THE LIMBAL PALISADES OF VOGT.

Their Cellular Organisation and Response to Hydrogel Contact Lens Wear.

A Thesis submitted by

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ABBREVIATIONS

A	Asian
ACA	Anterior Ciliary Artery
AEC	Aminoethylcarbazole
APES	3-Amino Propyl Tri-Ethoxy Silane
BrdU	Bromodeoxyuridine
C	Caucasian
CDK	Cyclin Dependent Kinase
DAB	Diaminobenzamide
DBP	Distance Between (adjacent) Palisades
DMEM	Dulbecco's Minimum Essential Medium
GMA	Glycolmethacrylate
HMAR	Heat Mediated Antigen Retrieval
mm	Millimetre
μm	Micrometer
LVC	Limbal Vascular Circle
N	Negro
PMC	Post Mitotic Cell
SC	Stem Cell
S-J	Sino-Japanese
SLR	Single Lens Reflex
TAC	Transient Amplifying Cell
TDC	Terminally Differentiated Cell

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ABSTRACT

This study sought to investigate the structure, cytology and possible function of the palisades of Vogt. These structures were found to be concentrated in the vertical meridian, where the conjunctival overlap of the cornea is greatest. Of the three primate groups studied, palisades were found to be exclusive to the limbal conjunctiva of man.

Palisades varied greatly in their form and degree of associated pigment. They were more regular inferiorly, whereas superiorly they often consisted of a number of interrupted papillae. Normally, in a Caucasian eye palisades were unpigmented and difficult to see against the white sclera. In an Asian eye, they were often outlined by thin lines of pigment giving the biomicroscopic appearance of a pair of pigment lines. In a Negroid eye palisades often appeared as thin white lines surrounded by diffuse melanin pigment.

Palisades appeared as a series of characteristic stromal elevations projecting into the overlying epithelium in histological section. A flat conjunctival surface was retained by epithelial cells filling the troughs between adjacent palisades. The thickness of the epithelial layer varied between 4-5 cells thick at palisade crests and 12-15 cells at the troughs. Dichotomy of basal cells of the palisade epithelium (and subsequently the remainder of the limbal conjunctival epithelium) was described in the current study for the first time. A heterogeneous distribution of melanin within the two basal cell types was found in pigmented conjunctivae. The more numerous small cells accumulated more cytoplasm within their perikaryon than their larger counterparts. Pigment donation was found to be the driving force in pigmentation of basal cells in primate limbal conjunctival epithelia.

Immunohistochemical staining was used to investigate the cell cycling time of the two native basal cell types. From the results of histology it was hypothesised that less densely pigmented cells have a quicker turnover than more pigmented cells. Large cells were found to be faster cycling than small cells, confirming the initial hypothesis. These findings have been correlated to corneal epithelium stem cell properties to suggest that small cells conform to the properties of these important progenitor cells whilst large cells conform to their immediate off-spring- transient amplifying cells.

Incidental to a quest to find palisades in the other primates, a peculiar native cell type was found in the limbal conjunctival epithelium of the cynomolgus monkey. "Star" cells were present in the basal and suprabasal layers. Their long, broad, insinuating processes suggested a capacity for movement. Coupled with the finding that these accumulated less pigment than their neighbours without processes, these cells would make ideal candidates for migration to the corneal epithelium.

The stability of the palisade pattern was investigated over a duration of one year under normal conditions and during hydrogel contact lens wear. Palisade morphology was found to be unaltered in both groups of subjects. The results may reflect the conditions and duration of the study. On the other hand, the ability to resist change in the short term may be an important property if the palisade zones are involved with generating cells for the corneal epithelium.

The apparent exclusiveness of the palisades of Vogt in man probably represents species variation. The palisade zones are highly organised areas that have been shown in the current study, to house cells conforming to the properties of corneal epithelial stem cells. Their presence at the corneo-conjunctival junction in man may serve to enhance the concentration of these important progenitor cells in this species.

DECLARATION

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CHAPTER 1

INTRODUCTION

1.1. Conjunctival Topography and Some Aspects of Structure

The conjunctiva is a thin semi-transparent mucous membrane that extends from the eyelid margins anteriorly, providing a smooth inner lining to the lids, and then reflects sharply upon itself at the fornix onto the anterior surface of the eye where it covers the sclera up to its junction with the cornea. It thus forms a sac whose mouth is bound by the free margins of the lids. The conjunctiva is conventionally described in six regions (Fig 1): marginal, tarsal, orbital, fornical, bulbar and limbal.

