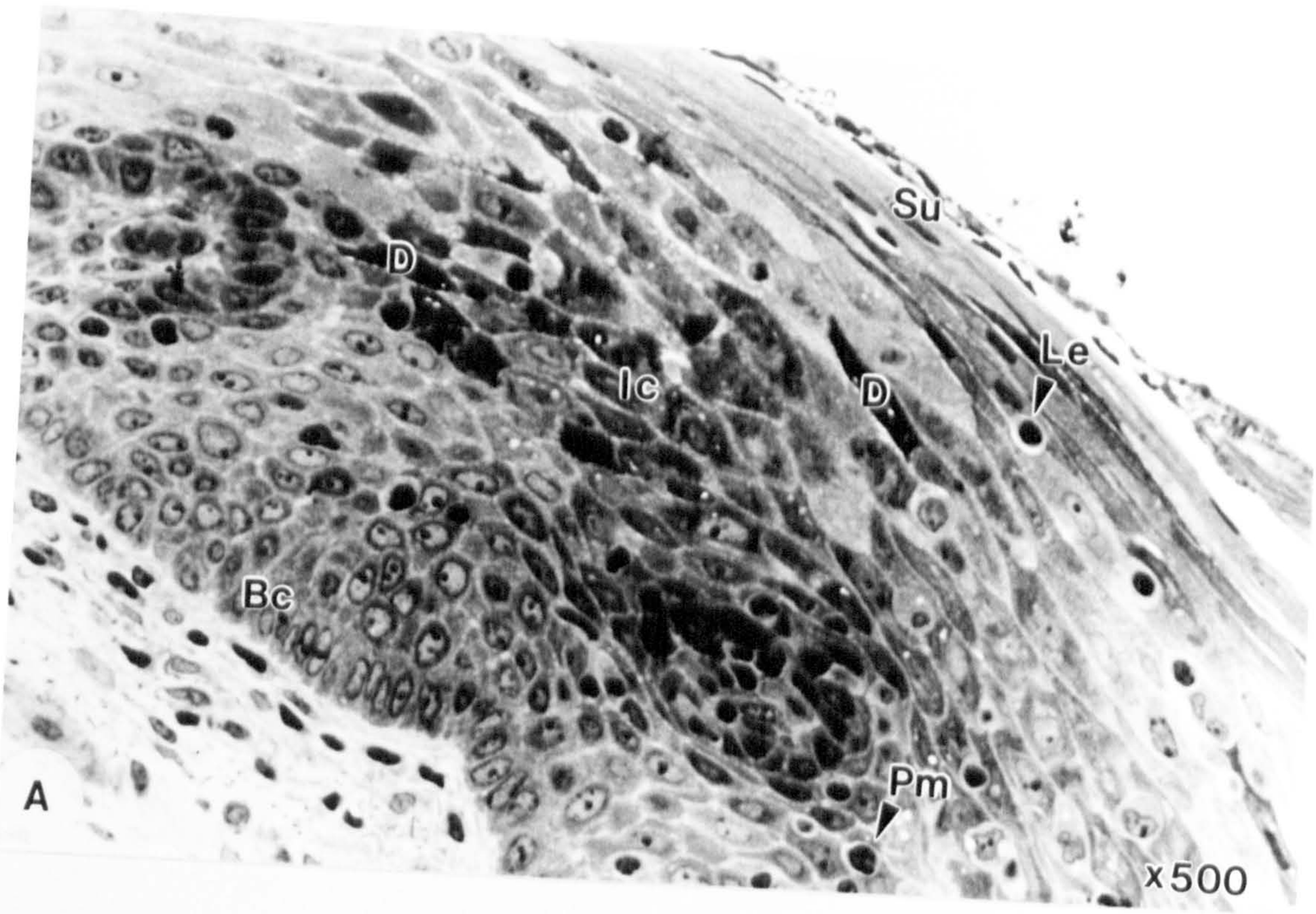

Su
D
Ic
G
Lb
Lb
Ic
D
L
V
Bc
D
Pl
Pmn
Ca
L

Plate 27a

LM photograph of vestibular epithelium (stained with methylene blue), four months post-treatment with ketoconazole. Dark staining cells are visible throughout the epithelium (D), individually, or in small foci particularly in the intermediate cell layer (Ic). Leukocytes are common in all cell layers (Le). A polymorphonuclear cell (Pmn) is visible in the lower part of the epithelium. x 500

Plate 27b

Semi-thin sections stained with methylene blue. Note the presence of dark cell (D) situated in foci along the basement membrane. x 800


Su
D
Ic
D
Le
Bc
A
Pm
x500

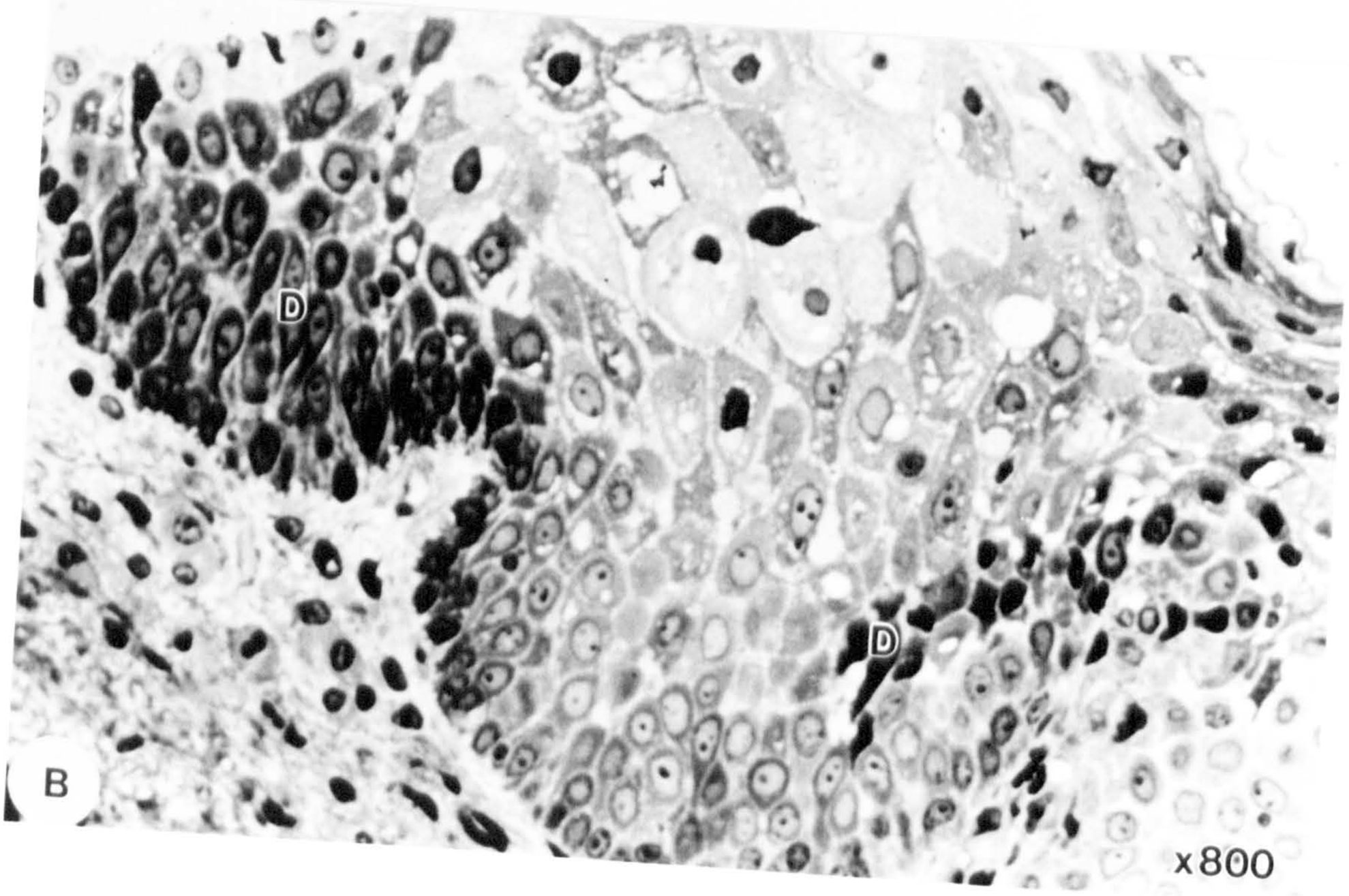
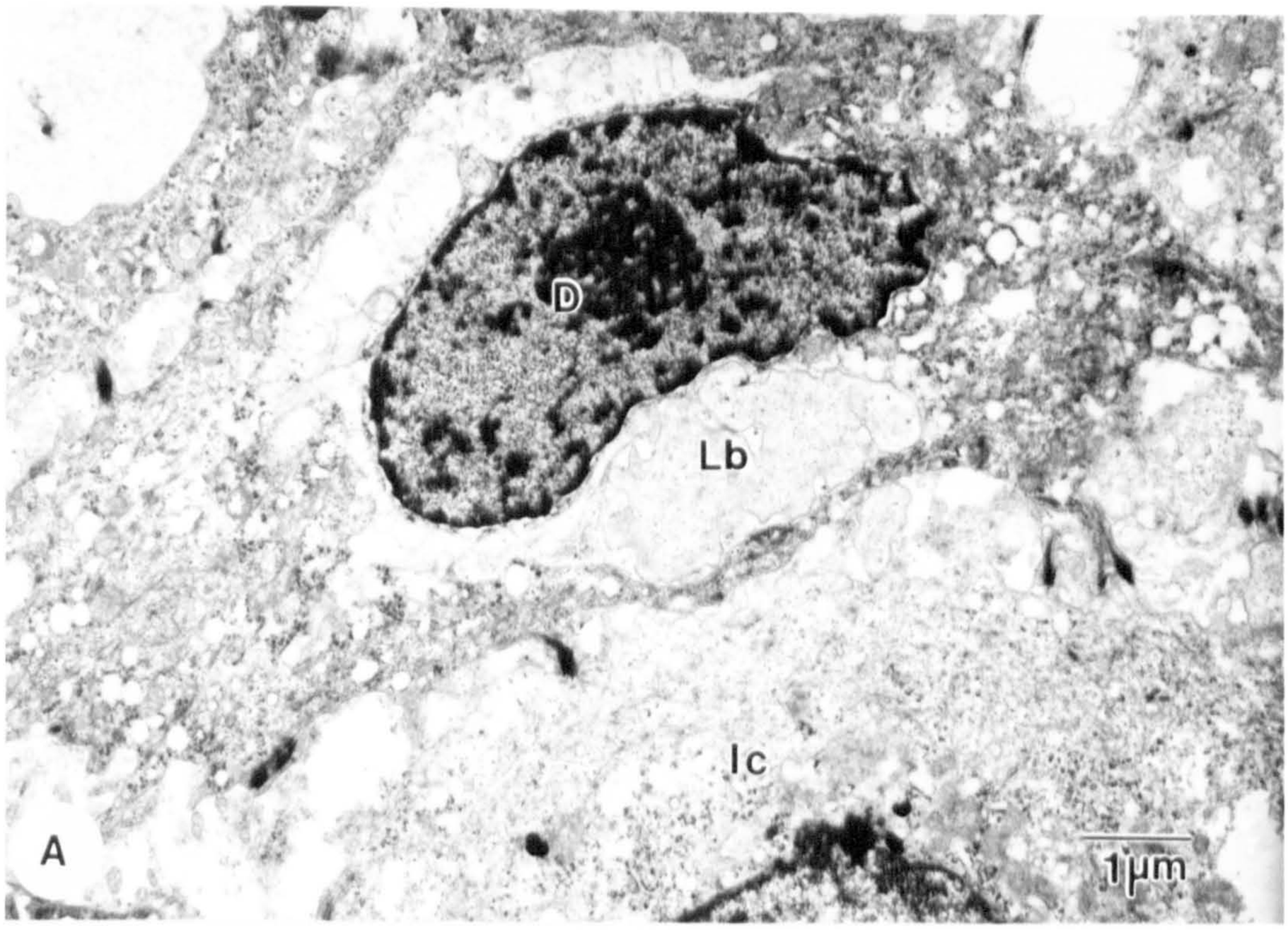

D
D
B
x800

Plate 28a A darkened, vacuolated cell in the intermediate cell layer (Ic) of vestibular epithelium (D). Cytoplasmic lobules are seen in close contact with this cell (Lb). x 10,200

Plate 28b Illustrating the highly vacuolated (V) cytoplasm of an apoptotic-like cell (D). Cytoplasmic lobules (Lb) are commonly associated with these dark staining cells; intact mitochondria are also present (M). x 10,000


D
Lb
Ic
A
1µm

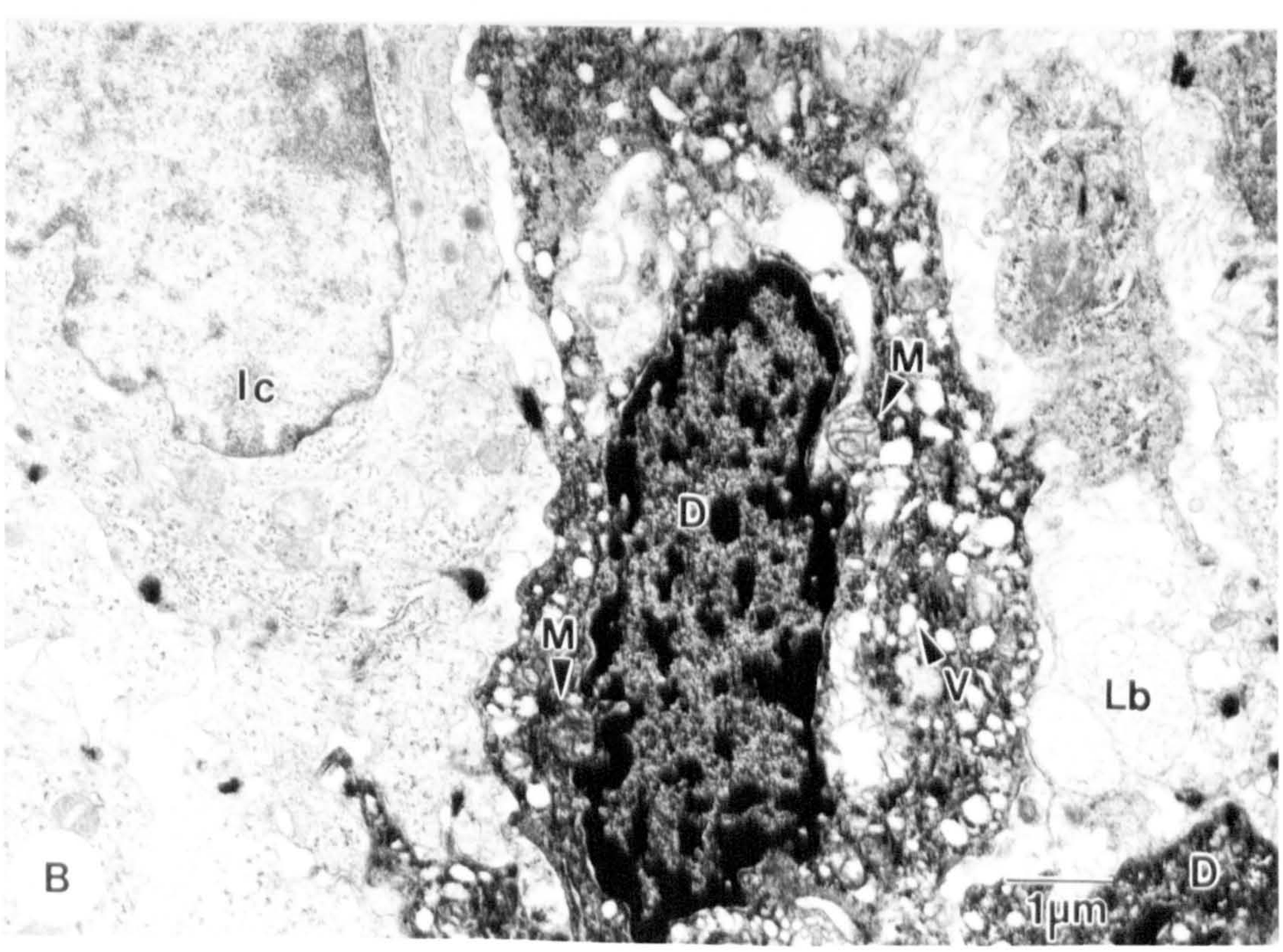
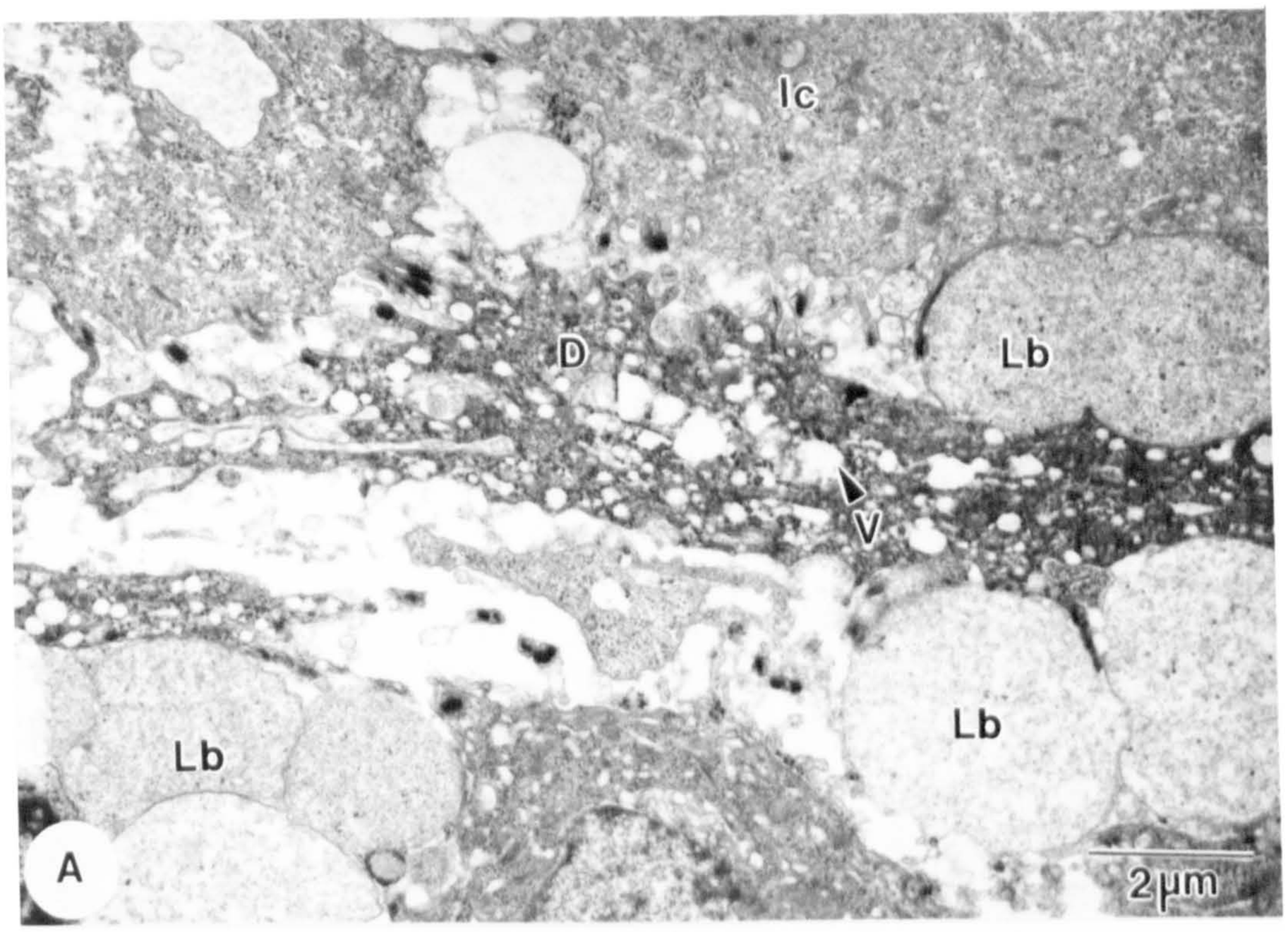

Ic
M
D
M
V
Lb
B
D
1µm

Plate 29a Illustrating upper intermediate cells which have begun to flatten. Cytoplasmic lobules are common (Lb), desmosomal junctions (De) are maintained between apoptotic-like cells (D), and normal appearing epidermal cells (Ic). x 8,000

Plate 29b Intermediate cells (Ic) in close contact with a group of cytoplasmic lobules (Lb) and a small lymphocyte (L). Surrounding epidermal cells are slightly vacuolated and dark staining (D) in appearance when compared with the intermediate cell in the top right of the micrograph (Ic). x 8,000