The marginal conjunctiva (mucocutaneous junction) extends from a line immediately posterior to the tarsal gland openings, passing around the marginal angle and extending as far as the subtarsal fold at the base of the tarsal plate. It possesses a thick non-keratinised stratified squamous epithelium that is sparse in goblet cells and a dense underlying fibrous connective tissue layer.

In the tarsal region the epithelium is reduced in thickness, surface cells become cuboidal and goblet cell density increases. In this region the substantia propria is thin and highly vascular, and the tissue is tightly adherent to the underlying tarsal plates.

The orbital conjunctiva extends from the limits of the tarsal plates to the fornix. Here the conjunctiva is much more loosely attached to the underlying tissues and tends to fold. The epithelium of the orbital and fornical conjunctiva is thicker than that of the tarsal region. Epithelial cells become more columnar and goblet cell density increases to reach a maximum at the fornix. The substantia propria varies in thickness and can be divided into a superficial adenoid and deep fibrous layer. Accessory lacrimal glands are often present buried deep in the subconjunctival tissues in the fornix region.

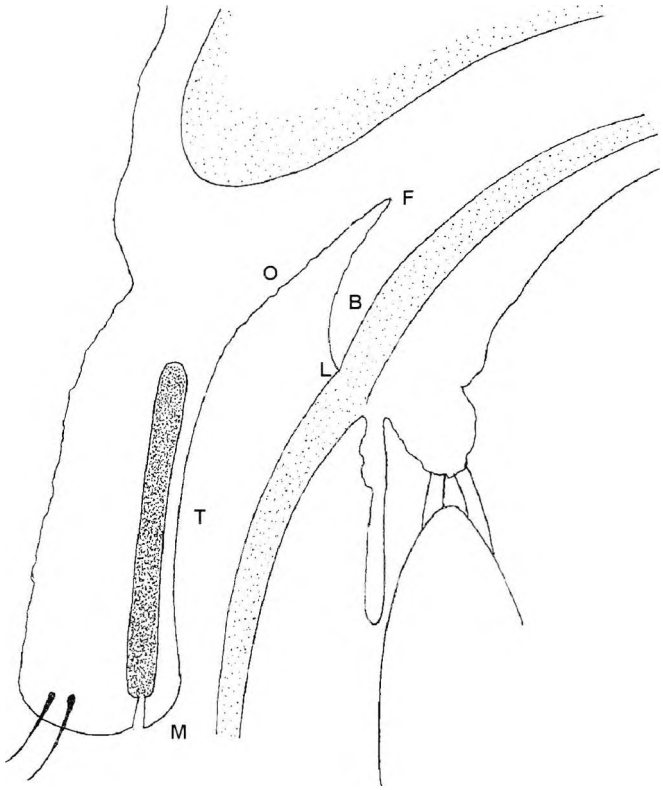


Figure 1. A sagittal section through the upper eyelid and anterior orbital structures showing the various regions of the conjunctiva. M= marginal, T= tarsal, O= orbital, F= fornical, B= bulbar, L= limbal.

Episcleral and conjunctival vasculature are readily visible through the bulbar conjunctiva. The epithelium is arranged in a similar manner to that at the fornix,

although there is regional variation in goblet cell density (Kessing 1968). The bulbar conjunctiva is freely movable due to its loose attachment to the underlying Tenon's capsule.

As the bulbar conjunctiva approaches the cornea, the epithelium thickens and superficial cells once again become more squamous. Goblet cell density gradually decreases until they become completely absent in the limbal conjunctiva. The limbus is demarcated anteriorly by a line joining the termination of Bowman's and Decemet's layer of the cornea and posteriorly by a line perpendicular to the distal aspect of the canal of Schlemn. It represents a transition zone, approximately 1mm in length between the opaque sclera and its overlying (bulbar) conjunctiva to the clear cornea. In man the junction between the limbal conjunctiva and the cornea is ill defined due to a conjunctival and scleral overlap of the peripheral cornea. This is greatest in the vertical meridian and so the cornea appears slightly oval with its long axis horizontal. The substantia propria of the limbal conjunctiva is richly invested with a plexus of small calibre capillaries. (Their distribution is discussed later in this chapter).