Ic
D
Lb
V
Lb
Lb
A
2μm

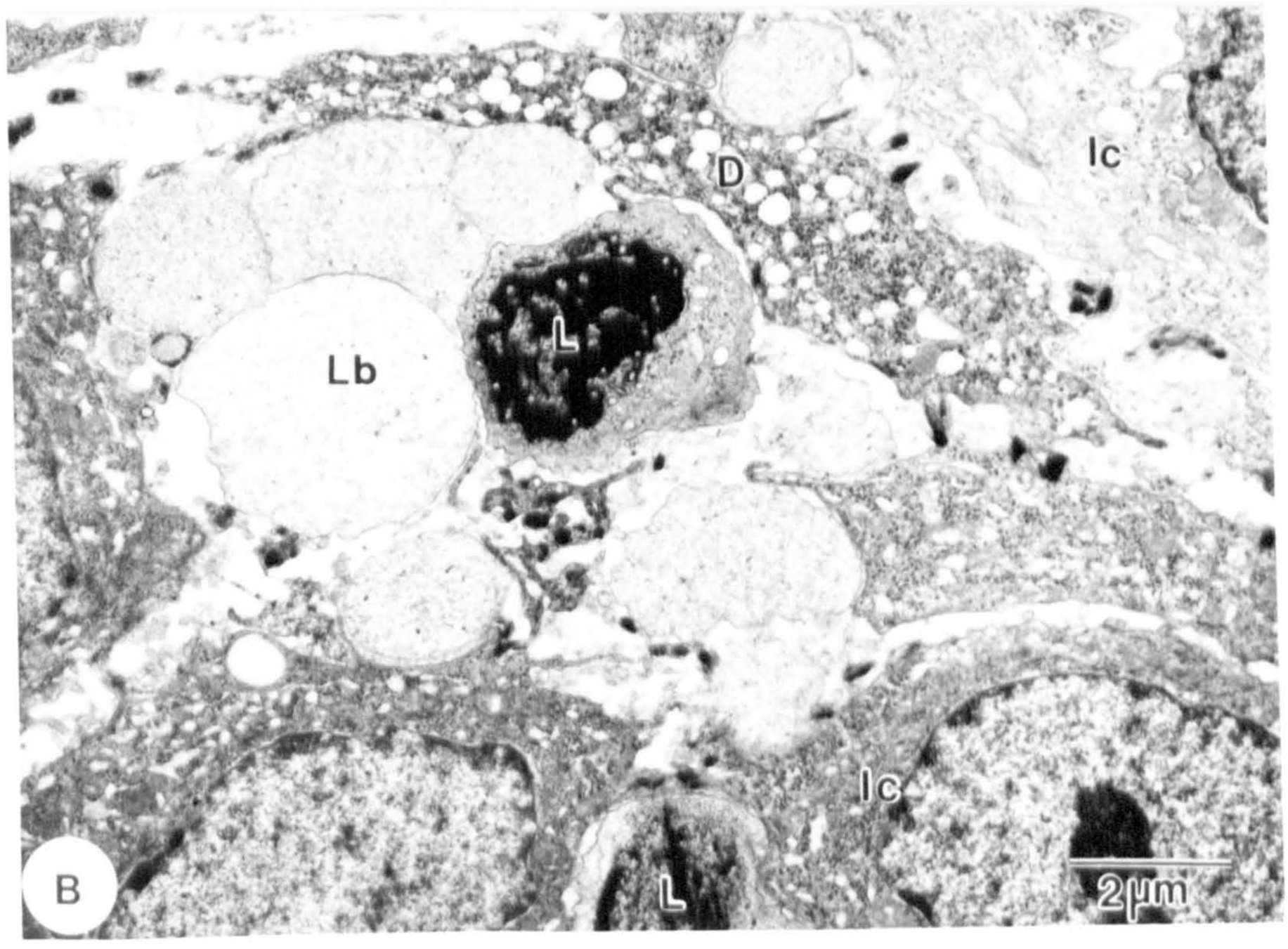
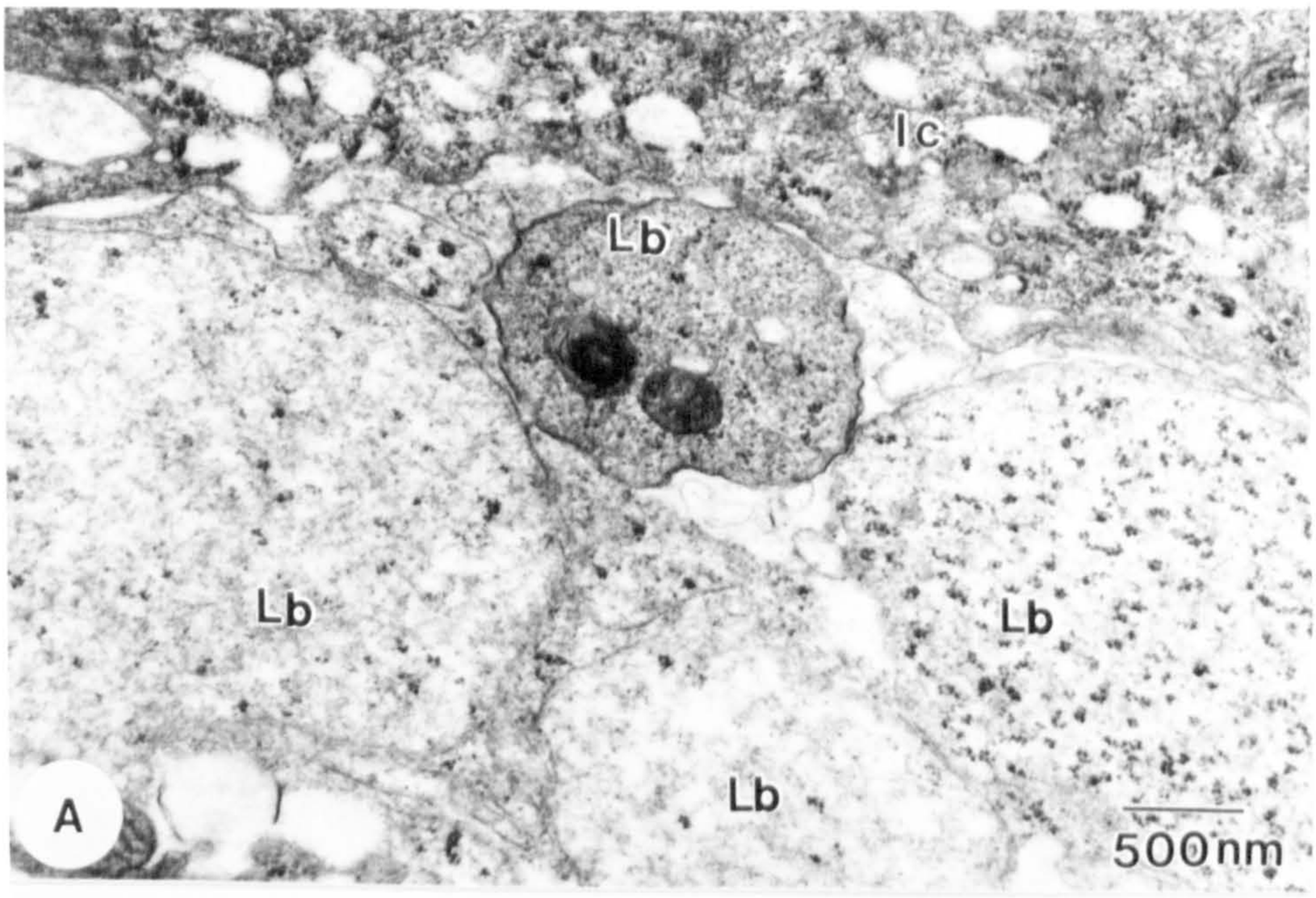
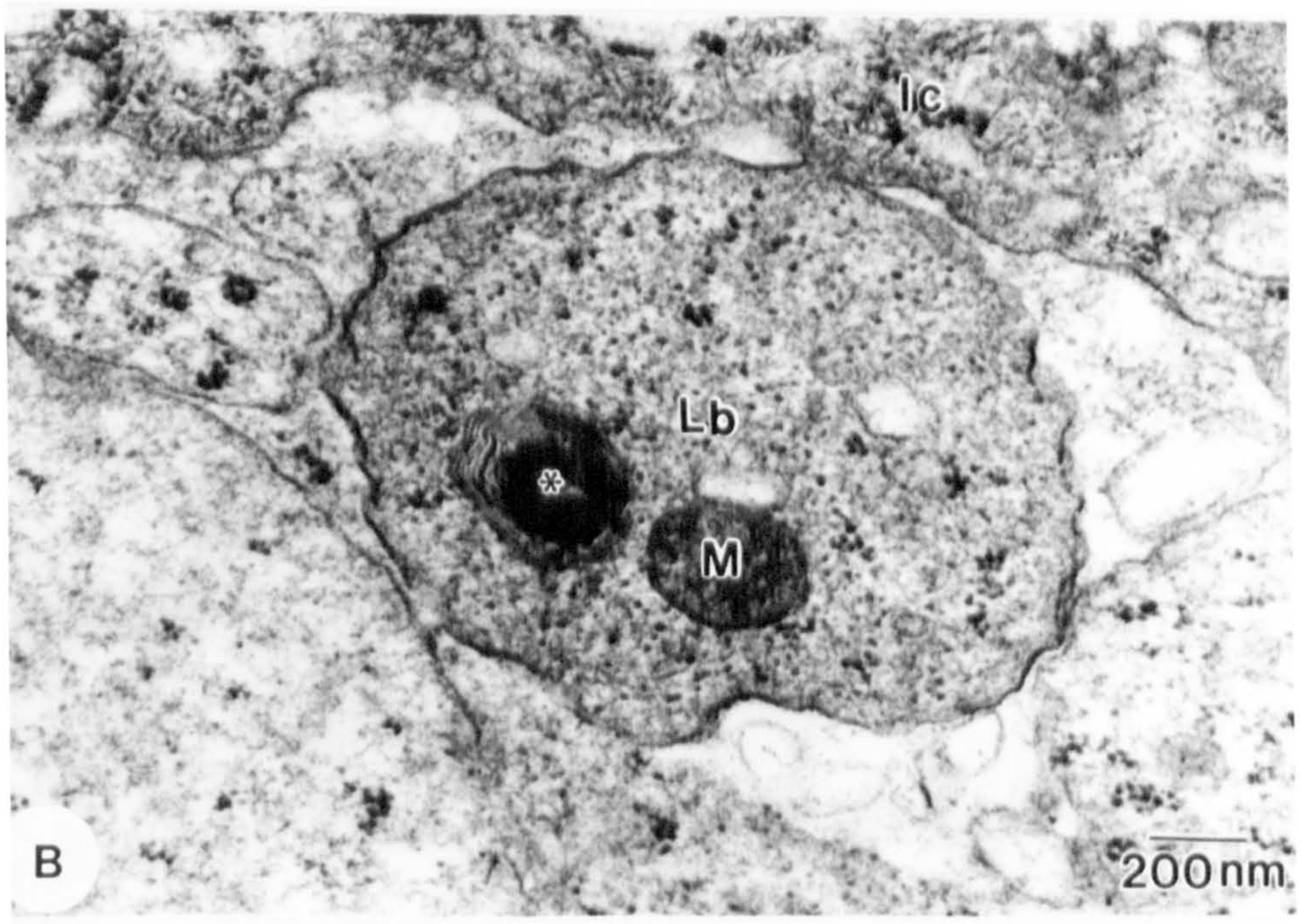

D
Ic
Lb
L
Ic
L
B
2μm

Plate 30a Cytoplasmic lobules (Lb) containing polyribosomes, fine cytoplasmic filaments in the epidermis of a post-treatment sample. An unusual whorled structure is present in one of these lobules (* Plate 30b), which has the appearance of a degenerating mast cell granule. This darker staining lobule, may in fact be a process of a nearby leukocyte / mast cell. x 22,000

Plate 30b A high magnification micrograph of the above cytoplasmic lobule (Lb), illustrating the whorled structure present (*), and a neighbouring mitochondria (M). x 42,500

Plate 30c Cytoplasmic lobules (Lb) situated in the dermis of a post-treatment sample. Nuclear material (N) is seen to be contained within one cytoplasmic lobule (Lb), no membrane is visible surrounding the electron dense nuclear material. A leukocyte (Le), which has the appearance of a NK cells makes close contact with several cytoplasmic lobules (Lb). x 34,000


lc
Lb
Lb
Lb
Lb
A
500nm


lc
Lb
M
B
200nm

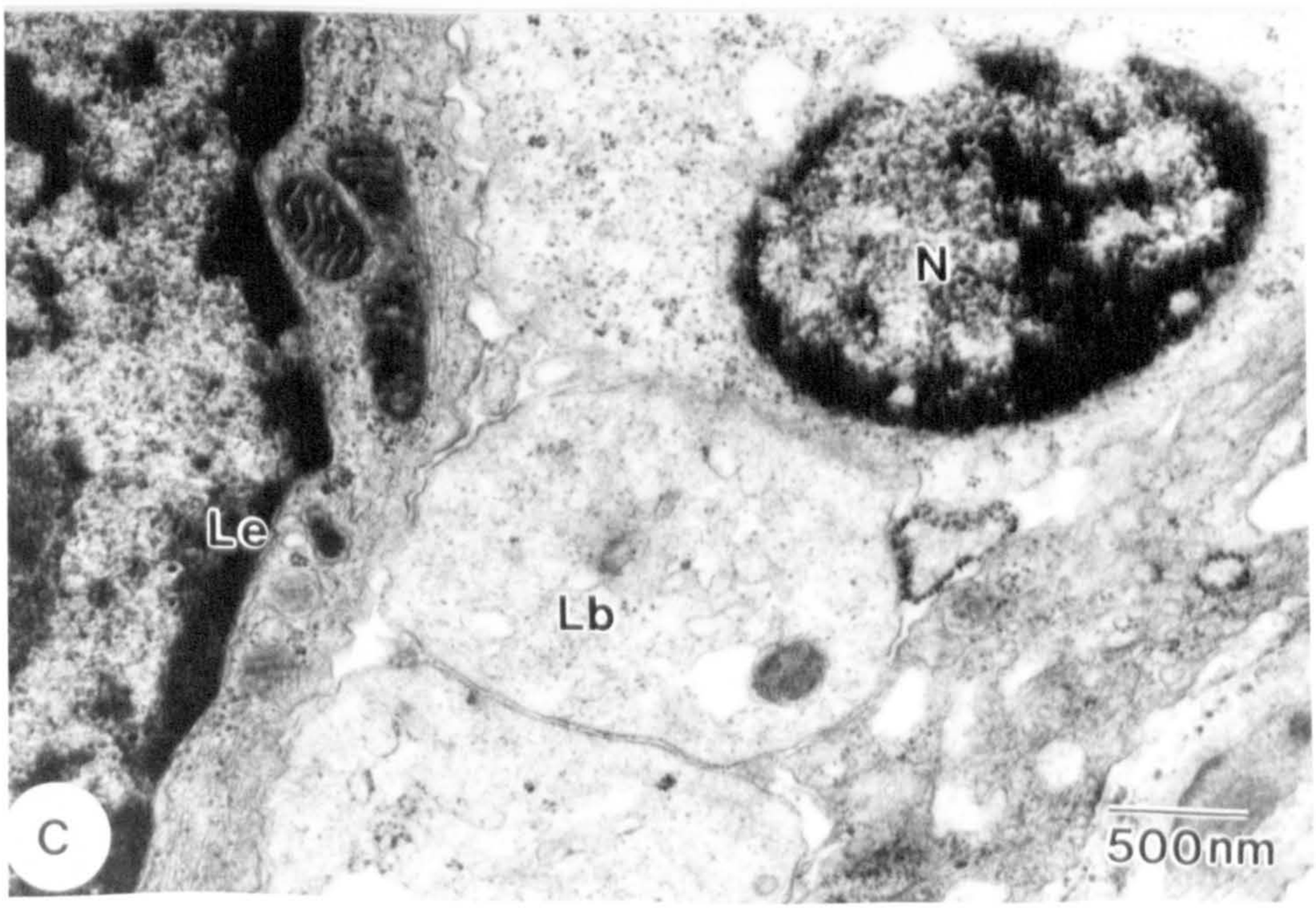

N
Le
Lb
C
500nm

Plate 31 Low power electron micrograph of the dermis, illustrating an area of loose connective tissue (C) close to a capillary, which is packed with cytoplasmic lobules (Lb). Nuclear material (N) is contained within one cytoplasmic lobule (Lb); the leukocyte (Le) present in this micrograph is large with a lobed nucleus and numerous mitochondria (M).

x 12,500

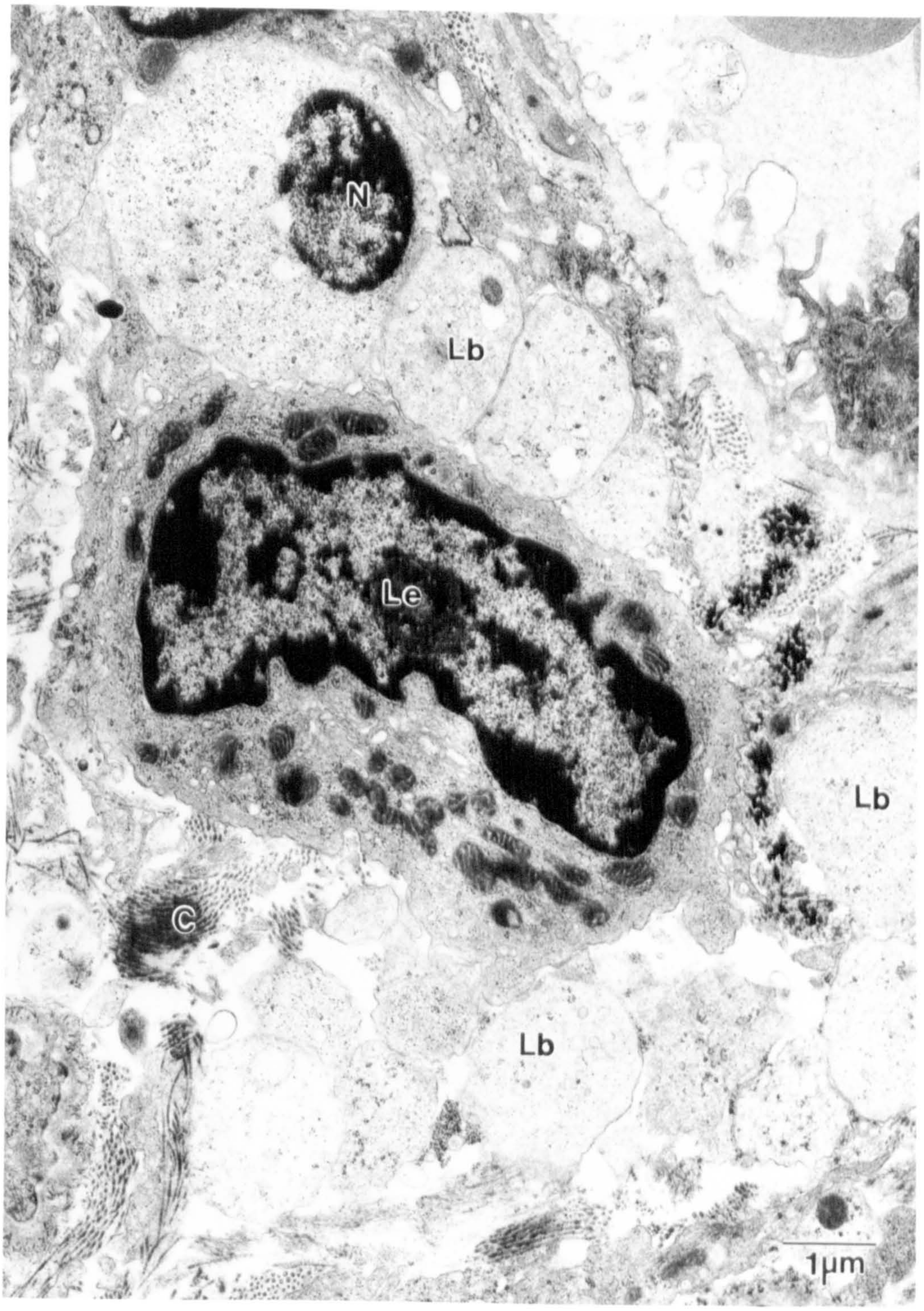

N
Lb
Le
Lb
C
Lb
1µm

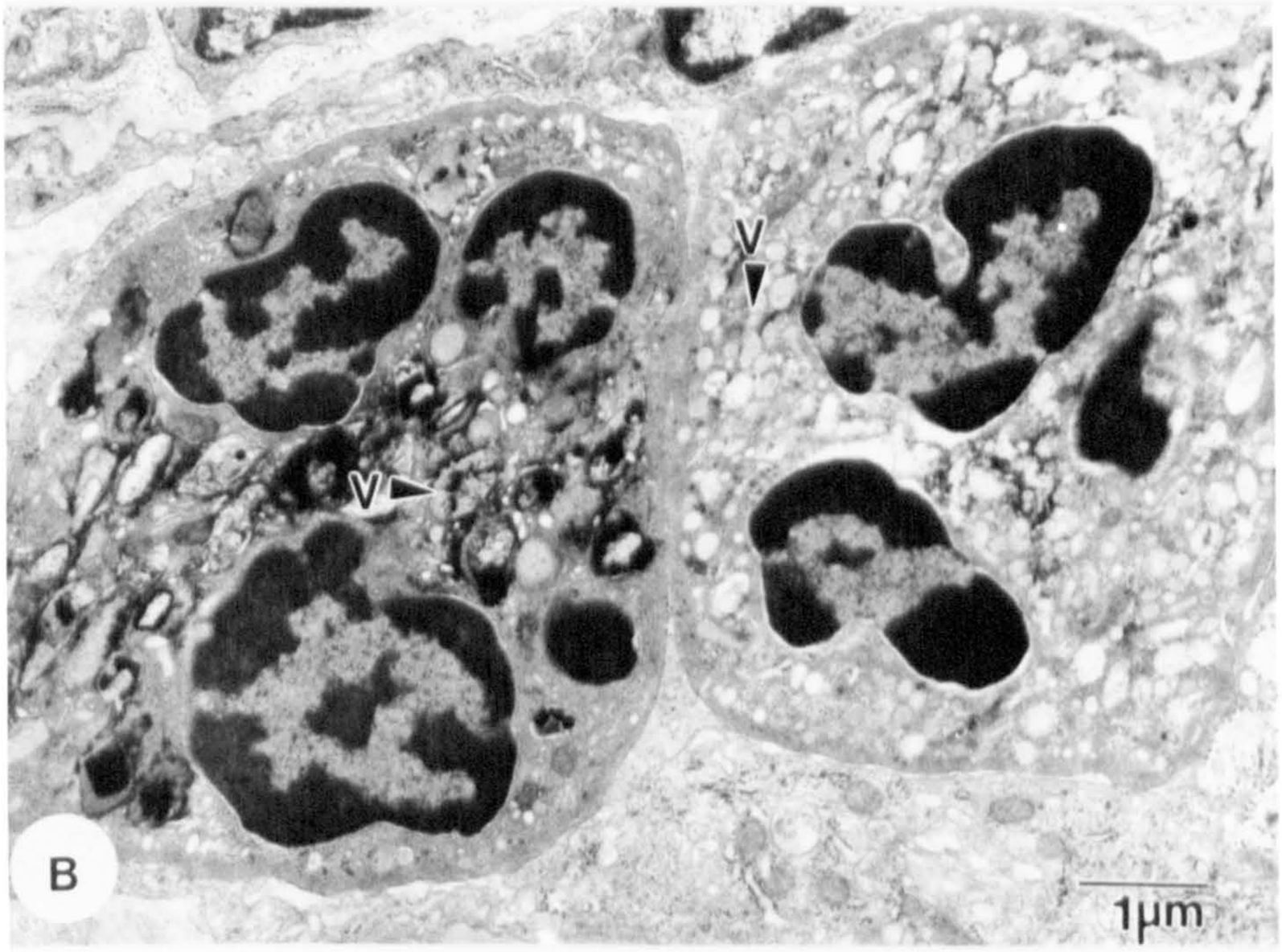
Plate 32a & b Macrophage (Ma) in the dermis of a post-treatment sample. Seen in close contact with the basement membrane, these cells were more common in the dermis then in the epidermis. Frequently these cells were found to contain angular, electron dense material in numerous vacuoles (V).

Plate 32 a x 6,800

Plate 32 b x 10,800

Plate 32 c Angular inclusions visible in the vacuoles (V) of a macrophage seen in loose connective tissue (C) close to the basement membrane. The nucleus of this macrophage is laterally displaced (Nu). x 15,000