Another feature of the limbal conjunctiva is the palisades of Vogt (Vogt 1921). It is commonly thought that palisades are exclusive to man. They constitute a series of radially orientated connective tissue ridges with a smooth epithelial covering which form a distinct zone adjacent to the corneal margin. Palisades are most marked in the vertical meridians where conjunctival/scleral overlap of the peripheral cornea is greatest. In histological section they are visible as stromal elevations into the overlying epithelium. They often act as conduits for nerves, blood and lymphatic vessels. The

true functional significance of these structures however remains speculative. Interest in the palisade zones increased with the introduction of stem cell concepts. These theories suggested that a proportion of basal cells of the limbal conjunctival epithelium act as stem cells for the provision of cells for the corneal epithelium (Tseng 1989). These concepts and the limbal palisades are discussed further later in this chapter.

1.2. Epithelial Cell Attachments.

As epithelial cells of the ocular surface are exposed to shearing forces during the blink and saccade, attachment between adjacent cells and that between basal cells and the stroma must be strong.

Epithelial cells of the cornea and limbal conjunctiva are attached in a similar manner (Gipson 1994). To prevent the passage of water and small molecules tight junctions (zonulae occludentes) are present between the apical surfaces of contiguous surface cells. Cells of the suprabasal and basal layer are attached to adjacent cells via membrane interdigitations and desmosomes.

Cells of the intermediate layer are also attached via membrane interdigitations and desmosomes. The intervening space between the membrane interdigitations, under normal conditions, is no more than 20nm (Bron et al., 1997). Arguably, the most important attachments however, are between basal epithelial cells and the underlying stroma. These two areas are joinedd via a series linked structures. Gipson et al (1987) described these as an adhesion complex (Fig. 2).