Ma
Ma
A
2μm

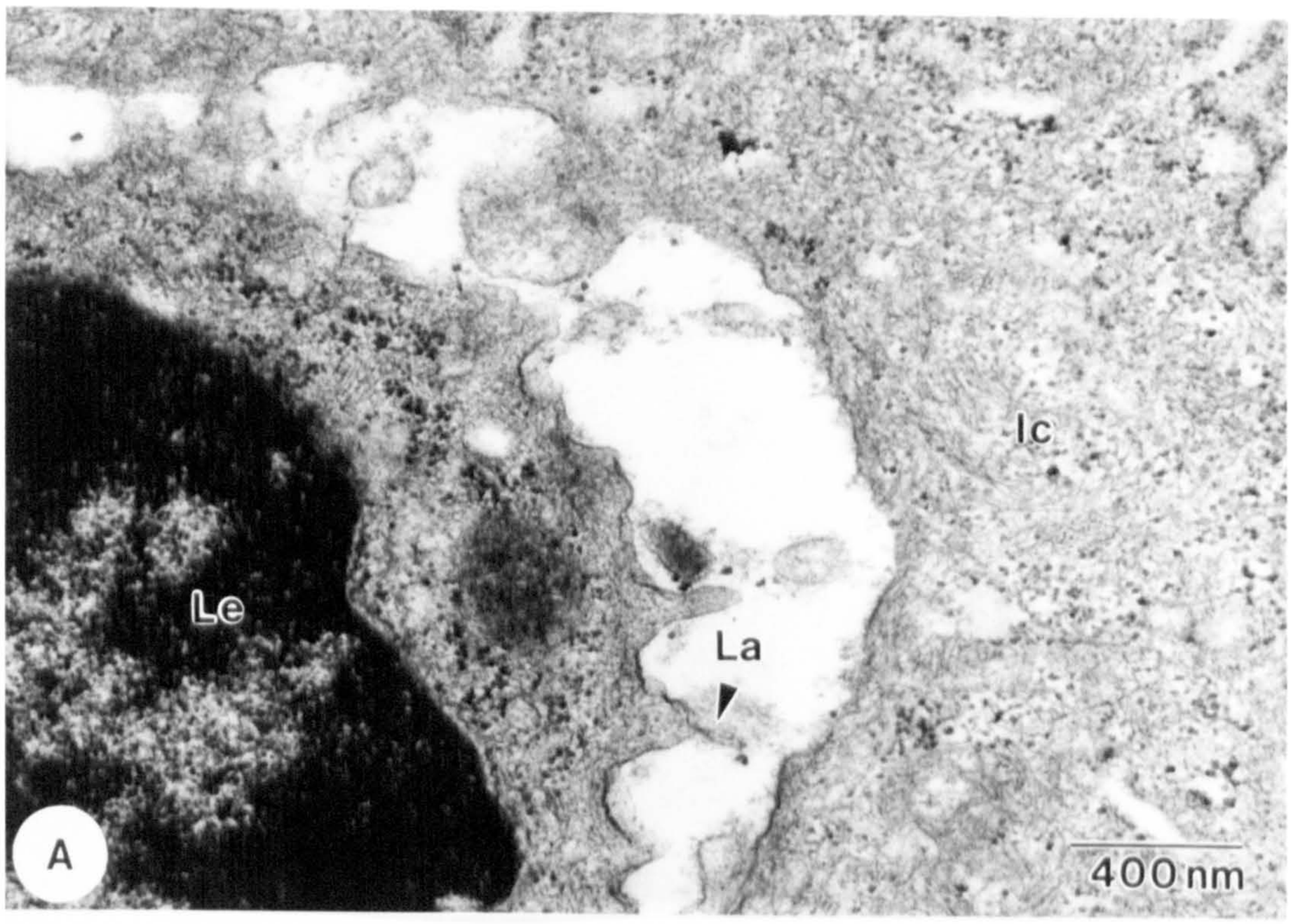

V
V
B
1μm

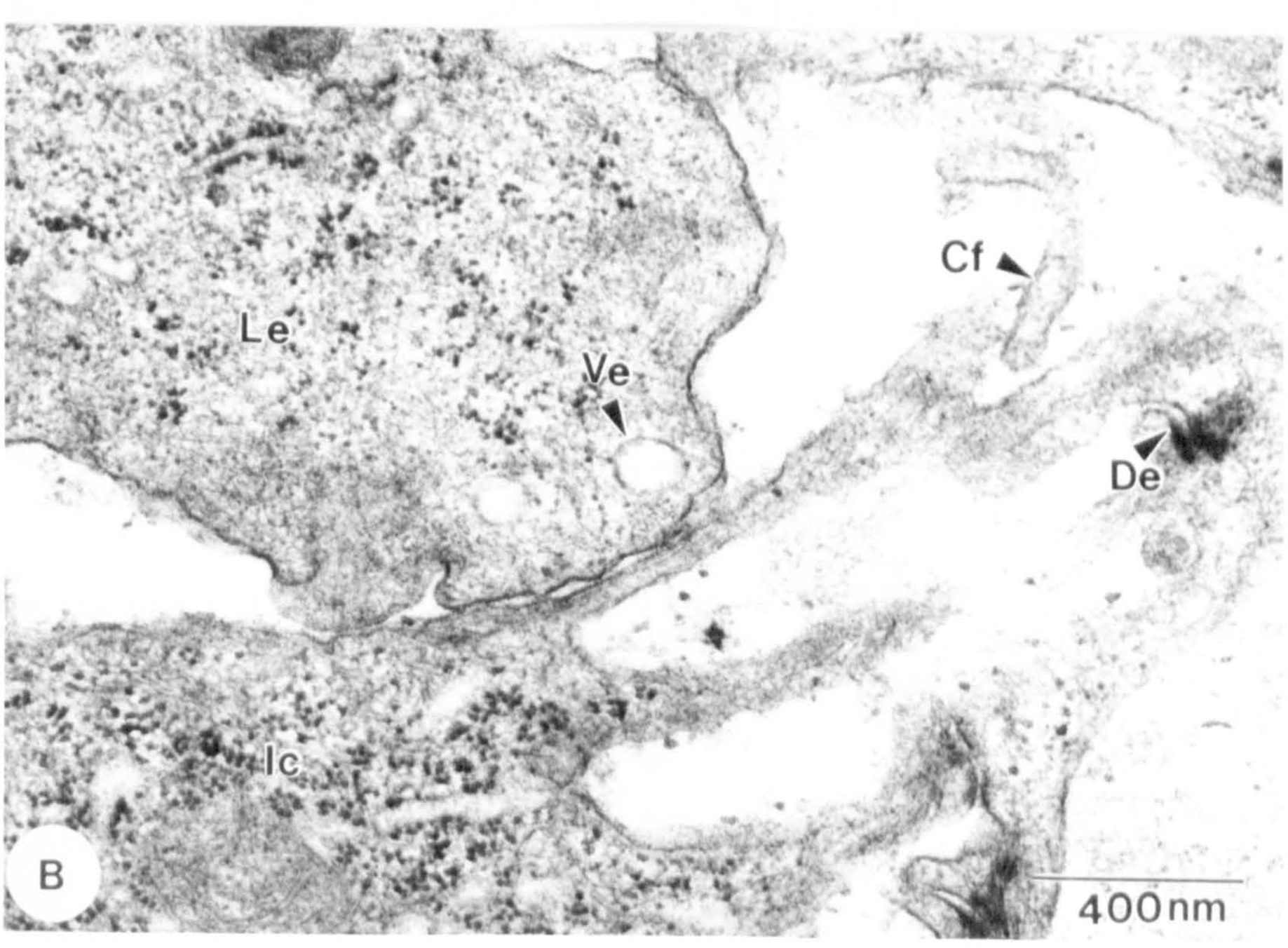

C
Nu
V
C
1μm

Plate 33a & b High power electron micrograph of leukocytes (Le) in the epidermis of a post treatment biopsy. Leukocytes (Le) are seen to make close cytoplasmic contacts with epidermal cells (Ic). Often, small vesicles (V) are visible fusing with the outer membrane of the leukocyte, dispelling their contents into the inter-cellular space. Cytoplasmic folds (Cf) and desmosomal junctions (De) do not appear to be damaged by the movement of leukocytes.

Plate 33 a x 40,000

Plate 33 b x 50,000


Ic
Le
La
A
400nm


Le
Ve
Cf
De
Ic
B
400nm

CHAPTER SEVEN

Quantification of histological parameters using image analysis

7.0 Abstract

TEM indicated three significant changes in the vestibular epithelium from VVS sufferers; a significant number of inflammatory cells, numerous apoptotic-like cells in all layers of the epithelium and undetermined membrane bound cytoplasmic bodies. Although these changes were quite pronounced in both the VVS and post-treatment biopsies, quantification using TEM was not able to be completed during this investigation. Therefore, light microscopy followed by image analysis, of control, VVS and post-treatment sections was undertaken using variations in the grey scale of the epithelium detected using a box transect. The analysis for the three groups indicated more dark staining cells in the VVS sections than in either the control or post-treatment samples. This was demonstrated by plotting the average grey scale values for each point along a transect line; areas of the epithelium containing densely stained cells resulted in a marked variation in vertical profile (i.e."spiky"), accompanied by a characteristic reduction in grey scale across the epithelium. This type of pattern was seen in all of the VVS samples, and to a lesser extent in the post-treatment biopsies. The control samples however, showed a smoother profile indicating a lack of densely stained cells.

Leukocyte profile densitywas estimated using a Weibiel stereological graticule to quantify differences in inflammatory cell numbers in each of the three sample groups. Normal vestibular epithelium, characteristically showed only a small number of leukocytes in the dermis and epidermis, but in the VVS and post-treatment biopsies there were many more leukocytes present, also, there was a distinct difference in the leukocyte counts in the control and VVS biopsies. However there was no significant difference in the counts for the pre and post-treatment samples, suggesting that the number of leukocytes may remain similar despite a reduction in vulvar sensitivity.

7.1 Introduction

Biopsies of vestibular epithelium, taken from VVS patients, were found to have distinct ultrastructural characteristics which were not evident in the control samples. These differences centred around the presence of a large number of dark staining, apoptotic-like epidermal cells. Associated with these cells were numerous lobules of cytoplasm which did not appear to have any cellular origin when serial sections were examined. Cytoplasmic lobules were found in close contact with apoptotic-like cells and with leukocytes. In post-treatment samples the number of apoptotic cells appeared to be very similar to the pre-treatment biopsies, however, these cells were more highly vacuolated than in the pre-treatment samples. This may be due to the natural progression of the process of apoptosis, or, alternatively, may be due to the effect of ketoconazole (see Chapter 6). The number of cytoplasmic bodies was greater in the post-treatment samples than in the pre-treatment samples, suggesting a continuation of apoptosis. The number of inflammatory cells appeared to be similar in both groups. In the pre-treatment samples, the inflammatory cells present were predominantly lymphocytes and plasma cells, which was consistent with the findings of Pyka *et al.* (1988) and Prayson *et al.* (1995). Pathological reports on VVS have described a mild to moderate, mixed inflammatory infiltrate, which is not indicative of a particular disease process. This is consistent with the observations of the present investigation where a moderate inflammatory response was a common finding.

It was not possible to estimate the number of intensely staining, apoptotic-like cells using TEM, as cells at various stages of change were present. However, it was obvious using TEM that there was a greater proportion of dark staining, apoptotic-like cells in the VVS samples when compared with the controls. Problems in quantification arose during the examination of post-treatment samples, which appeared very ultrastructurally similar, to the pre-treatment samples. It was important that any changes in the number of dark staining epidermal cells be quantified in some way, as the effect of ketoconazole may manifest itself as a change in the proportion of

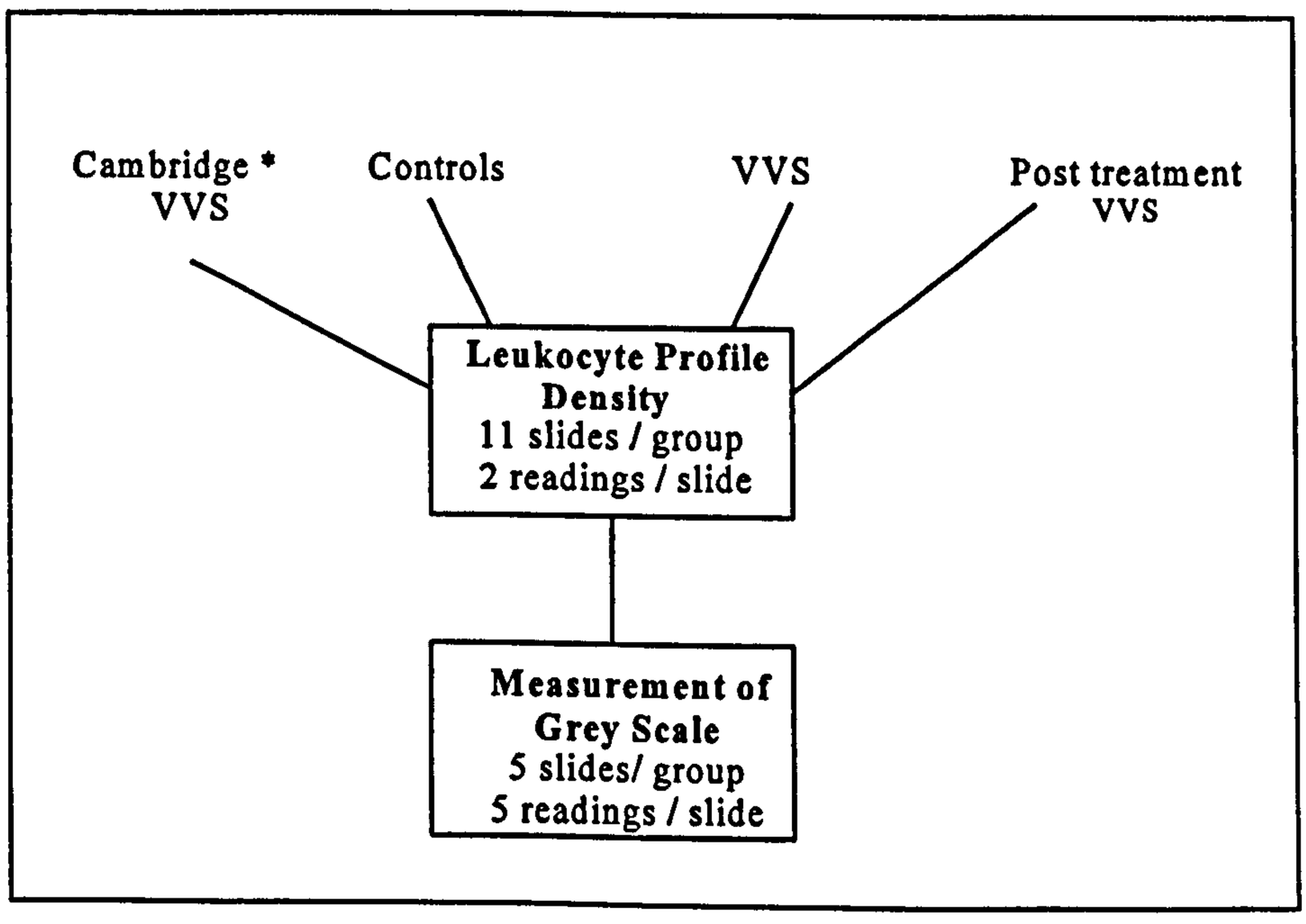
intensely staining cells in the epithelium. The number of leukocytes in the tissue sections, also needed quantification, as this may indicate the intensity of the cellular immune response. Quantification of the individual leukocyte types was not undertaken, as this had already been attempted by Pyka *et al.* (1988) and Prayson *et al.*, (1995).

In summary, the principal aim of this part of the investigation, was to quantify any difference in staining intensity evident between control, VVS and post-treatment biopsies, which may indicate the frequency of apoptotic-like cells present. Additionally, in order to demonstrate any differences in the inflammatory response in the three groups, the leukocytes profile density of the tissue sections was also quantified.

7.2 Methods

Semi-thin (0.4 - 0.5μm) sections taken from TEM blocks were used for the quantification process. Sections were collected on slides, and stained with 1% methylene blue, for exactly 1 minute. Three groups of slides were examined using a Quantimet 570 image analyser; controls, VVS, and post-treatment VVS samples. A programme was written (see Appendix 4) to measure the grey scale through the epithelium (Fig. 7.0). Images were acquired by attaching a Fujitsu General CCD, black and white camera to a Zeiss photo-microscope II, at an objective magnification of x20 and a photo eyepiece of x3.3. The images were digitised as a 256 grey scale, 512 x 512 pixel image using the Quantimet 570, and analysed using a macro (Appendix 4). This allowed a box transect to be positioned over the section. Using the box transect, a horizontal profile of lines was created and used as a mask to detect grey levels (Fig. 7.1)

Figure 7.0 Schematic diagram of the quantification procedure

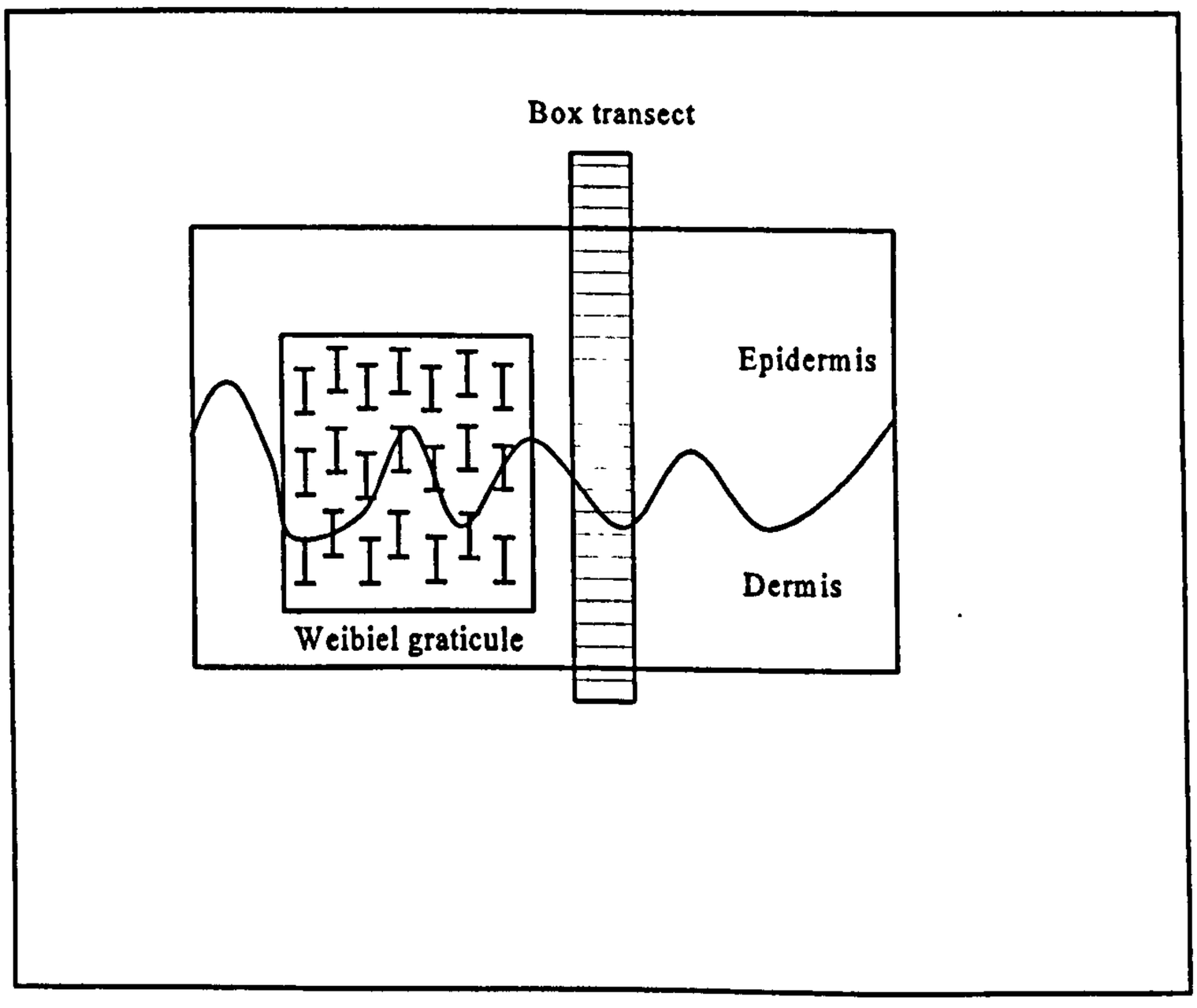


(* Cambridge samples were not included in the measurement of grey scale)

7.21 Leucocyte counts

An estimation of the leukocyte profile density of a section (N_A) was calculated using a Weibiel stereological graticule (Williams, 1977). The number of leukocytes was estimated for control, Cambridge (VVS), Plymouth (VVS), and post-treatment (Plymouth) samples. Patients from Cambridge had been diagnosed using the same criteria for VVS as established by Friedrich (1987). These samples were also examined by a pathologist to ensure no other pathological problems were evident. Using a light microscope, a Weibiel stereological graticule was superimposed over the section, positioned randomly to cover approximately an equal area of the epidermis and the dermis (Fig. 7.1) (Appendix 5).Two counts were completed for each slide, and the mean of these readings was recorded. A positive count was recorded only if the point sample intersect of the graticule bar was positioned over a leukocyte. If the graticule was positioned so that part of it was not covering the section, the number of intersections was reduced accordingly (see Appendix 5).

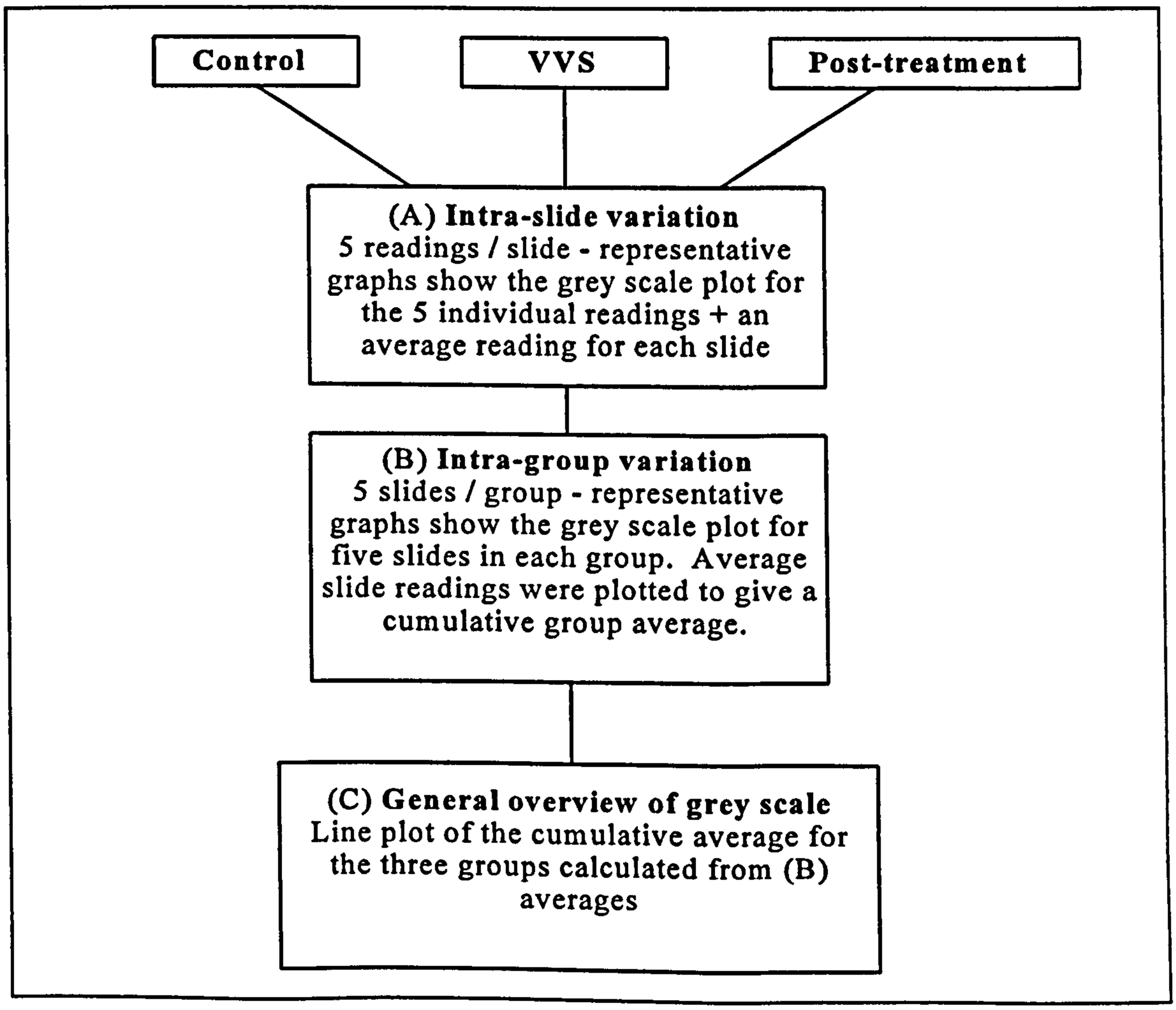
Figure 7.1 Schematic diagram of grey scale estimation using a box transect, and leukocyte counts using a Weibiel stereological graticule.



7.22 Grey scale estimation

Analysis of grey scale was completed to determine the frequency of intensely staining, apoptotic-like cells. The image analysis programme, involved the measurement of grey scale along a 13.92μm wide line of 30 pixels to produce an average value. This measurement was repeated in 1 pixel steps of 0.464μm, giving a set of 324 readings for each box transect. Three groups of slides were used, VVS, post-treatment and controls (Fig. 7.0). Seventy five box transects were completed, twenty five for each of the three groups (five / slide) (Fig. 7.2). The data sets for each slide were imported onto a Quattro Pro spreadsheet and the mean calculated for each grey scale point. The mean values for each of the five slides in a group were plotted on a stacked line graph, and the cumulative average of these calculated (Fig. 7.2). Statistical analysis was completed using Excel 5.0 and Statsgraphics for Windows.

Figure 7.2 Grey scale analysis using the image analyser



7.3 Results

7.31 Leukocyte profile density

One-way analysis of variance, illustrated highly significant differences between the numbers of leukocytes in the four groups ($p>0.0001$). There was a significant difference between the control slides, and all three groups of VVS slides, however, there was no difference in the pre and post-treatment leukocyte profile density (Table 7.0, Fig. 7.3). The control samples gave an average profile density of 2.4 leukocytes / graticule ±1.4, compared to 8.4±3.18 for Cambridge (VVS), 5.22±2.09 for Plymouth (VVS), and 4.77±2.48 for the post-treatment samples (Table 7.0). Control data was greatly influenced by one profile density of 7 leukocytes / graticule (Fig. 7.3). Apart from this outlying result, control biopsies demonstrated counts of between 0-4 leukocytes / graticule, the majority of samples demonstrated counts clustered around 2 and 3 leukocytes / graticule (Fig. 7.3). The standard deviation from the mean was lower in the control samples than in the other groups (Table 7.1).

In comparison to the controls, the VVS and post-treatment groups showed more variation, particularly variation within the groups. The samples from Cambridge demonstrated the highest average count (8.4 leukocytes / graticule), however, the standard deviation from the mean was also the highest in this group. The pre and post-treatment samples showed similar average values, and no statistical difference was seen between these two groups. The post-treatment samples showed a wide range of readings from 1-10 leukocytes / graticule, which was very similar to the range of counts obtained from VVS samples (2-10). The range of counts from Cambridge VVS patients was different, 4-16 leukocytes / graticule. The coefficient variation (CV), i.e., the relationship of the mean and standard deviation demonstrated that the post-treatment group and the control group were similar in respect to leukocyte profile density. In comparison the VVS samples from Plymouth and Cambridge illustrated similar coefficient variation, which was lower than the post treatment and control groups (Table 7.1).

Table 7.0 Table of mean values for leukocyte counts (leukocytes / graticule)

Identification	Mean (Leukocytes / graticule)	Lower Limit	Upper Limit	Group
Control	2.40	1.69	3.12	1
Cambridge	8.40	7.69	9.12	2
VVS	5.22	4.51	5.94	3
Post-treatment	4.77	4.05	5.48	3

Table 7.1 Standard deviation of leukocyte count illustrating maximum and minimum values

Identification (Group)	Standard deviation from the mean	Coefficient Variation (CV)	Minimum Leukocytes/ Graticule	Maximum Leukocytes/Graticule
Control	1.40	58.3	0.0	7.0
VVS Cambridge	3.18	37.8	4.0	16.0
VVS Plymouth	2.09	40.0	2.0	10.0
Post-treatment	2.48	51.9	1.0	10.0

7.32 Grey scale

The type of grey scale plot obtained using this technique is illustrated in Figure 7.4. The position of the epithelial surface and the basement membrane are indicated. The depth of the area plot is an indication of the grey scale (i.e., the average recorded across a 30 pixel line), and therefore the staining intensity of the epithelium along the horizontal transect. The y-axis represents the grey scale, 200 and above, being pale staining (i.e., background resin), and values below 200 being a shade of grey (i.e., part of the tissue section). The x axis is distance along the line of analysis (0 - 150μm). The plots for each of the readings are stacked and the average is shown as the bottom plot of the graph in contact with the x-axis (black).The differences in grey scale for the three groups of samples, which were calculated using the image analyser, are demonstrated in Figures 7.5 - 7.7.

(A) Intra-slide variation - Individual readings.

The grey scale plots obtained from the five readings / slide were very similar in all three groups of slides. Individual readings from VVS slides demonstrated a marked variation in vertical profile, producing a 'spiky' grey scale plot (Fig. 7.5). The average grey scale plot for this representative VVS slide is illustrated at the bottom of the area graph. The 'spiky' appearance of this representative plot is characteristic of all of the VVS samples measured. The appearance of the average plot, illustrates a change in staining intensity, which is quite considerable from the surface of the epithelium to the basement membrane. There are areas in this average plot, where many dark staining cells were encountered during the measurement of grey scale, however, this is more clearly demonstrated in the individual plots (Fig.7.5).

The representative post-treatment sample also demonstrated an uneven profile, and some intra-slide variation, which was a common observation in this group of samples (Fig. 7.6). The average plot for this slide is quite uneven, however the profile of the plot is uniform, and does not appear to taper significantly.

The control sample illustrated a relatively uniform staining intensity throughout the epithelium, illustrated by the smooth plot profile (Fig. 7.7). There was a slight increase in overall staining intensity from the surface to the basal cells, illustrated by a slight taper towards the middle of the grey scale plot. Towards the left hand side of this plot, the pattern becomes slightly uneven, this represented an area of dermis in the control sample (Fig. 7.7).

(B) Intra-group variation - Average plots /slide

The uneven, 'spiky' appearance of VVS data plotted from individual slides (A), was evident in all five of the VVS slide averages, but was not as pronounced as the profiles of the individual plots (Fig. 7.8). Some of the average slide readings when plotted, showed a very tapered profile, indicating the presence of a dark staining area of epithelium. The most tapered areas, of the VVS plots, seen in the middle part of the graph, were quite smooth in appearance in a majority of the VVS slides. Towards the left hand side, the plots became slightly uneven, this was followed by a decrease in grey scale and flattening of the vertical profile. The cumulative average plot of the five slides is seen at the bottom of the graph. Despite being an average plot, variations in the grey scale representative of staining intensity are evident. The average plot of the VVS slides, illustrates an uneven, and slightly tapered plot towards the basement membrane, after which there is an increase in grey scale (decreased staining), representing the dermis and the surrounding resin.

The post-treatment samples clearly demonstrated some intra-group variation (Fig. 7.9). Some of the slides illustrated an uneven plot, with a jagged profile, whereas others, demonstrated an even plot, and had a smooth appearance. The area profile of the cumulative average plot, from post-treatment samples is illustrated at the bottom of the graph. Although being slightly uneven where dark cells were encountered, this plot was very uniform, and did not appear to be tapered.

Control slides demonstrated only minimal variation within the group. The pattern of the plot was similar in all cases, however, some slides demonstrated a more jagged plot than others. The cumulative average plot from the control slides, is smooth in appearance and slightly tapered towards the middle of the plot followed by an increase in grey scale towards the right side of the plot (Fig. 7.10)

(C) General overview of grey scale plots from VVS, post-treatment and control biopsies.

The area cumulative average plots (C) for VVS, post-treatment, and control samples are illustrated in Figs. 7.11 - 7.13. It can be seen from the line plots, that the post-treatment average plot, illustrates minimal change in grey scale through the epithelium. However, the tapering pattern, from surface to basal cells is evident in all three of the line plots to various extents, and is seen most clearly in the control plot. The tapering of this plot illustrates the change in staining intensity in a normal epithelium, which involves an increase in staining intensity moving from surface cells towards basal cells. In contrast the VVS line plot shows considerable variation in the grey scale, and the profile of this line plot is extremely uneven.

A correlation was completed for the average, and cumulative average data from control, VVS and post-treatment samples. The correlation coefficient between samples was calculated:

$$P\,x, y = \frac{\mathrm{Cov}\,(X,Y)}{\sigma_x \cdot \sigma_y}$$

Relationship of x & y = Covariance of the two data sets / Product of their standard deviation

(From Excel 5.0)

It was demonstrated that the cumulative average readings for VVS and post-treatment samples were quite closely correlated (correl. 0.893), whereas, the control and VVS did not show much similarity (correl. 0.36). The post-treatment and control data was not closely correlated (correl. 0.499). However, there was a variation in the correlation within each of the three groups of samples examined. The control group demonstrated two samples, C and E which produced readings which were not closely correlated with the other samples in the group (Table 7.2). These two samples were however, quite similar to each other (correl. 0.672). Samples in the VVS group were quite closely correlated, correlation coefficients of 0.7 and 0.8 were common (Table 7.3). In the post-treatment group the correlation between the samples showed considerable variation, generally a correlation coefficient of 0.5-0.7 was obtained. However, sample B was found to show very little correlation with the other samples in this group (Table 7.4).

(C) General overview of grey scale plots from VVS, post-treatment and control biopsies.

The area cumulative average plots (C) for VVS, post-treatment, and control samples are illustrated in Figs. 7.11 - 7.13. It can be seen from the line plots, that the post-treatment average plot, illustrates minimal change in grey scale through the epithelium. However, the tapering pattern, from surface to basal cells is evident in all three of the line plots to various extents, and is seen most clearly in the control plot. The tapering of this plot illustrates the change in staining intensity in a normal epithelium, which involves an increase in staining intensity moving from surface cells towards basal cells. In contrast the VVS line plot shows considerable variation in the grey scale, and the profile of this line plot is extremely uneven.

A correlation was completed for the average, and cumulative average data from control, VVS and post-treatment samples. The correlation coefficient between samples was calculated:

$$P\,x, y = \frac{\mathrm{Cov}\,(X,Y)}{\sigma_x \cdot \sigma_y}$$

where $-1 \leq p\,x\,y \leq 1$

$$\mathrm{Cov}\,(X,Y) = \frac{1}{n} \sum (x_1 - \mu_x)(y_1 - \mu_y)$$

It was demonstrated that the cumulative average readings for VVS and post-treatment samples were quite closely correlated (correl. 0.893), whereas, the control and VVS did not show much similarity (correl. 0.36). The post-treatment and control data was not closely correlated (correl. 0.499). However, there was a variation in the correlation within each of the three groups of samples examined. The control group demonstrated two samples, C and E which produced readings which were not closely correlated with the other samples in the group (Table 7.2). These two samples were however, quite similar to each other (correl. 0.672). Samples in the VVS group were quite closely correlated, correlation coefficients of 0.7 and 0.8 were common (Table 7.3). In the post-treatment group the correlation between the samples showed considerable variation, generally a correlation coefficient of 0.5-0.7 was obtained. However,

A correlation was completed between each individual slide in a group, the data used for this correlation is an average of 5 grey scale plots / slide. Each table represents a group of samples either VVS control or post-treatment. In each group there are 5 slides (n=5) A - E, F is the average of all of the 5 slides in the group.

Table 7.2 The correlation coefficients for control samples.

CONTROL	A	B	C	D	E	F
A	-	0.943	-0.212	0.959	0.277	0.908
B	-	-	-0.339	0.928	0.194	0.835
C	-	-	-	-0.209	0.672	0.183
D	-	-	-	-	0.195	0.902
E	-	-	-	-	-	0.556

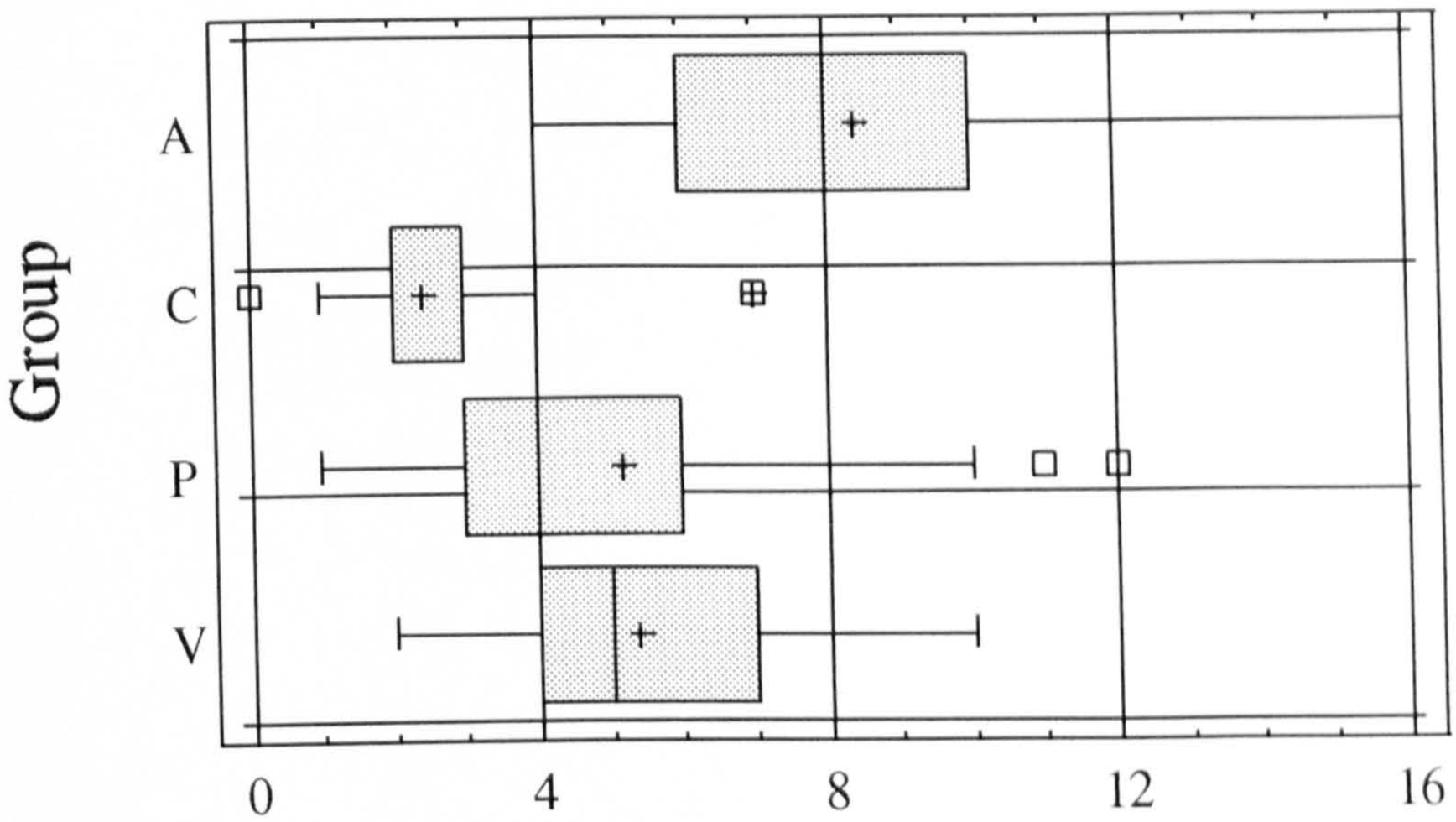
Table 7.3 The correlation coefficients for VVS samples

VVS	A	B	C	D	E	F
A	-	0.550	0.749	0.611	0.551	0.880
B	-	-	0.393	0.849	0.746	0.829
C	-	-	-	0.654	0.589	0.774
D	-	-	-	-	0.851	0.906
E	-	-	-	-	-	0.886

Table 7.4 The correlation coefficients for post-treatment samples

POST TREAT	A	B	C	D	E	F
A	-	0.560	0.151	0.723	0.630	0.755
B	-	-	-0.266	0.168	0.240	0.316
C	-	-	-	0.456	0.587	0.706
D	-	-	-	-	0.778	0.840
E	-	-	-	-	-	0.910

Figure 7.3 'Box & Whisker' plot of leucocyte counts from VVS (Plymouth & Cambridge), control, and post-treatment samples. Illustrating the range, and mean leukocyte counts .Refer to Appendix 6 for an explanation of the Box & Whisker Plot.



A = VVS Cambridge
C = Control
P = Post-treatment
V = VVS Plymouth

Figure 7.4 Schematic illustration of a grey scale plot, indicating the extent of the epithelium, the position of the epithelial surface and the basement membrane. The grey scale and the intensity of staining are shown on the y-axis. The length of the box transect is represented by distance (μm) along the x-axis.

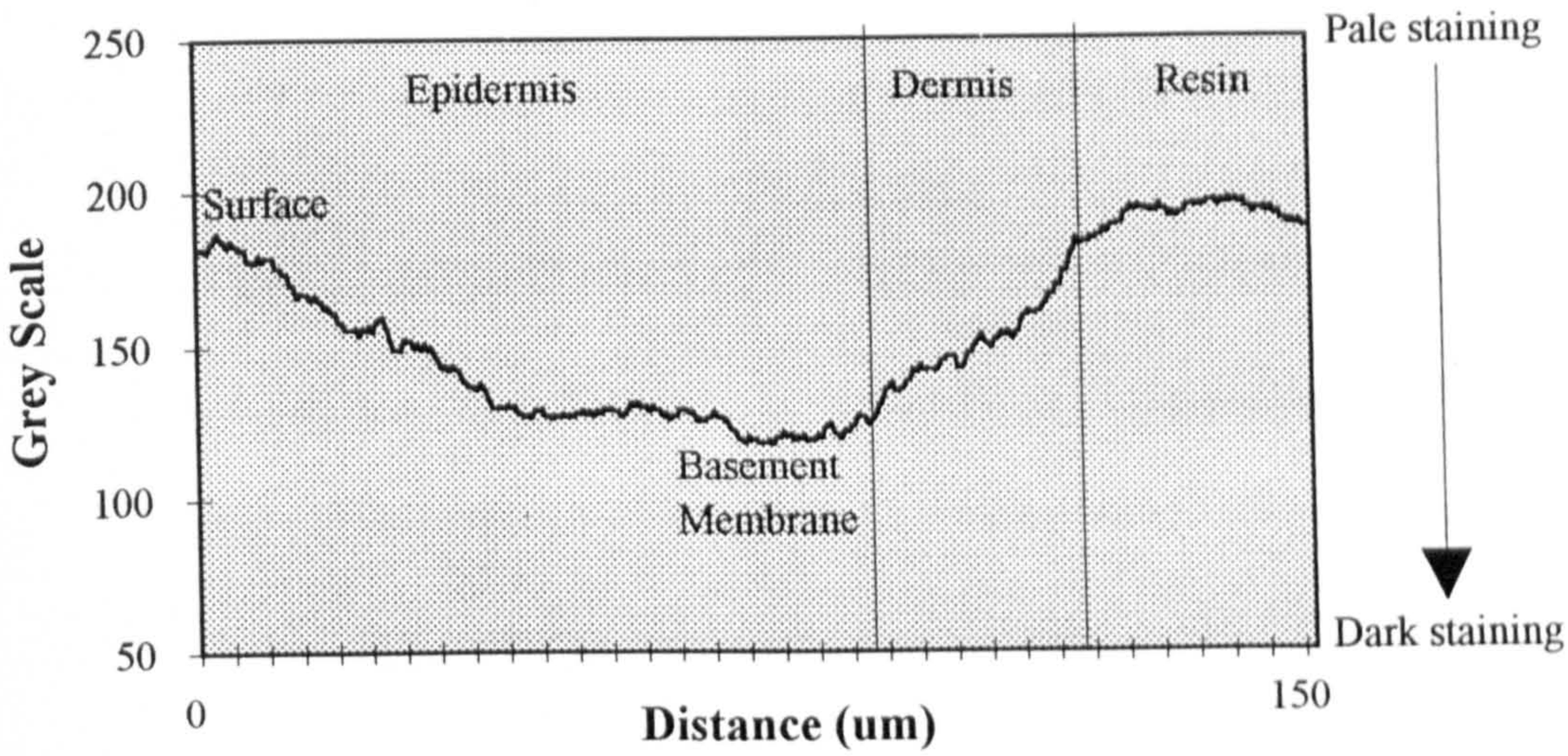
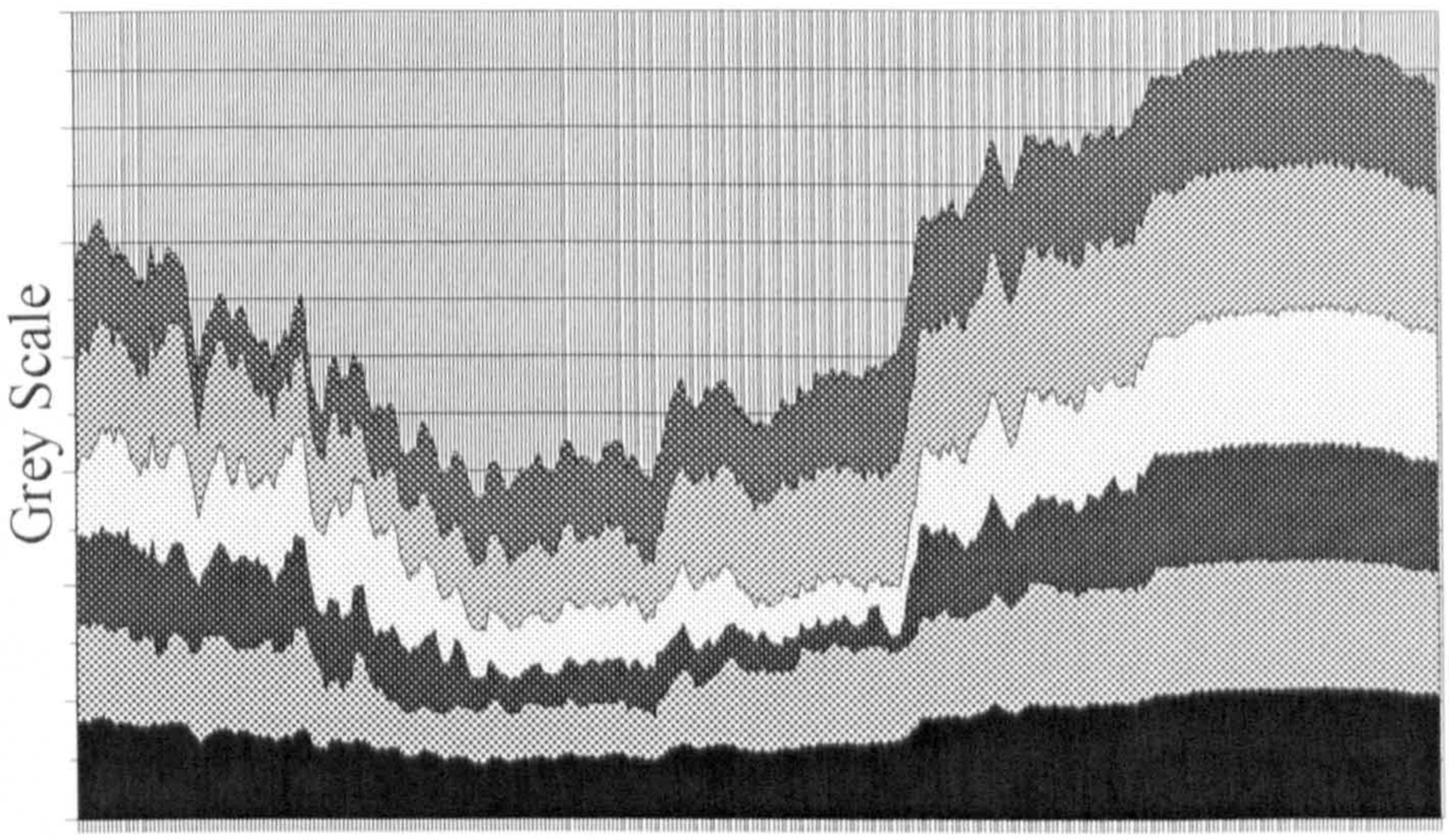

250
200
150
100
50
0
Grey Scale
Epidermis
Dermis
Resin
Surface
Basement
Membrane
Pale staining
Dark staining
150
Distance (um)

Figure 7.5 Representative grey scale plot of a VVS sample. Five individual readings were taken for each slide, and an average of these readings was calculated. The six resulting plots are shown stacked on top of each other, with the average at the bottom along the x-axis (black). The y-axis relates to gray scale, the range for each plot is 0-200 approximately.

Figure 7.6 Representative grey scale plot of a post-treatment sample from a VVS patient. Five readings were taken, and the average calculated, the five plots are shown stacked on top of each other in order to compare the profiles. The average plot is shown at the bottom of the graph in contact with the x-axis (black).

VVS (Sample 3)



Post Treatment (VVS7+)

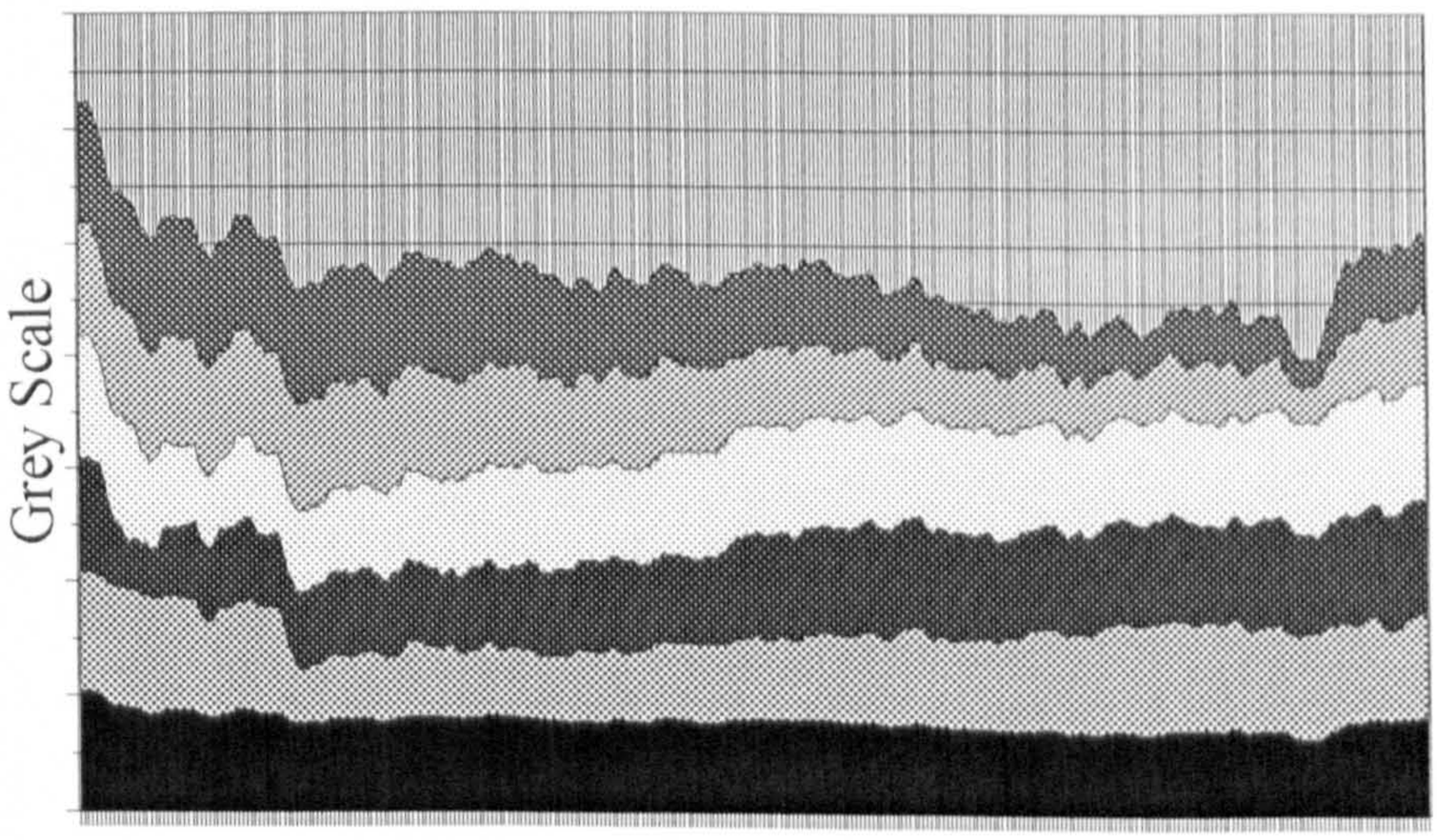


Figure 7.7 Representative grey scale plot from a control sample. The five individual readings were averaged to produce the plot which is situated at the bottom of the graph, in contact with the x-axis. The five plots are shown stacked together, to demonstrate differences in the profiles.