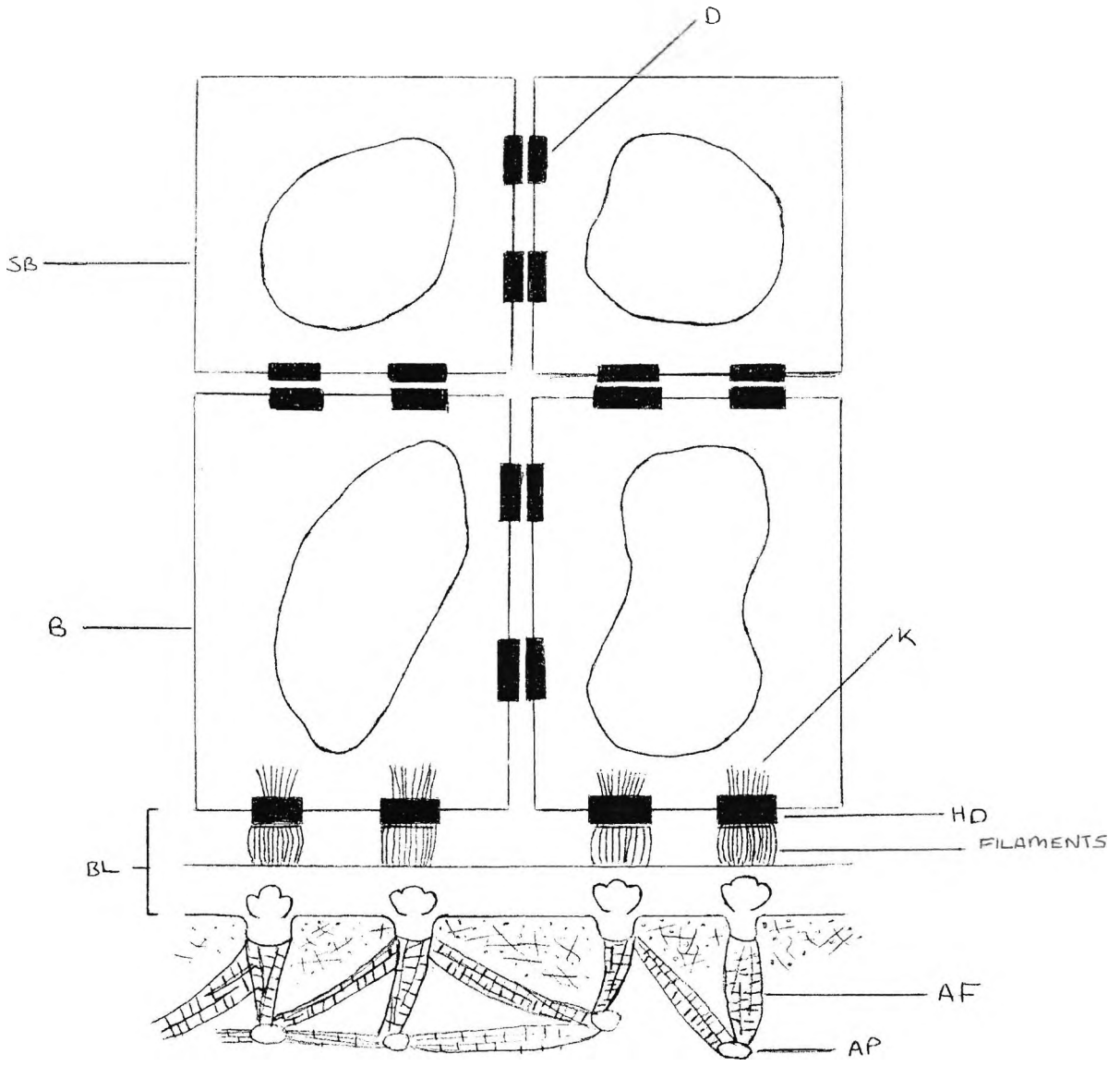


Figure 2. A schematic representation of epithelial cell attachments in the cornea and limbal conjunctiva. SB=suprabasal cells; B=basal cells; BL=basal lamina; AP= anchoring plaques; AF=anchoring fibril; K=keratin filaments and D=desomsomes. After Bron et al (1997).

Bullous pemphigoid antigens and $\alpha_6\beta_4$ integrin are present on hemidesmosomes. In skin, Watt (1994) suggested that not only do integrins, which are found in all basal cells, mediate adhesion of these cells to the underlying basement membrane but they also play

a role in cell migration, stratification and regulate the onset of terminal differentaition. In the context of the current study this observation could be highly applicable as not only are epithelial cells thought to migrate from the limbus to the cornea but this observation may also be relevant to the concept of the limbal location of corneal epithelial stem cells (see later). In the case of the latter point, Watt suggested the levels of the integrin β_1 in basal epidermal cells was highest in cells with the highest potential for proliferation (stem cells and transient amplifying cells) and was down regulated as the cell differentiated and matured, until it was completely lost in cells that were moving away from the basal level.

From the deep aspects of the hemidesome, fine anchoring filaments extend through a clear zone (lamina lucida) towards the electron dense zone (lamina densa) of the basement membrane. The molecular constiuent of the anchoring fibril has been dentified as a variant of laminin. The molecular components of the basement membrane include laminin, heparin sulfate and the globular domain of collagen type VII (see below) (Gipson 1987).

Anchoring fibrils, composed of cross-banded, collagen type VII, insert into anchoring plaques inthe anterior stroma of the limbal conjunctiva and Bowman's layer of the cornea. An associated molecule is laminin. Anchoring fibrils extend from the basement membrane throughout the anterior 1 to 2 μm of the stroma, forming a complex network that is intervoven with stromal collagen. This serves to hold the epithelium to the stroma.

1.3. Vascular Supply to the Limbal Conjunctiva

The limbal area is supplied by the anterior ciliary arteries (ACA's). They are forward extensions of the muscular arteries and are easily recognisable without the aid of a slit-lamp biomicroscope as they appear larger than neighbouring vessels. ACA's appear to terminate abruptly as they penetrate the sclera to join the major iridic circle (Hogan et al 1971). Frequently, a dark circumferential pigment ring may be visible where an ACA enters the sclera.

There are 7 or 8 ACA's in each eye. Each gives rise to episcleral branches that move forward in a radial manner towards the cornea. Approximately 2-3mm from the cornea, in the region of the palisades of Vogt, these turn sharply to lie more superficially in the conjunctiva (Vogt 1921; Graves 1934). Fine recurrent capillaries issue from these vessels and move closer to the cornea, forming the corneal vascular arcades or "limbal loops".