Control (Sample 7)

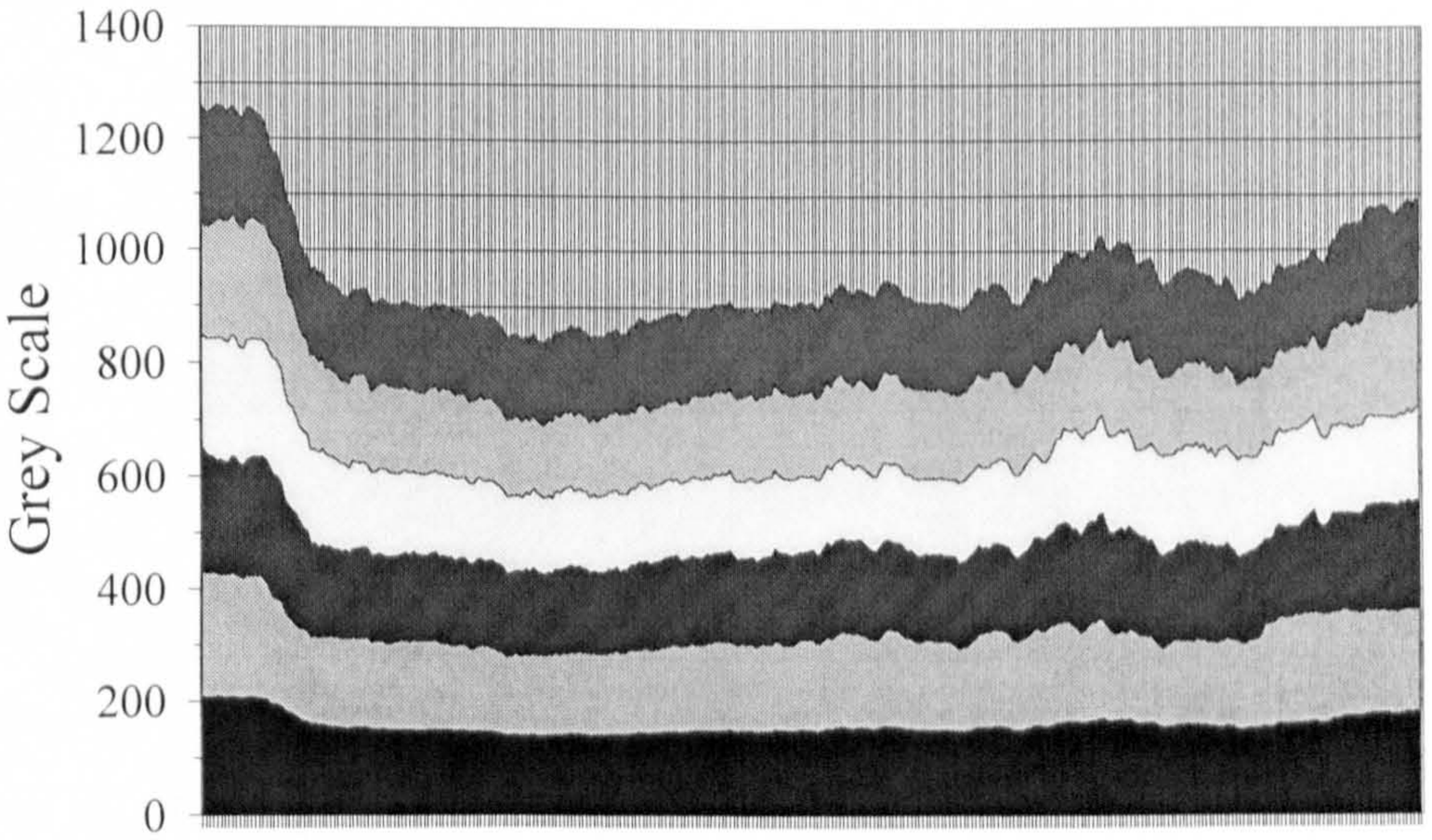
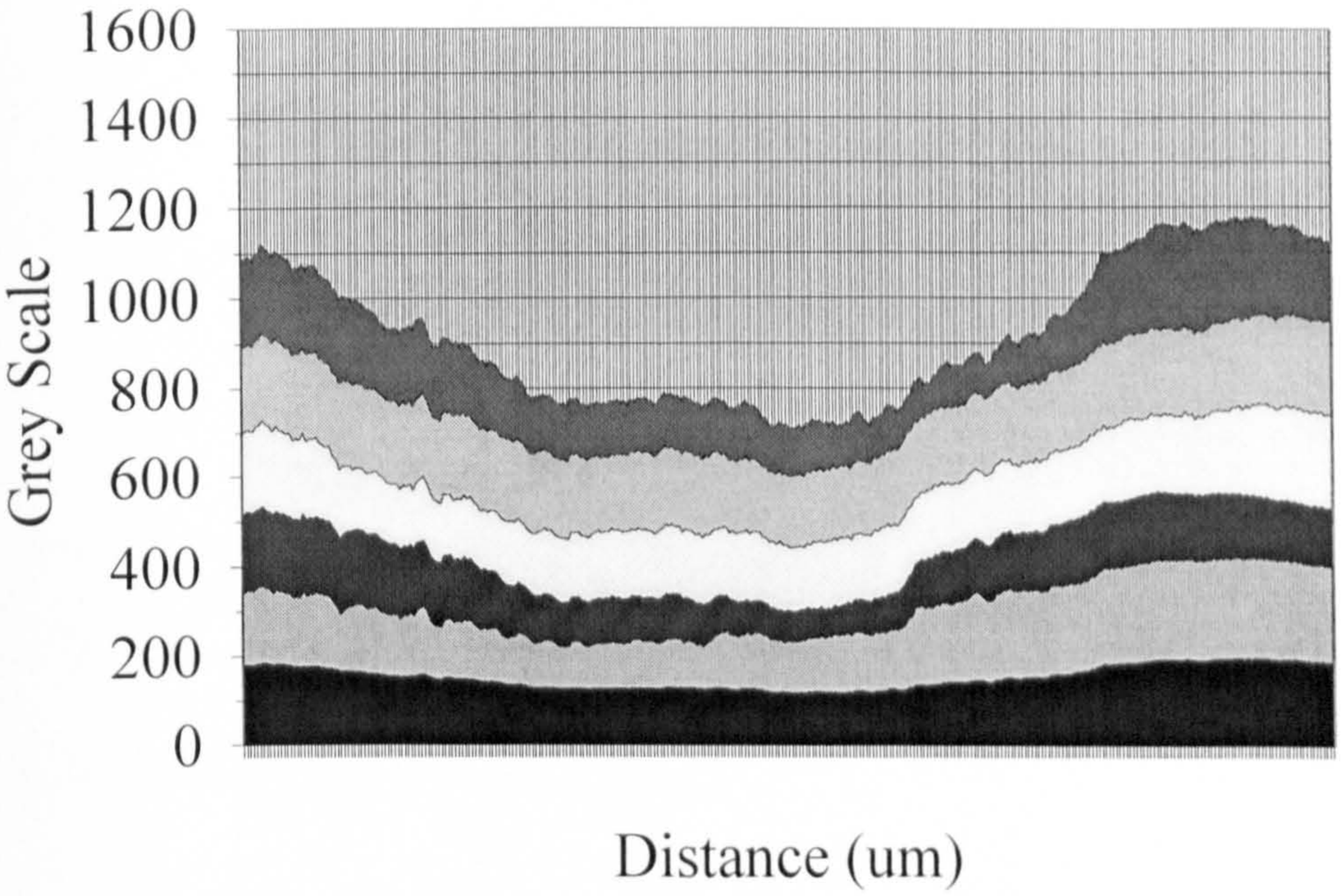


Figure 7.8 Grey scale plot of five VVS samples. The average values for each slide were plotted and the cumulative average calculated. The cumulative average is shown in contact with the x-axis.

Figure 7.9 Grey scale plot of five post-treatment samples. The average values for each slide were plotted and the cumulative average calculated. The cumulative average for the post-treatment samples is shown at the bottom of the graph.

VVS Cumulative Average



Post Treatment Cumulative Average

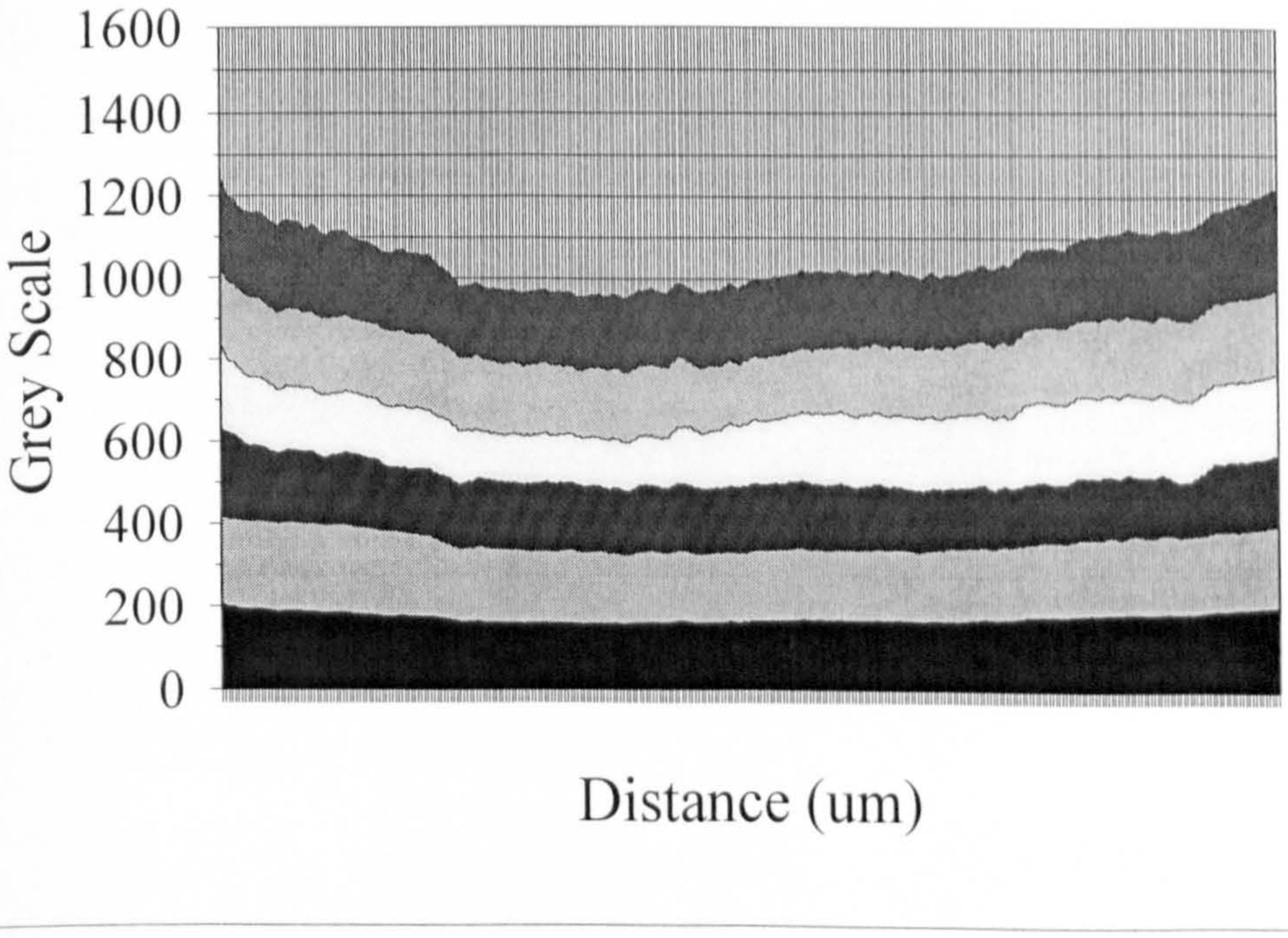


Figure 7.10 Grey scale plot of five control samples. Grey scale was measured using a box transect, the average readings for each slide were plotted and the cumulative average calculated. This plot is shown at the bottom of the graph in contact with the x-axis.

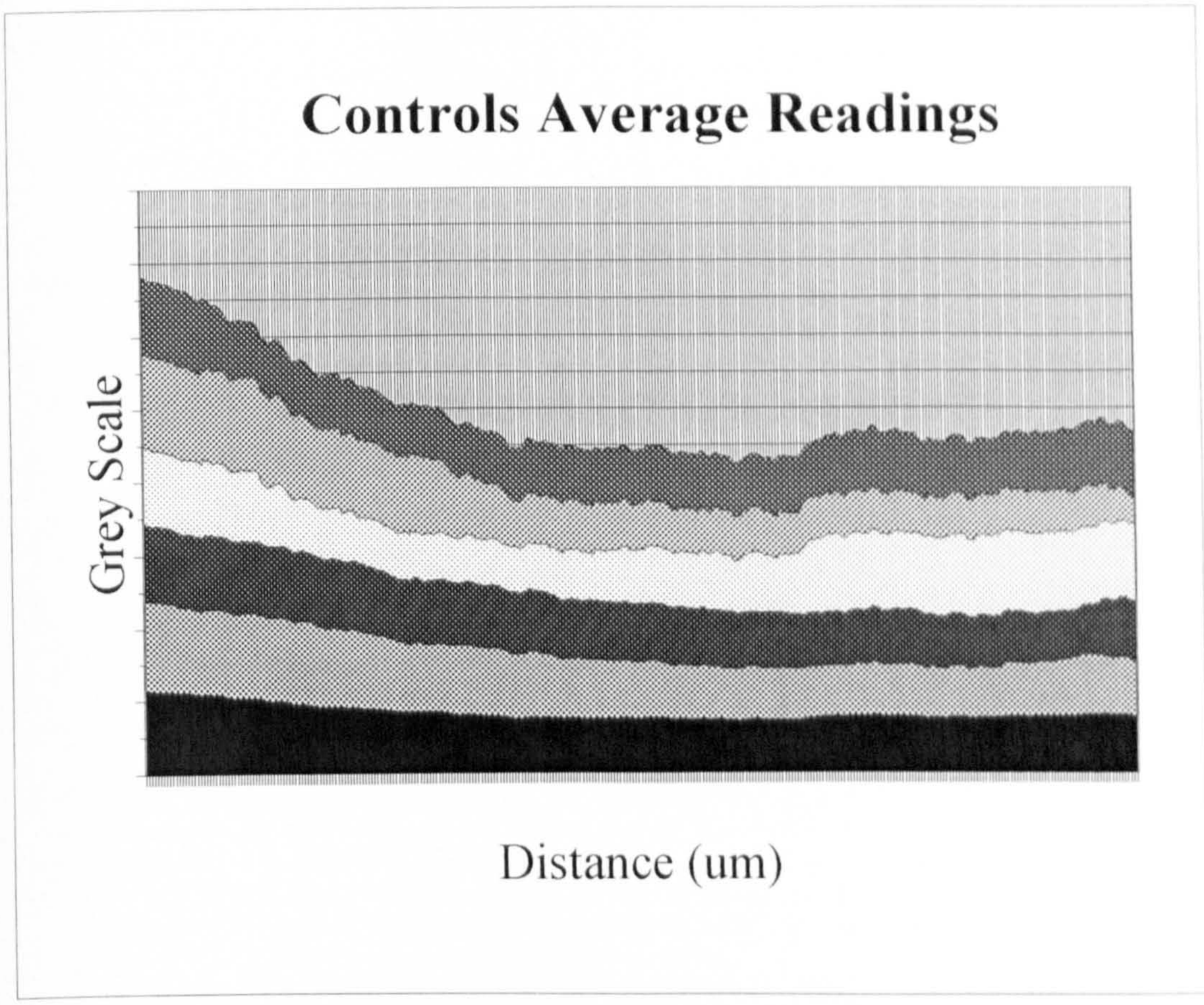
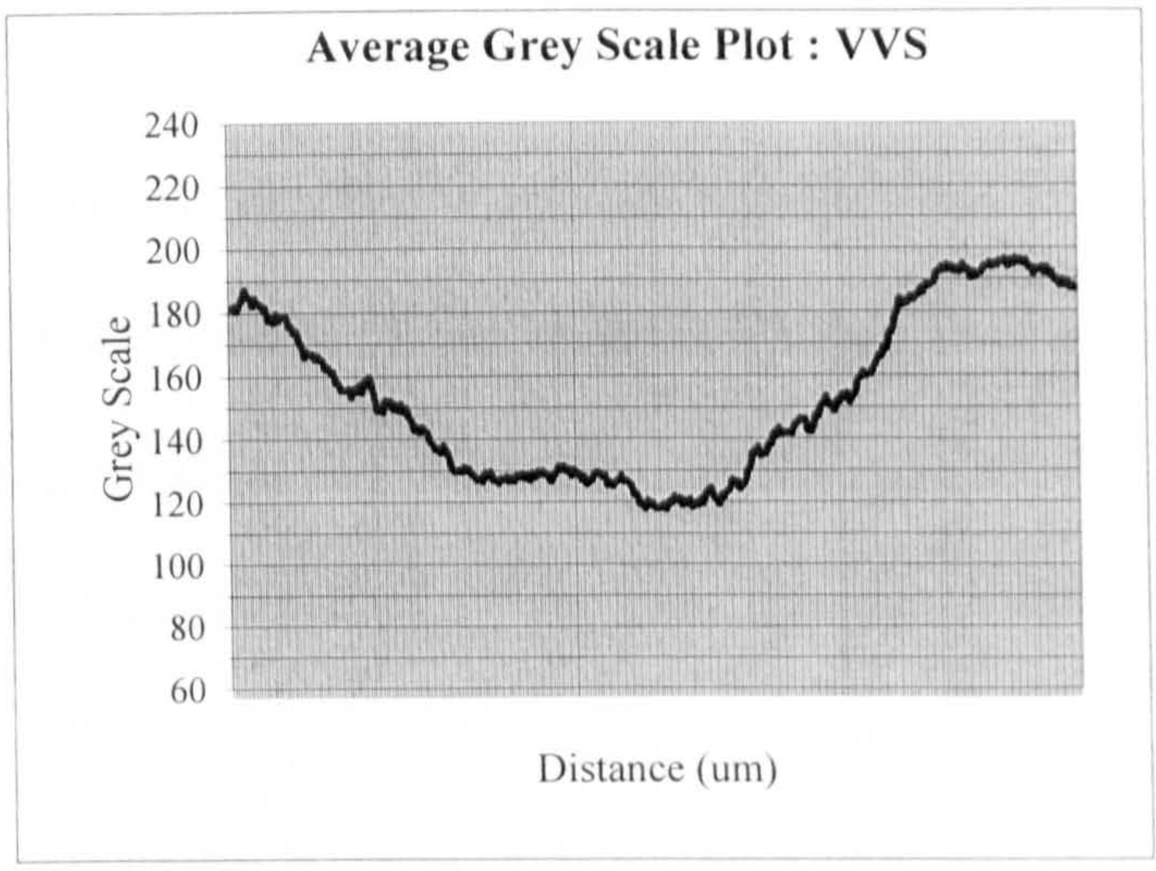
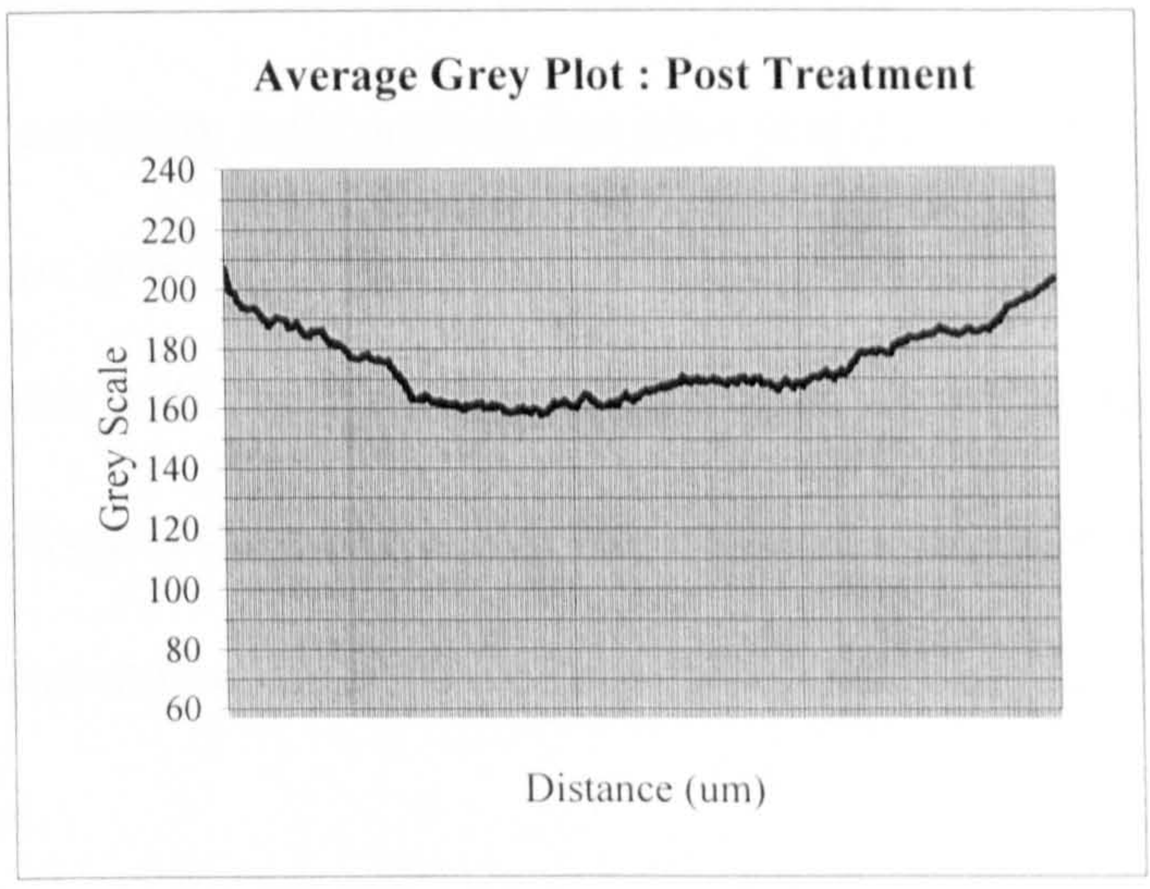

Controls Average Readings
Grey Scale
Distance (um)

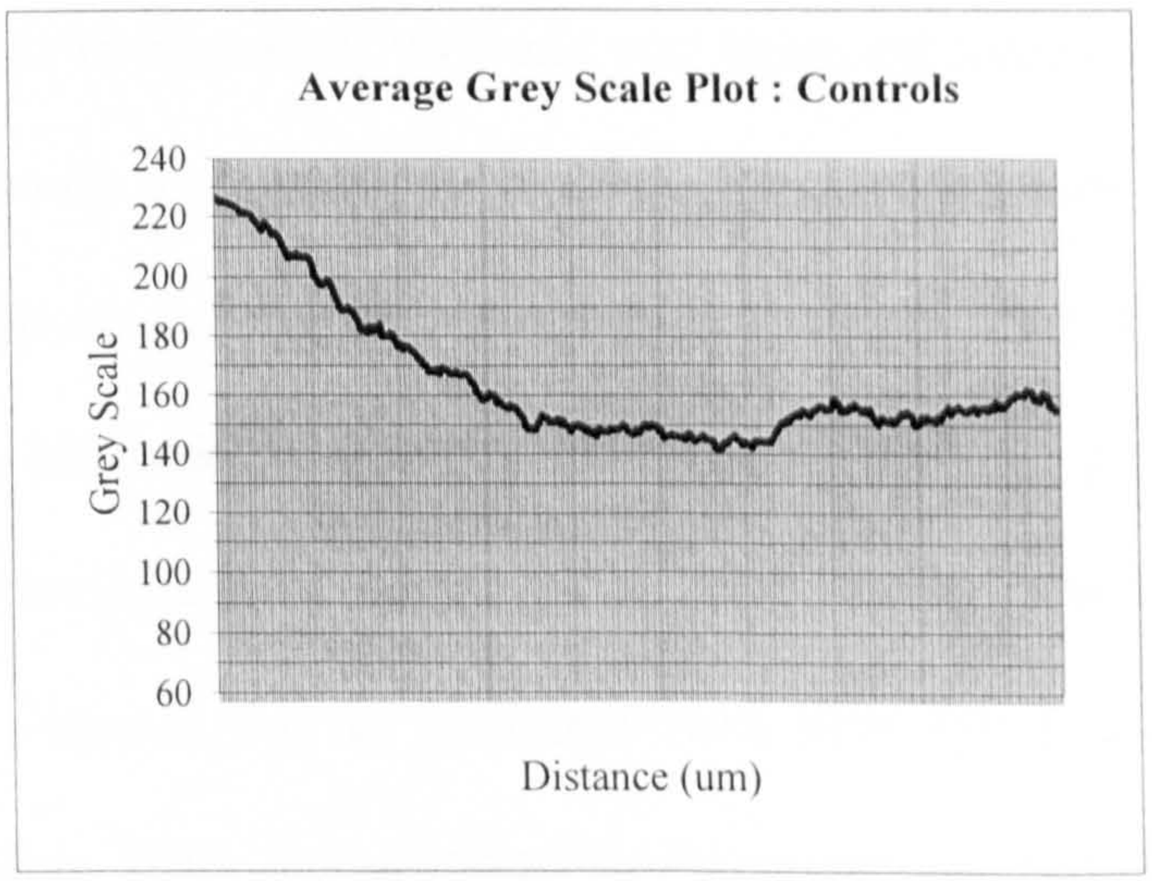
Figure 7.11 Line plot, illustrating the profile pattern obtained when the grey scale was measured across the epithelium. Representing the cumulative average reading for the VVS group.

Figure 7.12 Line plot of grey scale, illustrating the cumulative average reading for the post-treatment group.

Figure 7.13 Line plot of grey scale, illustrating the cumulative average reading for the control group of samples.


Average Grey Scale Plot : VVS
Grey Scale
240
220
200
180
160
140
120
100
80
60
Distance (um)


Average Grey Plot : Post Treatment
Grey Scale
240
220
200
180
160
140
120
100
80
60
Distance (um)


Average Grey Scale Plot : Controls
Grey Scale
240
220
200
180
160
140
120
100
80
60
Distance (um)

7.4 Discussion

The changes in vestibular epithelium due to VVS, which have been reported in the present investigation (Chapters 4 & 5),were unable to be quantified using TEM. As these were novel findings, not previously described in association with VVS, it was important to attempt some quantification of these results. It was anticipated, that the intensely staining cells seen using TEM, were apoptotic-like, and that these cells and associated structures were linked to the development or maintenance of vulvar pain associated with VVS. Investigations of the pathology of VVS are sparse; however, past investigations have found that VVS is characterised only by having a mild to moderate inflammatory cell infiltrate close to the basement membrane and surrounding the minor vestibular glands. Some authors have attempted to type the inflammatory cells present, but have found no evidence to link this condition to a particular disease process (Pyka *et al.*, 1988; Prayson *et al.*, 1995). The visualisation of apoptotic-like cells in this epithelium is, therefore, an important observation, and may be a key link to the pathology of the syndrome. It was necessary to demonstrate that these cells were in fact extremely common in the VVS and post-treatment samples, but were rarely visible in the control samples.

Leukocyte counts from control, VVS and post-treatment samples, demonstrated a significant difference between the groups ($p<0.0001$). It was confirmed that VVS samples had a significantly greater number of leukocytes in the epithelium than was evident in control samples. It was interesting to note that there was no difference in the number of leukocytes in the VVS and post-treatment samples. This may indicate, that there was no change in the inflammatory response due to treatment with ketoconazole (see Chapter 6). However, examination using TEM, demonstrated that the type of inflammatory cells present prior to treatment were different from those seen in the post-treatment samples (see Chapter 6). The use of immunological markers for various B and T cell types, and for Langerhans cells, may be

useful in elucidating change in the inflammatory response over time in this condition.

The pattern of the grey scale plots varied between the three groups, and also within the groups. The control plots were smoother in appearance than the VVS and post-treatment plots, and were generally tapered in appearance, becoming thinner through the transect. All of the control slides demonstrated a tapering of the plot consistent with a gradual increase in staining intensity towards the basement membrane. Some of the control samples did show areas where the plot was uneven, and where dark cells were expected to be found. However, this was not as pronounced as in the VVS samples where the plot had an extremely 'spiky' and uneven appearance (Fig. 7.5 - 7.7). The post-treatment samples showed similar grey scale plots to those obtained from the VVS samples. However, these plots demonstrated less variation in the vertical profile, i.e., were less 'spiky', and the change in staining intensity not so pronounced as in the VVS samples.

Samples from VVS patients produced an uneven plot, indicative of an epithelium with many dark cells. The average plot of the five slides in the VVS group demonstrates this extremely uneven 'spiky' appearance (Fig. 7.8). However, the variation in profile of the plot, indicative of change in grey scale and staining intensity, is seen more clearly in the individual slide plots, and in the line plot (Figs. 7.5 & 7.11). From these plots it can be seen, that there are areas in all of the VVS slides where dark cells are concentrated, this is illustrated by a decrease in the profile of the plot, which indicates a decrease in grey scale. In the VVS samples, the majority of the plots demonstrated a decrease in the profile, in the middle of the plot. This was also evident in the post-treatment and in the control samples (Figs. 7.6 & 7.12). However, the degree of change in the grey scale was the greatest in the VVS samples. All of the line plots were tapered in appearance, this was particularly clear in the control plot (Fig. 7.13), where a gradual decrease in staining intensity was observed moving from superficial cells to basal cells,

i.e., moving from the left, to the middle of the plot. This confirms the findings of light microscopy, where slides stained with 1% methylene blue, demonstrated a gradual increase in staining intensity from the surface towards the basement membrane. This change in staining was only affected by the presence of leukocytes in the epidermis, which stained intensely with methylene blue.

The variation in staining within a slide did not appear to be significant, however a statistical analysis would be required to confirm this observation. A one-way analysis of variance could be completed on the individual readings for each slide, to determine whether the variation within each slide is greater than the variation between slides. A statistical analysis would also be useful to determine whether the differences observed in the control and VVS samples were significant or due to chance.

The presence of leukocytes may also affect the grey scale and, therefore, the pattern of the area plot. The presence of a significant number of leukocytes in both the epidermis and the dermis in VVS and post-treatment samples, may result in an increase in the grey scale. Leukocytes were particularly evident in the dermis, immediately below the basement membrane, and in the basal and lower intermediate cell layers of the epidermis. This may account for the large increase in staining intensity illustrated in the cumulative line plot for VVS. The cumulative line plot for the post-treatment samples, was not however, consistent with this theory. The average post-treatment plot showed very little variation in staining intensity, and produced a more uniform plot than the control samples. It is possible that the intra-group variation in the post-treatment group was so great, that the cumulative average is a false representation of this group of samples. It was anticipated that the slides from the post-treatment group may produce a confused picture, as some of the response to treatment with ketoconazole was not uniform. Some patients responded quickly, and made a complete recovery over several months.

However, there were several patients in this investigation who did not see any decrease in vulvar sensitivity over four months. It may be the case, that samples from patients who responded well to treatment with ketoconazole, result in plots similar in profile to those obtained from control patients. In comparison patients showing a poor response to treatment may demonstrate a plot similar to that obtained from VVS samples. This is in part, consistent with the findings of the ultrastructural investigation, which indicated that there was a similar number of apoptotic-like cells present in the pre and post-treatment samples. However, it is obvious from the results of the ultrastructural investigation of the effects of ketoconazole, and the results of the image analysis, that post-treatment samples may be more variable than first anticipated. This finding is also consistent with the correlation data, which demonstrated that there was only moderate correlation within the post-treatment group when compared with the VVS and control groups (Tables 7.2 - 7.4).

Image analysis has proved useful for both the quantification of leukocytes in the tissue sections, and for the determination of grey scale indicative of dark cells. There are however, several drawbacks to the use of image analysis in this context. The resin sections, although stained for the same amount of time and viewed using the same level of illumination, demonstrated variations in the staining intensity of the resin. This may be due to the vagaries of resin polymerisation, and it was anticipated that this would affect the results obtained. Consequently a large sample number would be required in order to sufficiently diminish variations due to variable resin staining. The measurement of grey scale to detect dark cells, will also detect leukocytes in the epidermis and dermis. These were more common in VVS and post-treatment samples than in the control samples, and consequently the presence of these leukocytes may accentuate the differences between these groups.

The use of the image analyser to quantify differences between control, VVS and post-treatment samples, may be improved by the use of wax embedded sections. Although sections from each group would need to be stained side by side, the use of stains for light microscopy, such as Giemsa or Mallorys trichrome would be more specific and easier to control. This may reduce the degree of staining variation, and prevent background staining which was observed in many of the resin sections in the present investigation. Alternatively methacrylate, LR white or Lowicryl resins could be used, which would provide excellent staining and contrast for image analysis. The use of histological staining techniques may also allow other parameters to be investigated, such as the number of Langerhans cells, the number and distribution of leukocytes, and also a more accurate analysis of the number of apoptotic-like cells. Fluorescence microscopy, or immuno-gold labelling may also be used in conjunction with image analysis, to quantify the amount of a particular protein or antigen in the tissue.

Another use of image analysis which may be viable in future investigations of VVS, included the estimation of the volume fraction (V_V) of the epithelia taken up by dark staining, possibly apoptotic cells. In order to do this, tissues could be embedded in methacrylate resin to enhance section staining. The area of the section occupied by the dark staining cells, could be investigated using a threshold, or detect setting after image processing. A high pass filter which exaggerates differences, and sharpens and accentuates the image would be useful for this technique, and would indicate the number and position of the dark staining cells in the epithelium. This would not have been of use for the measurement of grey scale, as high pass filter accentuates differences which were not really apparent, therefore, producing false data by increasing noise.

Image analysis may also be utilised for the measurement of other features visualised using TEM. One characteristic feature of VVS samples which may be quantified using image analysis is the number of cytoplasmic lobules present, as these structures appear to be related to the presence of dark staining, apoptotic-like cells. These membrane bound cytoplasmic lobules may be by-products of apoptosis, however, they may also be dendritic processes of Langerhans cells. Image analysis may permit the quantification of these structures in TEM negatives from VVS samples, and allow a correlation to be made between the presence of dark staining cells and the number of cytoplasmic bodies. If no correlation is evident, and the cytoplasmic bodies are not positive for immunological markers for apoptosis, staining for Langerhans cells may elucidate their origin. This could be performed using fluorescence microscopy, which provides excellent contrast for image analysis. Alternatively, this analysis could be performed by direct image analysis on the Jeol 2000FX TEM, using the facility on the X-ray analysis package. This could also be completed by collecting digitised images on the TEM, and analysing them using the Quantimet 570.

In conclusion, the use of image analysis has permitted the quantification of the inflammatory response in terms of leukocyte numbers, and has demonstrated differences in the grey scale in control and VVS samples which may be due to the presence of apoptotic-like cells in the VVS samples. The presence of apoptotic-like cells should be investigated further with the use of immuno-cytochemistry, and should involve an larger study group in order to minimise the variation within this group of patients. Markers for apoptosis, such as Bcl2 or Bax could be used in order to detect cells which are prone to programmed cell death, or cells that are in the process of dying. As outlined above, image analysis will be important in further investigations of VVS, where it may be used for the quantification of cytoplasmic bodies, leukocyte types, and for the quantification of immunochemistry of apoptotic cells.

CHAPTER EIGHT

General Discussion

A review of the current literature on VVS illustrated that a majority of the work on VVS has emanated from the USA, and that a high percentage of these studies were treatment orientated. A majority of past investigations, have focused on the detection of HPV in VVS biopsies, and subsequent treatment with interferon (Friedrich, 1988; Horowitz, 1989; Hatch, 1991; Bornstein *et al.*, 1991, 1993; Waltzman & Wade, 1991). Very few of these investigations intended to examine the vestibular tissue in detail, which has resulted in a paucity of reports describing the inflammatory response associated with this condition. Pyka *et al.*(1988), and Prayson *et al.* (1995), have completed the most extensive studies of the pathology of VVS, but have found very little to indicate the involvement of a particular infectious agent or allergen. Past reports of the inflammatory response are consistent, and indicate that VVS is characterised by a mild to moderate inflammatory cell infiltrate, which is dominated by lymphocytes and plasma cells. The degree of inflammation, the type of inflammatory cells present, and the distribution of these cells has not been attributed to a particular disease process, or immunological reaction (Pyka *et al.*, 1988).

One of the initial aims of the investigation was to ensure that the study cohort of VVS patients described similar symptoms, and gynaecological histories when compared with VVS patients described in previous investigations (Goetsch, 1991; Marinoff & Turner, 1991, 1992; Bazin *et al.*, 1994). The epidemiological characteristics of the VVS patients in this study were described in detail in Chapter 3, this chapter also demonstrated the value of assessing vulvar sensitivity using the 'Vulvar Algesiometer '.

Past investigations have concentrated mainly on the epidemiological characteristics of their VVS patients, and only one of these studies has included a control group for comparison of data (Goetsch, 1991). Despite being a small pilot study, the present investigation included a control population of women, and explored several new areas which have not previously been examined. New approaches have included an examination of the use of self treatment, the perception of pain, and treatment by clinicians. The VVS patients were found to be similar biographically to patients described in other studies; all were white, an average age of 27.7 years, and commonly in stable monogamous relationships. The VVS population in the present study did not commonly develop VVS post partum, and 68.2% of the VVS patients were nulliparous. This investigation demonstrated several differences between the control and VVS group, particularly in relation to the history and location of past irritations, the description of the pain experienced, and inconsistencies regarding the diagnosis by GPs. It was concluded, that many of the questions included in this pilot investigation, may be omitted in a larger investigation, as the questionnaire covered too broad an area. Areas of particular interest, which could be expanded in a full scale investigation, included: the type and use of oral contraceptives, the incidence of cystitis and urogenital syndromes, a detailed history of past infections and irritations, and the treatments prescribed. Vestibulitis patients would be asked more detailed questions regarding their perception of pain, and would be provided with charts and pain scales, to map cyclic variations and pain intensity over several months.

The utilisation of a pain assessing instrument, the 'Vulvar Algesiometer', in accordance with the questionnaire, and anatomical investigation, formed a novel and balanced approach to the study of VVS. Although the measurement of vulvar pain is extremely subjective, there is no alternative means available as yet, for assessing the severity of VVS, or the improvement in vulvar sensitivity in response to treatment. Despite variations in pain perception between patients, and in individual patients over time, this means of assessment provided excellent

results. The initial, cumulative readings from control and VVS patients were significantly different, the mean cumulative score for control patients was 26.5 ± 4.85, whereas VVS patients demonstrated an average score of 6.9 ± 5.89. Vestibulitis patients were assessed over a four month period, during which time they were treated topically with 2% ketoconazole cream (Nizoral, Janssen Pharmaceuticals, Belgium). The cumulative readings over three months were significantly different, and vulvar sensitivity was seen to improve steadily over this time. However, after three months treatment with ketoconazole, there was no further significant decrease in vulvar sensitivity. There was also no significant difference in the readings of patients who received treatment with imipramine in addition to ketoconazole, when compared with patients who received just ketoconazole. This was surprising, as it was anticipated that even a small dose of imipramine may be sufficient to alter the patients perception of pain, or the degree of stress, both of which may alter the reported level of pain. Algesiometer readings from the Skenes' and Bartholins' glands also showed variation. Commonly readings were significantly lower on the Bartholins' glands than on the Skenes' glands ($p=0.0034$). This is consistent with past investigations, which have often described 'membraneous hypertrophy' or 'acute sensitivity' of the Bartholins' glands in cases of VVS (Michelwitz *et al.*, 1989; Barbero *et al.*, 1994). The information obtained from the assessment of patients using the algesiometer was valued by both the clinician and the patient. Consequently, the assessment of VVS patients is continuing in several G.U.M. clinics in the S.W. region, where some patients have been assessed for eight months or more.

This study has indicated many of the potential difficulties involved in the study of a gynaecological condition, particularly one with an unspecified cause. Despite this, several interesting pathological characteristics of VVS have been described, which have not previously been reported. These findings centre around the presence of apoptotic-like cells, which were visualised using electron microscopy. The description of these cells and

associated ultrastructural characteristics, may provide a platform for future studies of VVS.

The ultrastructure of the normal vulva had not been described previously, and as this investigation required a detailed knowledge of normal vestibular epithelium, this became a primary aim of the investigation. The ultrastructural of the normal human vulva, and the differences in appearance due to the effect of VVS formed the main focus of this investigation the results of which are illustrated in Chapters 4 & 5. As detailed in Chapter 4, the vestibular epithelium from control patients was similar in appearance to mucosal-like epithelia (Burgos & Roig de Vargas-Linares, 1978; Squier, *et al.*, 1976; Landay & Shroeder, 1977, 1979; Squier, 1977; Shroeder, 1981; King, 1983; Corbeil *et al.*, 1985). There was no keratinisation of the surface cells, and no granular cell layer or spiny cell layer as in true skin (Breathnach, 1971, Ghadially, 1988). The epithelium of the vestibule was found to be composed chiefly, of a large population of intermediate cells, which had sparse desmosomal junctions, and pale staining cytokeratin filaments. Surface cells were flattened, and the cytoplasmic membranes of these cells slightly thickened. Large glycogen aggregates were found in upper intermediate and superficial cells of this epithelium, which were characteristically seen in a halo around the nucleus. This was an unusual finding, as glycogen is rarely stored in skin, except in fetal tissue or in response to a disease process (Falin, 1961). Leukocytes, which were seen in all cell layers were isolated cases; the predominant inflammatory cell present in a majority of vestibular biopsies was the lymphocyte. Scanning electron microscopy illustrated surface cells which were polygonal in shape, with fine, raised intercellular junctions. The epithelial surface was covered with a complex interdigitating network of rounded microridges, of varying length, orientation and distribution.

When examined using TEM, vestibular epithelium from VVS patients demonstrated several striking attributes which were not evident in vestibular tissue from control women; the

characteristic features of VVS affected epithelium were described in detail in Chapter 5. The presence of dark staining, shrunken epithelial cells, visible throughout the epithelium were particularly distinctive; these cells were found to correspond to dark staining cells evident in semi-thin sections stained with methylene blue. Using TEM, these cells appeared individually, and in foci, and were commonly associated with leukocytes. In close proximity, membrane bound, cytoplasmic lobules were evident, which were not commonly seen in the control biopsies. It was initially anticipated, that these cytoplasmic bodies may be dendritic processes of Langerhans cells. However, on further examination of VVS samples, these structures were numerous, and when serial sections were examined these lobules did not appear to have a cellular origin.

An alternative, and exciting hypothesis for the presence of these intensely staining cells in VVS biopsies, involves the process of apoptosis (Wyllie *et al.*, 1984; Arends & Wyllie, 1991; Cohen, 1993a, 1993b; Collins & Rivas, 1993). Dark staining epidermal cells, demonstrated many of the features characteristic of apoptotic cells. Numerous epidermal cells in the VVS biopsies appeared shrunken, with condensed nuclear material, but with intact organelles and cytoplasmic membranes. Cytoplasmic lobules could also be accounted for within the theory of apoptosis, as during this process, dying cells are packaged into membrane bound bodies to prevent the initiation of an immune response. Despite several features in common with apoptosis, the nuclear changes characteristic of programmed cell death were not evident in the majority of the dark staining epithelial cells found in VVS biopsies. However, consistent with apoptosis, cytoplasmic membranes remained intact; and membrane bound cytoplasmic bodies were commonly found engulfed by epidermal cells, and in the process of being phagocytosed by leukocytes.

The main factor contradictory to the demonstration of apoptosis, was the presence of a mild to moderate inflammatory cell infiltrate, which was significantly more pronounced in the VVS samples than in the controls (Chapter 7). The initiation of an immune reaction is not a characteristic feature of apoptosis, a process which does not cause any adverse, or long term damage to the tissue. Therefore, the presence of a significant number of inflammatory cells may suggest an alternative or adapted form of apoptosis which permits a mild inflammatory reaction. Alternatively, the presence of apoptotic-like cells, and numerous leukocytes, may suggest two different processes operating independently in response to the same, or different stimuli. These processes may be associated, as in some VVS samples with a mild infiltrate, the number of apoptotic cells was not as great as in biopsies with a large inflammatory cell population. No attempt was made to establish a correlation between the intensity of the inflammatory response, and the number of apoptotic-like cells present, however this may be attempted in future investigations.

Scanning EM of VVS samples illustrated a similar pattern of microridges as seen in the control samples. However, the vestibular biopsies demonstrated slightly shrunken microridges, and areas where these structures were absent. In addition, small pore-like structures were seen, which resembled the openings of goblet cells. Using light microscopy, these structures were seen to be glandular in appearance. It was concluded that these structures were probably small minor vestibular gland openings, which were not evident in control samples. Biopsies taken from VVS patients were removed very accurately from beside the Skenes' glands with the aid of a colposcope. This may account for the increased number of these structures in VVS samples. This was a particularly interesting observation, as there have been few reports on the frequency of minor vestibular glands in the vestibule, or the appearance of these structures using SEM. Low temperature SEM, although not successful

in this study, may be useful in future investigations to clarify any changes in the microridge appearance in response to VVS.

Past pathological investigations of VVS have involved the assessment of vestibular biopsies only when patients initially present with idiopathic vulvar pain. There are no reports commenting on the change in the inflammatory response over time, or in response to treatment. In this investigation, patients were treated with a topical antifungal preparation, ketoconazole, which was reported to be a mild LTB_4 inhibitor (Beetens *et al.*, 1986). Treatment with ketoconazole appeared to have very little effect on the ultrastructural characteristics of VVS, however, some subtle changes were detected (Chapter 6). Dark staining cells were seen in similar numbers when compared with the pre-treatment samples, however, these cells were extremely vacuolated and were commonly surrounded by abundant cytoplasmic bodies. Lymphocytes and NK-like cells were frequent in the epidermis, in close contact with both cytoplasmic bodies, and with apoptotic-like epithelial cells. NK-like cells had a slightly lobed nucleus, abundant mitochondria and a well developed Golgi body, occasionally dark staining granules were seen in these cells. Alternatively, these leukocytes may have been large granular lymphocytes. In the dermis, plasma cells were less frequent, and macrophages were more common when compared with the pre-treatment samples. Frequently, macrophages were seen in the dermis, with pseudopodia extended, and in contact with groups of cytoplasmic bodies. The inflammatory response may be the result of a cell signalling error, resulting in the initiation of an inflammatory response out of context, as no etiological agent was evident in a majority of cases. Alternatively, an etiological agent or antigen may be present which has not yet been detected.

The frequency of dark staining cells and the number of leukocytes in the control and VVS samples were examined in Chapter 7. It was demonstrated, that there was a highly significant

difference in the numbers of leukocytes in the VVS and control samples. However, there was no difference in leukocyte numbers in the biopsies from VVS patients removed pre and post-treatment. This was anticipated, as the inflammatory cell infiltrate was seen to be of a similar intensity in a majority of VVS biopsies before and after treatment. However, the type of inflammatory cells present appeared to be different. This substantiates past reports which reported an inflammatory cell infiltrate in VVS biopsies, and refutes claims that VVS is predominantly a psychosexual problem (Schover *et al.*, 1992).

The assessment of the staining intensity of vestibular epithelium was problematic. Once the presence of apoptotic-like cells was established, the main intention of this experiment was to quantify the number of dark cells in the epidermis using image analysis. It was anticipated that an increased number of dark staining cells in the VVS samples would alter the staining intensity, and subsequently the pattern of the grey scale plot obtained. Although the sections were stained for an equal time, and at a preset level illumination, there were variations in resin staining which were unavoidable. If larger tissue samples were available, wax sections of vestibular tissue from control and VVS patients would be examined using alternative staining techniques, allowing various parameters to be measured more accurately, such as leukocyte types, numbers of Langerhans cells, and glycogen content of the epithelium. Image analysis has good scope for the assessment of VVS biopsies using TEM. Other approaches using this technique, may include an assessment of the density of cytoplasmic lobules present in the VVS samples. Additionally, the microridge structure and distribution could be investigated using the quantification facility available on the Jeol 2000 FX transmission electron microscope.