Many of the finest limbal loops are normally closed to the formed elements of blood. This can give the misleading appearance of vascular spurs where attenuated columns of blood cells stop at constrictions. Dobree (1950) suggested that obstructed channels may represent up to 50% of those available at the limbus. When the conjunctiva is mildly irritated, the capillaries lumen widen, allowing the passage of the formed elements of blood. The net result is that a fuller picture of the limbal vasculature is apparent.

Drainage at the limbus occurs via the limbal venous circle (LVC) (Meyer 1990). Vessels of this system are situated immediately behind the forward most limbal loops. The LVC drains into radial episcleral channels which pass towards the rectus muscles from where they join the principle veins of the orbit.

1.4. The Palisades of Vogt

Perhaps somewhat surprisingly there is very little literature regarding the morphology, cytology and stability of the palisades of Vogt, therefore the current study is designed to provide a clearer understanding of the anatomy and morphology of the these structures, with a view to suggesting a possible function. Additionally, the variety of palisade forms is examined and their stability is investigated under normal and experimental conditions. In a complimentary study, the possible presence of palisades in other primate species is also investigated. If present, these would serve for further investigations that cannot be preformed in man.

The appearance of the transition zone between the conjunctiva and the cornea has been of interest since 1859, when Manz described a series of peculiar structures, in the limbal conjunctiva of swine (Manz 1859). This worker attributed a glandular function to these structures and attached the title "limbar glands" He found no equivalent structures in the limbal conjunctiva of man but described how at the upper and lower corneo-scleral junction the conjunctival stroma formed radially orientated ridges. This description led to a series of studies investigating the possible existence of "Manz glands" in man. Ciaccio (1874) stained human conjunctivae with carmine and confirmed the

observations of Manz, as did Virchow (1910) who also demonstrated the presence of radial connective tissue papillae in man. These authors demonstrated that these stromal structures had an epithelial covering which ensured a smooth ocular surface but failed to conclude whether or not they served a glandular function. The nature of these limbal structures remained unclear until the authoritative work of Aurell and Kornerup (1949) who finally dismissed the possibility of Manz glands in man.

Vogt (1921) gave the first adequate biomicroscopic description of this area, although he accredited Streiff (1910) with recognising a series of "radial stripes" a few years earlier. He described two narrow crescent-shaped bands at the upper and lower limbus which consisted of fine radially orientated connective tissue ridges that often served as conduits for radial capillaries which were frequently the source of the limbal arcades. He named these connective tissue ridges "palisades". Graves (1934) used different terminology to describe palisades, referring to them as "trabeculae". In his extensive biomicroscopic study of the limbal vasculature, he described how radial blood vessels coursed through and between the trabeculae. He suggested that the unique nature of the vascular system implied that this zone served a specialised function.

Based upon similarities in the nature of pigment slide into the peripheral cornea from the limbus and the palisade pattern in Negroid eyes, and wound healing observations in guinea pigs, Davanger and Evensen (1971) were the first to suggest a possible function of the palisade zone. They hypothesised that they may be involved in the generation and replenishment of the corneal epithelium. This study was the first to speculate a specific function to this area. Davanger and Evensen's hypothesis was supported by Bron

(1973) who correlated similarities between the vortex patterns in some corneal epithelial disorders and the arrangement of the limbal palisades. Schermer et al (1986) proposed that the cornea is unable to generate its own epithelium and that it is derived from corneal epithelial stem cells (see later) which are located in the basal layer of the limbal conjunctival epithelium. Davanger and Evensen's hypothesis acquired new relevance with the introduction of these concepts. Tseng (1989) suggested that the palisades are modifications present to enhance the local concentration of stem cells.

Bron and Goldberg (1980) discussed the variety of the palisade pattern. They found an approximate symmetry between the eyes of an individual but noted that palisades varied between different regions in the same eye. They showed that palisades were most prominent in the vertical meridian, in particular the 6 o'clock position, where they were generally linear but found that they were absent on the whole from the horizontal meridian. Palisade stability was also questioned by these workers, who suggested that the palisade pattern may undergo senescent changes and that this may be a factor in the increased incidence of corneal pathology in the elderly. The variety and stability of the palisade pattern is investigated in the current study. Also the relationship between the stability of the palisade pattern and corneal integrity is examined when the palisade pattern is challenged with hydrogel contact lenses.