The use of TEM has proved invaluable in the study of VVS, and has illustrated significant changes in the ultrastructural characteristics of this epithelium, which appear to be due to

VVS. However, with the information resulting from this investigation, it is now essential to develop histological techniques to investigate these characteristic findings in more detail. Immuno-cytochemistry may be of particular use in future investigations of VVS. In particular, the presence of markers linked to apoptosis should be examined, such as Bcl2 and Bax, to determine whether the dark staining cells are actually undergoing apoptosis. The application of LTFS for TEM, once perfected, has the benefit of maintaining the antigenicity and natural structure of the tissues. This technique will permit the use of immuno-gold labelling for TEM in order to accurately locate markers of apoptosis. The nature of the cored and electron lucent vesicles seen in VVS samples could also be investigated using this technique (Bailly *et al.*, 1992). The change in the inflammatory response should also be characterised, as this may provide clues to the progress of the syndrome, and the action of the treatments used. As in the studies completed by Pyka *et al.*(1988), and Prayson *et al.* (1995), the characteristic cells of the immune response could be detected using a range of T and B cell markers, however, these studies have indicated this approach to be of little value. The presence of NK cells and macrophages in post-treatment samples could also be quantified to demonstrate any trends present. The origin of the membrane bound lobules should also be investigated to ensure that they are of epidermal cell origin. If they do not prove to be remnants of epidermal cells, the possibility of dendritic or Langerhans cell processes should be investigated further using LM or immuno-gold labelling at the TEM level (Hanau *et al.*, 1986).

Another interesting observation ensuing from this investigation, has involved the initiation of symptoms associated with a 'urogenital sinus' syndrome, in patients who have been treated successfully for VVS. Patients with VVS were found to suffer with cystitis at a higher frequency than control patients (see Chapter 3). The correlation between interstitial cystitis, urogenital syndrome and VVS has been investigated by several authors, however, the results were inconclusive (McCormack, 1990; Fitzpatrick *et al.*, 1993; Foster *et al.*, 1993). Several

samples of trigone epithelium were obtained during the course of this investigation which were processed for TEM. The trigone of the bladder is derived from the same embryonic origin as the vestibule of the vulva, and consequently may show similar changes in response to VVS. These biopsies demonstrated some unusual findings, however, no control biopsies were available for comparison. Future investigation of patients with VVS would include the assessment of patients for urogenital problems; patients who did demonstrate urethral discomfort with no obvious etiological cause should be biopsied, and the tissue investigated using LM and TEM.

This study has resulted in a step towards the understanding of the pathological process involved in VVS. However, as in a majority of investigations, many more questions have been raised than have been answered. It is probably true that the initiating factor in the development of VVS is multifactorial. However, the maintenance of the immune reaction, resulting in vulvar pain associated with VVS may be due to a common factor. The possibility of an autoimmune type reaction, such as in bullous skin conditions affecting the vulva, should not be ignored (Marren, 1993). It may be the case that apoptosis is occurring in response to an autoimmune stimulus, and is affecting large numbers of epidermal cells which express a particular antigen (Tan 1994). The presence of a moderate inflammatory cell infiltrate, may not be linked to the process of apoptosis. If apoptosis is operating on a large scale as anticipated, why are inflammatory cells more common in VVS than in control samples? Is the cellular damage a result of the inflammatory response, and if so, why does the tissue show no effects of disease on a macroscopic scale?

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APPENDIX ONE

Ethical committee approval

PLYMOUTH
HEALTH AUTHORITY

DERRIFORD HOSPITAL
DERRIFORD ROAD
PLYMOUTH
PL6 8DH

Telephone: Plymouth: (0752) 777111
Extensions: HMSC Chairman : 2640
HMSC Hon. Secretary : 52045
HMSC Committee Clerk : 2641

Your Ref: *Our Ref:* MTI/kw/LREC 8 February 1994

Please address all correspondence to the above-named Department

Dr G D Morrison
Consultant in Genito-Urinary Medicine
Freedom Fields Hospital
Plymouth

Re: Investigation of Vulvar Vestibulitis Syndrome for Ultramicroscopy pre and post treatment with Ketoconazole.
Plymouth Study No 446

Dear George

Thank you for your letter of 31 January 1994 enclosing copy of the DDX certificate for the above-named study and for confirming that the University of Plymouth will meet the histopathology costs.

I am pleased to take Chairman's action to give approval for this study to commence in Plymouth.

The Committee would be pleased to have a report on progress and outcome in due course.

Best wishes.

Yours sincerely

M T Inman
Chairman
Plymouth Local Research Ethics Committee

[redacted] (for the attention of Clinical Trials Officer)

APPLICATION TO THE PLYMOUTH LOCAL RESEARCH ETHICS COMMITTEE FOR APPROVAL OF A STUDY INVOLVING HUMAN SUBJECTS
APRIL 1992

INSTRUCTIONS

Investigators are advised to check that this is the current version of this application form.

(a) Please complete in TYPESCRIPT.

(b) Send 11 COPIES (collated) to: The Chairman
Local Research Ethics Committee
HMSC Office
Level 7
Derriford Hospital
Plymouth PL6 8DH

The Plymouth Local Research Ethics Committee meets on the second Tuesday of each month, except August. Applications should be with the Chairman at least two weeks before that date.

(c) If there is insufficient space on the form to answer any question(s), please attach your answer(s) on separate sheet(s). All applicable questions should be answered; it is not sufficient just to say "see Protocol." Please use the symbol "N/A" where a question is not applicable.

1. TITLE OF PROJECT

A pilot study of vulvar vestibulitis (vvs) its prevalence, social sequelae and associated conditions.

2. (a) Applicant - names and addresses, position, qualifications and functions of all involved.

Dr E J Chandler, Principal Lecturer in Sociology, University of Plymouth, Department of Applied Social Science, Drake Circus, Plymouth, PL4 8AA. Ph.D. Director of research project.

(b) ALL other workers' names and addresses etc.

Ms. Carole Sutton, Lecturer in Computing, University of Plymouth, Department of Applied Social Science, Drake Circus, Plymouth, PL4 8AA.

Ms P Sargent, Postgraduate Research Student, Department of Biological Sciences, 410 Davy Building, University of Plymouth, Drake Circus, Plymouth, PL4 8AA.

(c) State the personal experience of the applicant (and, if different, that of team members involved) in the field of investigation concerned.

Extensive experience in conducting and managing social science research projects.

(d) Which of the above is co-ordinating the study?

(e) If the applicant is not a Consultant (or equivalent) give the name and status of the person taking responsibility for the study.

Dr. George Morrison, Consultant in Genito-Urinary Medicine, Freedom Fields Hospital, Plymouth, PL4 7JJ.

3. Where will the research be done?

The GU Clinic, Freedom Fields Hospital & 2 family Planning Clinics in GP Practices.

4. Purpose of the research:

(a) To which of the following does it relate: Diagnosis, therapy, prevention or understanding of disease or function?

The understanding of the disease and the effectiveness of treatment.

(b) Is it a pilot study or a definitive one? Single centre or multi-centre?

Pilot study in a single centre.

(c) Approximately how long will the study take to complete?

9 months

(d) Please state the precise aims of the study (what questions are to be answered and what is the value of answering them).

i) to gain more information about the prevalence of VVS in the general population.

ii) to establish the relationship between VVS, obstetric history, sexual behaviour, hygiene regimes and associated conditions of Candida infections & papilloma warts.

iii) to test/develop an interview schedule to be used in a larger scale study.

5. Design of the study - what will be done, what results are expected, and how the results will be analysed or evaluated. Include 11 copies of the full Protocol, including any questionnaires to be administered. (Please note Draft Protocols are not acceptable).

Copies of protocol and questionnaire enclosed.

6. Give the inclusion and exclusion criteria for choosing patients/subjects.

As there will be a census of women attending the GU clinic, all subjects will be included. The sample of women aged 20-35 in the Family Planning Clinics will be randomly selected. No criteria other than age gender will be used for selecting the sample.

7. How many patients/subjects do you expect to enrol locally?
Total number of subjects = 75.

8. How was the number of patients/subjects decided on?

There are currently 25 patients registered with this disorder at the GU clinic. These are to be matched with 2 groups of 25 subjects, with each group drawn from family planning clinics located in different socio-economic areas. This should permit an analysis of sufficient statistical significance to support a larger study.

9. How will the patients/subjects be recruited:

(a) What sources of patients/subjects will be used?

The GU Clinic and 2 Family Planning Clinics.

(b) How will they be approached?

They will be written to, informed about the research and invited to participate.

(c) Do they have a special relationship with the investigator? e.g. student or subordinate staff.

No

(d) Is written information to be given to them? (Please attach).

Yes - Enclosed in protocol.

10. (a) Please attach the consent form to be used. Consent should normally be WRITTEN. If consent is to be verbal, please give a justification.

The consent form will be incorporated in the information given to subjects and this is enclosed in the protocol.

(b) Please give a justification if, in any circumstances, consent is to be given by someone OTHER than the patient/subject.

11. If the subjects are in an especially vulnerable group (e.g. Children under 16, mentally handicapped etc.) please complete the following sections:

(a) In what way, if any, can the proposed study be expected to benefit the individual patient/subject on whom it is to be performed?

not applicable

(b) In the case of a study which cannot be expected to benefit the individual patient/subject -

(i) What are the potential risks, if any, to the persons taking part in the study?

not applicable

(ii) Is parental or guardian agreement to be obtained, and if so, in what form?

not applicable

(iii) Is the patient/subject capable of giving assent? ("ASSENT" is used here to imply a willingness that does not necessarily carry the greater understanding and legal implications that are generally understood by 'consent').

not applicable

12. If the study involves a drug or drugs, what stage of evaluation applies to each drug? Does it have a product licence for the purpose for which it is to be used?*

Not applicable

13. Is any drug being supplied by a company with a clinical trial exemption certificate or in response to an investigator with a clinical trial exemption?*

Not applicable

14. Product liability and consumer protection legislation make the supplier and producer (manufacturer, or any person changing the nature of a substance, e.g. by dilution) strictly liable for any harm resulting from a consumer's (subject or patient) use of a product.

(a) What arrangements have been made for indemnity for unforeseen adverse reactions? If applicable, the arrangements involving a drug supplied by a company should conform to the ABPI code of practice. (Copies of the ABPI Indemnity Form are available from the Research Ethics Committee Office).

not applicable

*See Appendix I at the back of the form.

(b) What arrangements will exist to indemnify investigators and the Health Authority against claims made against them? Medically qualified investigators are advised against entering indemnity agreements in which the indemnifying body takes control of claims arising out of alleged negligence by the investigators; defence of these claims should always remain in the hands of the defence organisations.

not applicable

15. What will be done to the patients/subjects? If arterial or venous blood samples are to be taken, please specify how, where, frequency and amount.

Not applicable

16. If the study is on patients:

(a) What differences are there from the usual management of such cases?

None

(b) If additional imaging procedures are being undertaken for the purpose of this Trial, please confirm that the Director of Imaging is satisfied that the risks are acceptable.

not applicable

17. What information is to be given to the patient's GP (especially if a drug is to be given or an invasive procedure is undertaken) and will the passage of such information be a condition of the subject's participation?

Not applicable

18. Multi-centre studies:

(a) Who takes overall responsibility for the study?

(b) Who has the control of the data generated?

(c) Are there other research ethical committees to whom this study is being referred? Indicate those who have already given approval.

19. The declaration of details relating to financial arrangements for research work is now a GMC requirement. Give <u>FULL</u> details of payments to be received and payments to be made to researchers, individuals or departments e.g. Pharmacy, chemistry etc. Details of payments to be made to subjects/patients should also be included.

The proposed research involves no payments/receipts.

20. (a) State any ethical problems or considerations that the investigators consider to be important or difficult with the proposed study.

The personal and sensitive nature of the infomation to be gathered in the interviews. All those involved in the research have been trained in interviewing skills. If personal help is requested by subjects in the course of the interview, they will be given information about confidential counselling service for women and a contact number for a nurse at the GU clinic.

(b) Are there any potential hazards to subjects/patients. If so what is their estimated probability and what precautions are being taken to meet them?

(c) Are the procedures being used in this study likely to cause discomfort or distress to subjects/patients? If yes, estimate the degree and frequency of discomfort or distress entailed.

21. Principal investigator's signature.....

22. Signature of Consultant (or equivalent) taking responsibility if different from Principal Investigator.

.....

Date .....

N.B. The Plymouth Local Research Ethics Committee requires a progress report on all studies given approval.

APPENDIX TWO

Questionnaire format

University of Plymouth
Genito-Urinary Clinic

Biographical details

Qu 1. What is your age ? ☐

Qu 2. What is your present marital status ? (Tick one)

1. Single ☐
2. Married ☐
3. Separated ☐
4. Divorced ☐
5. Widowed ☐

Qu 3. How many children do you have ? ☐ If none, go to Qu. 5

Qu 4. How old are your children ?

First child ☐
Second child ☐
Third child ☐
Fourth child ☐
Fifth child ☐

Qu 5. What is your ethnic origin ? (Tick one)

1. White ☐
2. Black Caribbean ☐
3. Black other* ☐
4. Indian ☐
5. Pakistani ☐
6. Chinese ☐
7. Other* ☐

* Please specify _____

Qu 6. What is your occupation ?

_____

Qu 7. What is your partner's occupation ? (if applicable)

_____

Obstetric History

Qu 8. Details of pregnancies.

Please complete the following table ticking all those applicable for each pregnancy starting with your first pregnancy.

SHOW CARD 1

	1. Forceps	2. Stitches	3. Caesarean section	4. Miscarriage	5. Termination	6. Normal
First						
Second						
Third						
Fourth						
Fifth						

Qu 9. In the six months following pregnancy did you have any difficulties with the following ?

	1. Using Tampons	2. Sexual Intercourse
First		
Second		
Third		
Fourth		
Fifth		

Qu 10. Are you having regular sexual intercourse with a man ? (Tick one)

1. Yes ☐
2. No ☐ Why not ? _____

If no go to Qu 18, page 3.

Qu 11. How long have you been in your current relationship ?

_____ Years _____ Months

Qu 12. Do you currently use any form of contraception ? (Tick one)

1. Yes ☐
2. No ☐ If no go to Qu 15, page 3.

Qu 13. Tick any of the following contraceptives that you are currently using.

SHOW CARD 2

1. Condoms	☐	Brand name _____
2. Pill	☐	
3. Cap	☐	What cream do you use ? _____
4. Coil	☐	
5. Contraceptive cream	☐	Brand name _____
6. Femidom	☐	
7. Rhythm / Natural Method	☐	
8. Contraceptive foam / Pessary	☐	Brand name _____

Qu 14. How long have you been using this method of contraception ? (Tick one)

1. Less than 6 months ☐
2. 6-12 months ☐
3. More than 1 year ☐

Qu 15. What was your last method of contraception ? (Tick one)

SHOW CARD 2

1. Condoms ☐
2. Pill ☐
3. Cap ☐
4. Coil ☐
5. Contraceptive cream ☐
6. Femidom ☐
7. Rhythm / Natural Method ☐
8. Contraceptive foam / Pessary ☐

Qu 16. Why did you change ?

Qu 17. In the last month how often have you had sexual intercourse ? (Tick one)

SHOW CARD 3

1. Not at all ☐
2. Once ☐
3. Two to Three times ☐
4. Once a week ☐
5. Several times a week ☐
6. Daily ☐
7. More often than above ☐

Qu 18. At what age did you start menstruating (periods) ? ☐

Qu 19. At what age did you first have sexual intercourse ? ☐

Qu 20. When you have a menstrual period do you use (Tick one)

1. Sanitary towels only
2. Tampons only
3. Both tampons & Sanitary towels

Go to Qu 24.

Qu 21. If Sanitary towel only . Have you used tampons in the past ?

1. Yes
2. No

If no go to Qu 24.

Qu 22. When did you last use tampons ? (Tick one)

1. Up to 6 months ago
2. 6 months to 1 year
3. 1-2 years
4. 2-5 years
5. Greater than 5 years

Qu 23. Why did you stop using tampons ?

_____
_____
_____

The following are a series of questions relating to the types of toiletries and washing products that you may use.

Qu 24. Do you wash your underclothes - underpants, bras, tights etc. - in: (Tick all applicable)

1. Biological washing powder
2. Non biological washing powder
3. Fabric Softners

Qu 25. When you have a bath do you use bubble bath or a similar product ? (Tick one)

1. Yes
2. No
3. Do not bath

Qu 26. When you have a shower do you use a shower gel or similar product ? (Tick one)

1. Yes
2. No
3. Do not shower

Qu 27. Do you wash your hair in the : (Tick one)

1. Bath ☐
2. Shower ☐
3. Wash basin / using a shower head ☐

Qu 28. Do you wash your vaginal area apart from when you are in the bath / shower ?

1. Yes ☐
2. No ☐ If no, go to Qu 29.

Qu 29. Do you use soap ?

1. Yes ☐ Brand name _____
2. No ☐ If no, go to Qu 31.

Qu 30. If yes, have you experienced any irritation after washing ?

1. Yes ☐
2. No ☐

Qu 31. Do you use any special cleansing products for the vaginal area eg. Femfresh, moist tissues, talcum powder ?

1. Yes ☐
2. No ☐ If no, go to Qu 32.

Qu 31. If yes, what are you using ?

_____

How often do you use it ? (Tick one)

1. Once a day ☐
2. Twice a day ☐
3. Every 2-3 days ☐
4. Less than the above ☐

Now go to Qu 33.

Qu 32. If no, have you used such products in the past ?

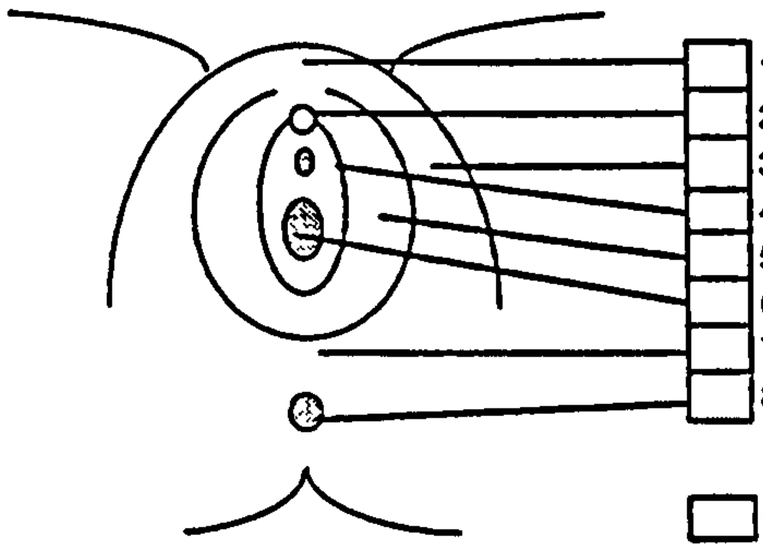
1. Yes ☐ What were they ? _____
2. No ☐ _____

Qu 33. Did you find that the special cleansing products caused you any irritation ?

1. Yes ☐
2. No ☐

Qu 34. Have you ever suffered any discomfort / irritation or other symptoms in the following areas ?

SHOW CARD 4



1. pubic area
2. clitoris
3. vulva (outside lips)
4. urethra (bladder opening)
5. labia (inside lips)
6. vagina
7. perineum
8. rectal area

☐ 9. None, go to Qu 39.

Qu 35. In your own words please describe the symptoms.

_____
_____
_____

Qu 36. Did you treat attempt to treat the symptoms yourself ?

1. Yes ☐ If yes, What did you do ? _____
2. No ☐ _____
_____

Qu 37. Did you consult any of the following ?

1. GP ☐
2. Chemist ☐
3. Genito-Urinary Clinic ☐
4. Other (please state) ☐
_____

Qu 38. What was the diagnosis ?

_____
_____
_____

Qu 39. Was your last smear normal ?

1. Yes ☐
2. No ☐
3. Do not know ☐

When was your last smear taken ?

____ months ago

Qu 40. Have you ever suffered from any of the following conditions ? (Tick all applicable)
SHOW CARD 5

1. Thrush /Yeast infections ☐ Please answer the questions on the GREEN sheet
2. Cystitis ☐
3. Herpes ☐
4. Genital Warts /Papilloma virus ☐ Please answer the questions on the BLUE sheet
5. Vaginitis - a fish smelling vaginal discharge ☐
6. Vulvar Vestibulitis ☐ Please answer the questions on the PINK sheet

University of Plymouth
Genito-Urinary Clinic

The following questions should be answered only by individuals who have suffered from thrush, candida and other yeast infections

Qu 41. When you first had the symptoms did you try to treat yourself ?

1. Yes
2. No ☐ If no, go to Qu 43.

Qu 42. What did you use ?

_____
_____

Did it work ? 1. Yes 2. No ☐

Qu 43. Did you consult your GP ?

1. Yes
2. No ☐ If no, go to Qu 46.

Qu 44. Did your GP examine you ?

1. Yes ☐
2. No ☐
3. Can not remember ☐

Qu 45. Did your GP take a swab (sample) that was sent to the laboratory for diagnosis ?

1. Yes ☐
2. No ☐
3. Do not know ☐

Qu 46. Did you go to the chemist ?

1. Yes ☐
2. No ☐

What were you given ?

_____

Qu 47. Did you go to the Genito-Urinary Clinic ?

1. Yes ☐
2. No ☐

What were you given ?

_____

Qu 48. How long did the treatment last for ? (Tick one)

1. One day ☐
2. 2-6 days ☐
3. One week ☐
4. Two weeks ☐
5. More than two weeks ☐

Qu 49. Did the symptoms reoccur ?

1. Yes ☐
2. No ☐ If no, go to Qu 51.

Qu 50. How many times have the symptoms reoccured in the last year ?

1. One to four times ☐
2. More than four times ☐

Qu 51. Have you taken any of the following in the last year ?

1. Antibiotics ☐
2. Steroids ☐

Thank you for completing this section of the questionnaire. If you have suffered from : Papilloma / genital wart virus please complete the questions on the Blue sheet (page 9) Vulvar Vestibulitis please complete the questions on the Pink sheet (page 11)

University of Plymouth
Genito-Urinary Clinic

The following questions should be answered only by individuals who have suffered with the papilloma / genital wart virus

---

Qu 52. Have you attempted to treat the warts yourself ?

1. Yes
2. No

☐ If no, go to Qu 54.

Qu 53. What did you do ?

Qu 54. On suspecting your condition did you first go to the

1. Your GP
2. Genito-Unnary Clinic
3. Other, please state

☐ Go to Qu 57.

Qu 55. Did your GP say they would go away on their own ?

1. Yes
2. No

☐

Qu 56. Did your GP refer you to the GU clinic ?

1. Yes
2. No

☐

Qu 57. Did you receive any treatment ?

1. Yes
2. No

☐ If no, go to Qu 59, page 10

Qu 58. If yes, what treatment did you receive ?
SHOW CARD 6

1. Laser treatment ☐
2. Electrical Cauterisation ☐
3. Topical treatment ☐
4. Freezing ☐
5. Interferon Injections ☐
6. Other, please state ☐

Qu 59. How long were you treated for ? _ _ _ _ _ _ months

Qu 60. Did the warts reoccur ?

1. Yes
2. No

Qu 61. Have you been for a check up in the last 6 months ?

1. Yes
2. No

Thank you for completing this section of the questionnaire. If you have suffered from :
Thrush / Yeast Infection please complete the questions on the Green sheet (page 7)
Vulvar Vestibulitis please complete the questions on the Pink sheet (page 11)

University of Plymouth
Genito-Urinary Clinic

The following questions should be answered only by individuals who have suffered with vulvar vestibulitis.

Qu 62. Did you attempt to self treat your symptoms ?

1. Yes
2. No

☐ If no, go to Qu 64.

Qu 63. If Yes, what did you do ? _____

Qu 64. When you first experienced vulvar vestibulitis did you first go to :

1. Your GP ☐
2. Genito-Urnary Clinic ☐
3. Other, please state ☐
_____

DO NOT answer any of the remaining questions on the pink sheets

Qu 65. Did your GP examine you ?

1. Yes ☐
2. No
3. Can not remember

Qu 66. Did your GP immediately diagnose vulvar vestibulitis ?

1. Yes ☐ If yes go to Qu 68
2. No

Qu 67. If no, was the initial diagnosis any of the following ?
SHOW CARD 7

1. Thrush /Yeast infections ☐
2. Cystitis ☐
3. Herpes ☐
4. Genital Warts /Papilloma virus ☐
5. Vaginitis - a fish smelling vaginal discharge ☐
6. Vulvar Vestibulitis ☐

Qu 68. Did your GP initially offer you any treatment ?

1. Yes ☐
2. No ☐ If no, go to Qu 70 (page 12)

Qu 69. What was the treatment ?
_____
_____
_____

Did the treatment work ?

1. Yes ☐
2. No
3. Only for a short period

Qu 70. Did you return to your GP for further help ?

1. Yes
2. No

Qu 71. If yes, what were the outcomes of your subsequent visits to your GP ?

SHOW CARD 8

1. Further treatment that worked
2. Further treatment that did not worked
3. Lack of help & gave up going
4. Seeing a counsellor/therapist
5. Refered to GU clinic

Qu 72. Did you try going to different GPs ?

1. Yes
2. No

The following statements relate to what you thought of your GP on presenting yourself with this condition. If you saw more than one GP please answer for the first GP that you saw. Tick the box for the answer that is the nearest to what you think. Ticking neutral means that you do not having any feelings either way to the question.

Qu 73. I was totally satisfied with my initial visit to the GP.

SHOW CARD 9

1.Strongly Agree
2. Agree
3. Neutral
4. Disagree
5. Strongly disagree

Qu 74. Some aspects of my consultation could have been better.

SHOW CARD 9

1.Strongly Agree
2. Agree
3. Neutral
4. Disagree
5. Strongly disagree

Qu 75. The Doctor was interested in how the symptoms were affecting me.

SHOW CARD 9

1.Strongly Agree
2. Agree
3. Neutral
4. Disagree
5. Strongly disagree

Thank you for completing this section of the questionnaire. If you have suffered from :
Papilloma / genital wart virus please complete the questions on the Blue sheet (page9)
Thrus / Yeast Infections please complete the questions on the Green sheets (page 7)

University of Plymouth
Genito - Urinary Clinic

Section two: To be completed by vulvar vestibulitis sufferers attending the Genito-Urinary Clinic only.

Qu 1. Were you refered by a GP to the GU clinic ? (Tick one)

1. Yes ☐
2. No

Qu 2. How many times have you been to the clinic to date ? ☐

Qu 3. On presenting yourself at the clinic did you already know that you were suffering with vulvar vestibulitis ?

1. Yes ☐ If yes, go to Qu 5.
2. No

Qu 4. If no, what did you think was a matter with you, in your own words ?

_____
_____
_____

Qu 5. On hearing the diagnosis which of the following best describes how you felt ?

1. Relieved ☐
2. Anger
3. No specific feelings

Qu 6. In your own words describe the symptoms that you are experiencing

_____
_____
_____

Qu 7. What bothers you most about this condition ?

_____
_____
_____

Qu 8. How long have you had these symptoms ? (Tick one)

1. Less than six months ☐
2. Six months to one year
3. One to three years
4. Five to ten years
5. More than ten years

Qu 9. Have you been free from the symptoms at any time ?

1. Yes
2. No

☐ If no, go to Qu 11.

Qu 10. If yes, when ?

Qu 11. What seems to make the symptoms worse ?

Qu 12. What can you do to get relief ?

Qu 13. How are the symptoms you have NOW related to your INITIAL symptoms ?
(Tick all applicable) SHOW CARD 10

1. Same ☐
2. Less intense discomfort ☐
3. More intense discomfort ☐
4. Less frequent ☐
5. More frequent ☐

Qu 14. Which of the following statements best describes the discomfort that you experience most often ?

SHOW CARD 11

1. Slight, I only notice it when I think about it ☐
2. Slight, I can ignore it by not thinking about it ☐
3. Moderate, I always know it is there but I can still perform most tasks ☐
4. Severe, I can only perform a number of tasks which require little concentration ☐
5. Severe, I find it imposible to do anything ☐

Qu 15. Do you find that your discomfort results in any of the following ?

SHOW CARD 12

1. No interference with daily routine ☐
2. Some interference with daily routine ☐
3. Interferes with daily routine ☐
4. Confinement to bed ☐
5. needs immediate medical attention ☐

Qu 16. Do your symptoms have any of the following affects ?

SHOW CARD 13

1. Does not affect sexual intercourse
2. Sometimes prevents you from having sexual intercourse
3. Completely prevents sexual intercourse
4. Causes discomfort but does not prevent sexual intercourse
5. Do not know - not sexually active. Go to Qu 18.

Qu 17. If you try to have sexual intercourse indicate any of the following that are applicable

SHOW CARD 14

1. Discomfort is worse with certain positions
2. Discomfort is mostly at the opening of the vagina
3. Painful on entry but then intercourse is tolerable
4. Discomfort / irritation after intercourse

Qu 18. Have you seen a counsellor since you have been attendng the GU clinic ?

1. Yes
2. No

Qu 19. Have you been given any advice on how to manage the symptoms and discomfort ?

1. Yes
2. No

What was the advice ?

Qu 20. Have you had any treatment since attending the GU clinic ?

1. Yes
2. No

What was the treatment ?

Thank you for completing this questionnaire.

APPENDIX THREE

Drug information - Ketoconazole

1

Title: **AN OPEN PILOT STUDY OF R 85355 A POTENT 5-LIPOXYGENASE INHIBITOR IN THE TREATMENT OF VULVAR VESTIBULITIS SYNDROME**

Protocol No: **R85355-GBR-001**

CIMS No:

Test Drug: 0.5% R85355 cream (F)

Comparator drug: None

Regulatory Status: CTX

Sponsor: Janssen Pharmaceutical Ltd
Grove, Wantage
OXON OX12 0DQ

Monitor: N.R.Blatchford,B.Sc.,
Senior Research Programme Manager,
Janssen Research Foundation,
Grove, Wantage,
OXON OX12 0DQ.

Tel no - Office: 0235 772966 (24 hr)
Car : 0836 310685

Principal Investigator(s):

Dr. G. Morrison,
Depertment of Genito-Urinary Medicine,
Freedom Fields Hospital,
Greenbank,
Plymouth.

Date of draft: 5th January 1993

PAC approval:

I confirm that any patients treated in the course of this study will be treated in accordance with this protocol.

Signed.....

I agree on behalf of Janssen Pharmaceutical Ltd to the terms and conditions in this protocol.

Signed..... Date:.....

2.

1. INTRODUCTION

Vulvar vestibulitis syndrome is a complex condition with symptoms and findings involving and limited to the vulvar vestibule. It consists of severe pain on vestibular touch to attempted vaginal entry, tenderness to pressure localised within the vulvar vestibule and vulvar erythema of varying degrees. Histopathological findings are consistent with a chronic, non-specific inflammatory response that is occasionally associated with metaplasia of the minor vestibular glands. To date the syndrome has been seen in association with sub-clinical human papillomavirus, chronic recurrent vaginal candidosis, chronic recurrent bacterial vaginosis, chronic alteration in the vaginal pH and the use of chemical and destructive therapeutic agents.

1.1 Treatment

The treatment for acute vulvar vestibulitis syndrome requires the eradication of any infection and all therapeutic modalities, local or systemic, that may continue to the problem should be discontinued. Topical steroids are applied as soon as all infection has been treated.

The treatment for chronic vulvar vestibulitis is much more complicated. Some success has been obtained when a specific etiologic condition can be identified and treated but even in these cases, such an approach will not yield a cure in many cases. When all treatment modalities have failed, surgery is then considered.

Recently a pilot study was completed looking at the efficacy of ketoconazole 2% cream in chronic vulvar vestibulitis. The intention was to utilise the weak 5-lipoxygenase activity of ketoconazole in controlling this condition. Response was defined as at least a 50% improvement in presenting symptoms. A total of 14 patients were treated with ketoconazole 2% cream applied twice daily to the vulva for one month. Of those treated 9 responded, 4 had no benefit from therapy and one patient failed to return for follow-up. This response rate was considered adequate to justify further research into chronic vulvar vestibulitis with much more potent and specific 5-lipoxygenase inhibitors.

1.2. R85355.

At submicromolar concentrations, R85355 suppresses the FMLP-induced leukotriene biosynthesis by intact human polymorphonuclear leukocytes with an IC50 value of 0.30mM. It inhibits the conversion of arachidonic acid to 5-HETE by RBL 5-lipoxygenase. This inhibitory effect is substantially reduced in the presence of 13-HpODE suggesting the ability of R85355 to interfere with the oxidative activation of this enzyme.

When tested at a concentration of 5mM, R85355 had no inhibitory effects on cyclo-oxygenase, phospholipase A2 and xanthine oxidase. A partial inhibition, (<35%), of platelet 12-lipoxygenase and soybean 15-lipoxygenase was observed.

3.
R85355 has been investigated for inhibition of radioligand binding in vitro to neurotransmitter receptor sites, drug receptor binding sites, ion channel ligand binding sites, peptide receptor binding sites, thromboxane A2, PAF, leukotriene D4 receptor binding sites and terbenazine sensitive release sites. It was also examined for inhibition of neurotransmitter uptake in rat brain synaptosome preparations. At the highest tested concentration, 10mM, R85355 was inactive in all these systems.

At 10-5 molar R85355 has no or little effect on alpha-1, alpha-2, beta-1 and beta-2 adrenergic responses, 5HT2, H1 and H2 histaminic responses, contractions induced by adrenergic nerve stimulation, calcium induced contractions, endothelium dependent relaxations or singlet oxygen-induced contractions. At high concentrations a slight decrease in cardiac output was observed in isolated working rabbit hearts. No protection against deterioration of heart function after ischaemia and reperfusion was observed.

These experiments indicate that R85355 is at present thought to be devoid of major effects other than the documented inhibition of 5-lipoxygenase enzymatic activity.

Leukotrienes formed from arachidonic acid by the 5-lipoxygenase enzyme are presumed to be involved in allergic and inflammatory processes. R85355 inhibits *in vitro* and *ex vivo* the 5-lipoxygenase activity in blood. *In vitro* R85355 inhibits the production of LTB4 in A23187 or zymosan-stimulated human blood. Similar results are obtained in canine and rat blood. At higher concentrations inhibition of the cyclo-oxygenase enzyme is observed.

Oral administration of R85355 to beagle dogs, (0.31 to 2.5mg/kg), produces a rapid and sustained inhibition of *ex vivo* LTB4 biosynthesis. At 2.5mg/kg this inhibition is almost complete, (>90%), for more than 8 hours. At this dose there is no effect on the cyclo-oxygenase activity confirming the selectivity of R85355 for 5-lipoxygenase.

R85355 has been studied in a large series of general *in vivo* pharmacological tests in mice rats and dogs at a dose of 40mg/kg. It was devoid of significant interaction with muscarine, nicotine, dopamine, D1 and D2), adrenaline (alpha 1 and 2), histamine (h1 and 2), serotonin, opoid and PAF receptors, monoamine oxydase inhibition and monoamine uptake. It was also devoid of toxic effects, aspecific effects on body temperature, muscle tone or respiration. No activity characteristic of CNS stimulants, sedatives, hypnotics, anticonvulsants, benzodiazipines, antihypnotic compounds, anti-diarrhoeals, laxatives, gastrointestinal motor stimulating compounds, antisecretory compounds, diuretics, antiemetics,emetics, narcotics, aspirin-like compounds or anti-inflammatory compounds was observed. R85355 was also devoid of interaction with liver microsomal enzymes.

In the rat one month toxicity study it was concluded that R85355 was well tolerated and did not result in overt signs of toxicity up to 80mg/kg/day. Laboratory examination revealed no target organ for overdosing. When administered for one month to dogs, R85355 was well tolerated up to 10mg/kg/day. At 40mg/kg/day toxicity was mainly evidenced by decreased body weight gain and changes in laboratory parameters that identified the urinary tract as the main target organ for overdosing. A similar tendecy was present at 10mg/kg/day with 2.5mg/kg/day non-toxic.

4.

In the reverse gene mutation test it was concluded that R85355 had no mutagenic properties.

The specific inhibition of the 5-lipoxygenase enzymes suggests that R85355 would be a suitable agent to treat vulval vestibulitis.

2. OBJECTIVES

The prime objective is to determine the efficacy of the 5-lipoxygenase inhibitor R 85355 in the treatment of vulvar vestibulitis and the speed of onset of action. Once this has been established a placebo controlled study will be considered.

Secondary objective is

To assess a new pressure application device and it's value in providing quantitative variables rather than the subjective one's used to date.

Assessment of vulvar vestibulitis has been extremely difficult in the past as only subjective criteria have been available. The standard methodology involved a swab test in which a water moistened cotton tipped swab is applied gently over the skin at 6 pre-determined sites. These 6 sites are 3 posterior to the vagina, 2 either site of the urethra and 1 posterior to the clitoris.

Response is graded as follows;

0 = normal sensation
1 = unpleasant scratchy sensation
2 = painful sharp sensation
3 = patient acknowledged pain and was noted to flinch
4 = patient noted significant pain and was recoiled

A specific piece of apparatus has been developed at The University of Plymouth in which a small disposable probe produces variable pressures on a 5 point scale. The pressures available are measured in mille Newton and range from 0 to 0.63 with the first grade of pressure being variable between 0 and 0.14mN and the other 4 fixed pressures. It is therefore possible to provide quantitative and reproducible pressure stimulation.

3. PATIENTS

10 patients with Vulvar vestibulitis will be recruited.

To be eligible for the study patients must fulfil the following criteria.

3.1. Inclusion Criteria.

1. Bartholin's and Skene's ductal erythema

2. Bartholin's and Skene's ductal tenderness

3. Hymenal ring tenderness

4. Entry dyspareunia e.g. intercourse, insertion of tampon/speculum

5.
5. Sensitivity to pressure with a variable pressure applicator on at least two of 6 predetermined vulval sites

3.2 Exclusion criteria

1. Pregnant patient or patients intending to become pregnant.

2. Women of child-bearing potential not receiving an adequate form of contraception.

3. Patients with microbiologically proven anaerobic vaginosis.

4. Patients with mycologically proven vaginal candidosis.

5. Patients unavailable for a 6 month follow-up period.

6. Apparently free from anaerobic vaginosis or vaginal candidosis for the past 3 months.

4. STUDY DESIGN.

An open non-controlled study. Two centres will be involved in the study.; PLymouth General Hospital and the Royal Devon and Exeter Hospital.

5. TREATMENT

Formulation : 0.5% R85355 cream
3 x 30g will be provided per patient

Route : Topical

Administration : Cream to be applied twice daily to the vulvar area

Treatment duration : 8 weeks

Medication :

Medication will be supplied by Janssen Pharmaceutical Ltd.The pharmacist will be responsible for dispensing and accounting for the clinical trial supplies. A record of the drugs dispensed must be maintained and recorded on the patients drug sheets and in the study case record form. Both should be available to the monitor for inspection.

Under no circumstances will the investigator allow the trial supplies to be used for a purpose other than as directed by this protocol.

Clinical trial supplies assigned to a patient participating in the trial and returned by that patient must be entered on the Drug Accountability form. These supplies must not be dispensed again, even to the same patient.

All unused clinical trial drugs will be counted by the investigator or pharmacist and monitor for assessment of compliance. Reasons for non-compliance should be ascertained by the investigator. An account of returned medication will be kept on the Drug Accountability form provided to the pharmacy.

6.

Storage : at room temperature.

LABELLING

R85355-GBR-001

Patient Initials _____
Randomisation No _____

Apply twice daily to the affected area

VARIABLES TO BE MEASURED.

On entry to the study then weekly for 4 weeks then monthly for a further 3 months.

The following will be assessed as

0 = absent
1 = mild
2 = moderate
3 = severe

1. Entry dyspareunia during intercourse, tampon insertion or speculum insertion.

2. Bartholin's ductal erythema

3. Skeen's ductal erythema

4. Hymenal ring tenderness

5. Pressure response for Bartholin's ductal tenderness and Skeen's ductal tenderness. This will be assessed on a 1 to 5 scale where 1 = 0 to 0.14mN and 5 = 0.63mN

On entry then at the end of the 8 week treatment period.

A biopsy will be taken from periductal tissue for electron microscopy.

7.

ADVERSE EVENTS

All adverse events experienced by patients treated will be recorded on the adverse event report forms. Information collected will include date and time of onset and cessation, frequency, and severity, action taken. A judgement by the treating physician on causality will also be obtained. Any serious adverse events (where 'serious' is defined as fatal, life threatening, disabling, incapacitating or resulting in hospitalisation or the prolongation of hospitalisation) should be reported immediately to the Adverse Reaction Monitoring Unit at Janssen Pharmaceutical Ltd, even if not considered to be related to the trial medication. These will be immediately notified by the unit to the DoH under the CTX scheme. All other adverse events will be notified to the DoH at the end of the trial.

10. MONITORING OF THE TRIAL

The study will be monitored according to the current JRF standard operating procedure for the Monitoring of Clinical Trials. This will involve an initial set up visit followed by monthly telephone contact and visits every 6-8 weeks as appropriate to patient recruitment rate.

The trial monitor will meet with the investigator and all staff involved in the study prior to the study start to review the procedures to be followed in conducting the study and recording the findings in the Case Report Forms. During the trial, the investigator will permit the trial monitor to control the progress of the trial on site as frequently as necessary. The investigator will make available the Case Report Forms, provide missing or corrected data, and sign the Case Report Forms. Key items of data transcribed onto the Case Report Forms, such as the patient's sex, date of birth, assessment dates and bloed oestrone concentrations will be reviewed again source documents in the presence of the investigator without compromising patient confidentiality. Any inconsistencies found at this review will be resolved and the Case Report Form amended.

Monitors will sign an undertaking to be bound by the confidentiality requirements of The Venereal Diseases act 1918.

CRITERIA FOR MODIFICATION OR TERMINATION OF THE STUDY

There are no criteria for modifying or terminating this study.

REVIEW & CONSENT PROCEDURES

CTX approval will be obtained. Approval will be obtained from the Plymouth Area Health Authority and The Royal Devon and Exeter Ethics Committees, from the Janssen Pharmaceutical Ltd. protocol approval committee and the international product co-ordinator in Janssen Beerse.

8.
Written informed consent will be obtained from the patients before commencement of the study.

Patients will be supplied with an information leaflet prior to obtaining that consent, and an information leaflet will be also sent to the patients' general practitioner.

The patient is free to withdraw from the study at any time without prejudice to her/his welfare or future treatment.

INDEMNITY COVER

Janssen Pharmaceutical Limited has endorsed the A.B.P.I. guidelines on compensation for medicine induced injury in clinical trials which were published in the British Medical Journal on 3rd September 1983. The Company carries liability insurance to cover such claims but this coverage is not extended to damages which the investigators or third parties may suffer by reason of acts of commission or omission on the part of such investigators and which are not in accordance with accepted common medical practices (state of the art).

INDEMNITY AGREEMENT

See Appendix 2

PUBLICATION POLICY

Investigators are free to publish the data generated from this study in any form, provided that Janssen Pharmaceutical Ltd., will first be given the opportunity to comment on the content. Publication of results will be initiated by the trial investigator. The data generated on the original clinical trial record forms will be the property of Janssen. These are required as some regulatory authorities, notably the FDA, require original data sets for examination. Copies of the originals will be provided by Janssen for the investigators to retain.

APPENDIX FOUR

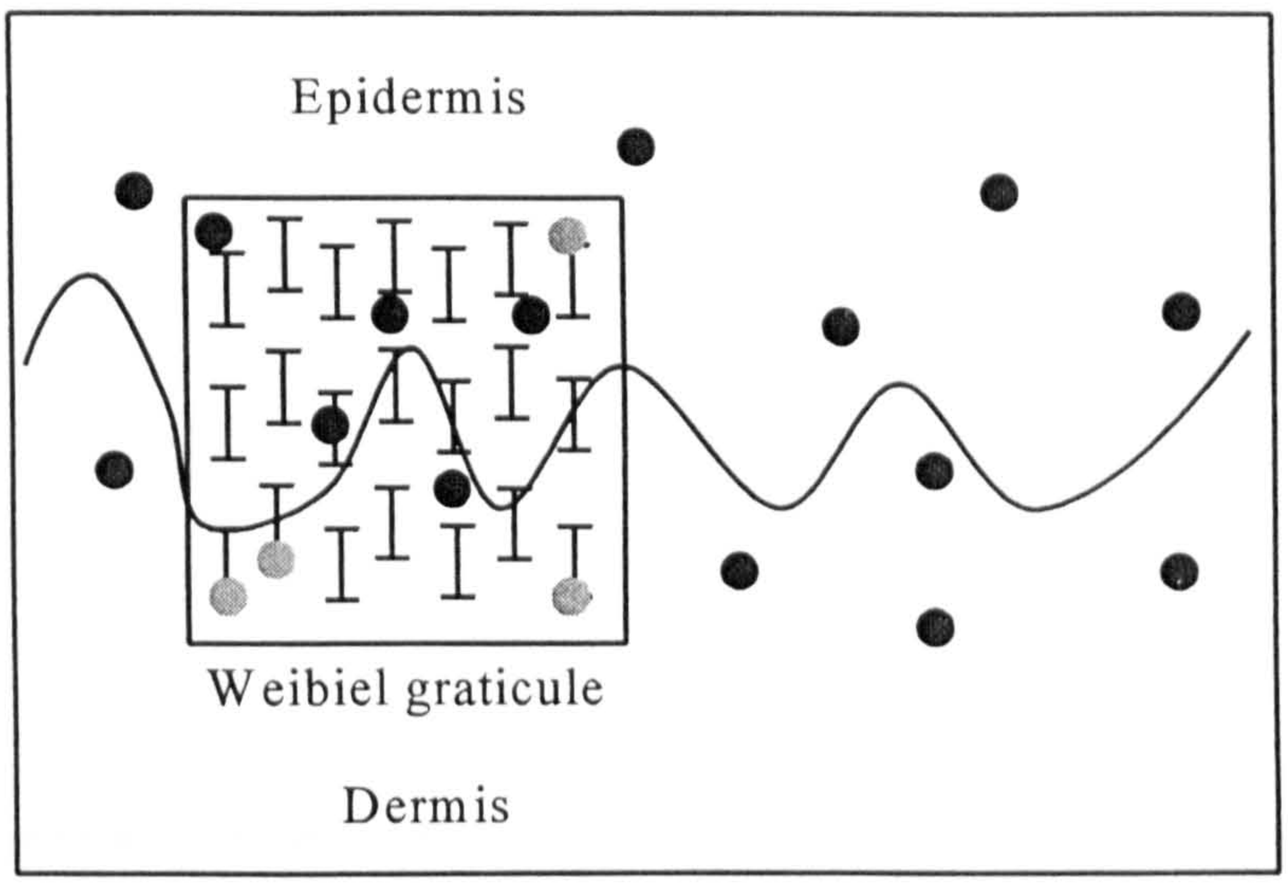
Image analysis programme

```
 5 dim g(325)
10 camera 1
11 scanner  30.45  51.16
12 setlamps  43.74 0
20 qmenu 'image_setup'
30 calibrate 3, 't'
40 mframe 175 122 30 325
41 iframe 0 0 512 512
50 multiacquire 5 0 4
60 delin 0 1 256 3
85 qmenu 'measure_grey'
86 vprof 1 175 122 30 325 g(1)
90 input 'name of file? 'n$
100 open #1 'b:'+n$+'.prn'
110 x=1:g=1
120 print#1:g, g(x)
130 g=g+1:x=x+1
140 if g=325 then 200
150 goto 120
200 close#1
210 wtoph 1 2 256 3
220 anamorph 2 3 6 9
230 greydetect 3 3 86 1 1 0
240 binmode 1 2
242 binsegment 2 3 256 1 5
250 setftrpar "8,28,15,1,2,3,29"
251 ftrgrey 1 : measfeat 3 1 4 300000 : clraccept
252 acceptxfer 3 4
260 qmenu 'feature_results'
300 end
```

APPENDIX FIVE

Estimation of Leukocyte Profile Density

Estimation of Leukocyte Profile Denisity using a Weibiel Steriological Graticule



- Negative count
- Positive count

Positive counts were recorded only if a leukocyte was positioned on a point sample intersection. The leukocyte profile density for this example would be 4.

APPENDIX SIX

Explanation of the Box and Whisker Plot

Explanation of a Box and Whisker Plot, illustrating the maximum, minimum, mean, median and outlying values.