Palisades are often difficult to see biomicroscopically as they appear in poor contrast against the white sclera. Their visibility is enhanced by the presence of melanin pigment. Ruskell (1989) offered an anatomical explanation for the biomicroscopic appearance of palisades in Asian eyes, where pigment is often seen to bracket the

stromal elevations. He found that pigment was concentrated within basal epithelial cells and it was most dense in cells forming the slopes of the palisades and practically absent in those at the peaks. The process of how primate limbal conjunctival epithelium cells obtain pigment remains undescribed. As the current study investigates the relationship between pigment and the palisade pattern, the process of epithelial cell pigmentation can be examined further.

In a recent study, Townsend (1991) examined the clinical and histological appearance of the palisade zones. Believing that the palisade zones were responsible for replensinhg the corneal eithelium this investigator set out to examine whether cells of this area conformed to the morpholgical characteristics of stem cells and their off-spring as described elsewhere in the body.

Using a conventional 30x magnification slit-lamp Townsend categorised 300 subjects into one of 6 sets; each group having 50 subjects. These were: less than 40 years of age, over 40 years, male or female and lightly or heavily pigmented individuals. Corroborating the observations of earlier workers he noted that palisades were more linear infereiorly whilst superiorly they tended to be less linear and often attenuated. In quantifying palisade dimensions he found a mean length of 0.31mm (range 0.09 to 0.86mm), mean width of 0.04mm (range 0.03 to 0.06mm) and mean separation between adjacent palisades of 0.09mm (range 0.06 to 0.12mm). Like Bron and Goldberg (1980), Townsend found symmetry in the palisade pattern of an individual but noted variation in the types of palisade. He categorised palisades into one of three patterns. The first, described as 'standard', were linear and conformed to the average dimensions stated

before; this pattern had little or no associated pigment. The second variety was described as 'exaggerated', these were wider and were associated with heavy pigmentation and branched often. Streaks of pigment slide were often associated with this variety. The final type was described as 'attenuated', here the palisade was broken and associated with little or no pigment; Townsend claimed that this pattern was most commonly observed in lightly pigmented females. He found that palisades were absent in between 10 and 20% of the subjects he examined and that they were particularly absent in lightly pigmented individuals and the elderly.

A puzzling aspect of this paper was that, contrary to his clinical findings, in flat preparations Townsend claimed that lower limbal palisades were 'rather stubby' wheras superiorly the palisades were thin and elongated. This observation seems to have been carried over to the cytological aspects of the study.

Townsend noted that at light microscopic level the basal cell population lining the troughs of the palisade epithelium appeared no different to those at the crests. Two types of palisade basal epithelial cell were observed using electron microscopy. One was large, more numerous, and characterised by a prominent large, central nucleus. These cells contained abundant melanin granules and other cytoplasmic organelles but only a moderate number of keratin filaments. The second cell type was less frequent and distinguished by virtue of its smaller size and greater electron density. Its nucleus occupied the most of the cell, leaving little room for melanin granules and other organelles.

In the light of his histological findings and the fact that palisades were not visible clinically in up to 20% of the subjects examined, Townsend reasoned that it would be difficult to postulate that the palisade epithelium is involved in the renewal of the corneal epithelium. He recognised however, that the inability to see palisades clinically did not confirm their structural absence. The cellular richness of the palisade zones, led this worker to leave open the argument that this area was involved in cellular replenishment.

1.5. The Concept of Limbal Stem Cells

There is now a strong body of evidence which suggests that the corneal epithelium is unable to generate its own cells and that they are derived from cells of a conjunctival lineage. It is widely accepted that these "stem cells" are resident in the basal layer of the limbal conjunctival epithelium. Evidence supporting the existence of these cells is apparent from early work, much of which concentrated on epithelial cell migration following wounding. Friedenwald and Buschke (1944) arrested cell division with colchicine and demonstrated a greater number of mitotic figures in the periphery of the corneal epithelium in rats in comparison to the centre. These observations suggested the generation of these cells is away from the central cornea. The ability to rapidly regenerate under normal conditions is now a widely accepted property of any tissue containing stem cells. The work of Hanna and O'Brien (1960) demonstrated this property. They found that the corneal epithelium is a rapidly self-renewing tissue which has the ability to regenerate under normal conditions over a period of 4 to 6 days.

Davanger and Evensen (1971) first speculated that the limbus may be involved in the production of cells for the corneal epithelium. The work of Schermer et.al. (1986) was the first to demonstrate that the corneal epithelium was maintained by stem cells. They showed in rabbit, AE5, an antibody marker against 64-kiloDalton keratin, an indicator of advanced stage corneal epithelial cell differentiation, was expressed in all corneal and limbal epithelial cells except limbal basal cells. They concluded that the latter must therefore be undifferentiated or "primitive". Correlating their findings to stem cell theory in other epithelia, these workers argued that this property suggested that basal

cells of the limbal conjunctival epithelium correspond to stem cells for the corneal epithelium. By a similar line of thought, positively stained basal cells of the corneal epithelium were thought to be the immediate offspring of stem cells- transient amplifying cells. Centripetal movement of epithelial cells from the limbus to the cornea in the normal eye is an established process. Schermer and co-workers reasoned that sequestration of stem cells meant that transient amplifying cells must be the cells which migrate.

A series of investigations attempting to identify and characterise corneal epithelial stem cells in man followed the work of Schermer et al. Ebato et al (1987) found that the central corneal epithelium replicated more slowly than the peripheral corneal and limbal conjunctival epithelium. They suggested the greater regenerative capacities of these areas was because of the presence of transient amplifying and stem cells. These workers supported their argument by demonstrating fewer mitotic figures in central corneal epithelial explants compared to the two other areas under investigation. Thoft et.al. (1989) presented more direct evidence for corneal epithelial stem cells in man. Using the same monoclonal antibody (AE5) as Schermer and co-workers, they examined regional variations in the staining pattern. They found most positive staining in the vertical meridian, in particular superiorly and negligible staining in the horizontal positions. They concluded that corneal epithelial stem cells in man were to be found predominantly in the vertical meridian, and in these areas the entire basal layer of the limbal conjunctiva represented corneal epithelial stem cells.

Stem cells in other self-renewing epithelia, such as skin, have been shown to be slow-cycling, however, they produce offspring that proliferate rapidly (Hume and Potten 1983). Cotsarelis et al (1989) demonstrated this property in basal cells of the limbal conjunctival epithelium of SENCAR mice using tritiated thymidine. They found that under normal conditions, only 10% of basal cells displayed stem cell labelling characteristics. However, in response to wounding and tumour promoting antigens many more basal cells acquired stem cell labelling characteristics. They concluded that under normal conditions the corneal epithelium is maintained by a relatively small proportion of stem cells that are located in the basal layer of the limbal conjunctival epithelium but during times of stress the remainder of this layer had the ability to acquire stem cell properties to fulfil the need for further cells.

Much of the current theories of corneal epithelium stem cells are based on similarities between the limbal conjunctiva and skin. Lavker and Sun (1983) examined monkey palm epidermis and found that presumptive stem cells were located deep in the rete-pegs, close to the dermal vasculature, and were characteristically densely pigmented. They argued that this arrangement ensured that these important progenitor cells were protected by the layers of cells above them and their close proximity to the dermal vasculature guaranteed ample nutrition. These workers reasoned that stem cells were densely pigmented in order to protect the DNA within their nuclei from harmful UV damage. By contrast they found that the immediate offspring of these important progenitor cells- transient amplifying cells accumulated very little pigment within their cytoplasm. They suggested this heterogeneity existed because slow-cycling stem cells were resident on the basal layer for long periods without cell division and so acquired

more pigment through donation by melanocytes than their faster-cycling offspring, which divide rapidly and move off basal level to more superficial layers. Cotsarelis and co-workers proposed (in their aforementioned study) that the limbal conjunctiva provided similar features to corneal epithelial stem cells and hypothesised that in pigmented conjunctivae, these cells would always be densely pigmented. The process by which limbal conjunctival epithelial cells acquire pigment is currently undefined. Therefore, the relationship between cell cycle time and density of pigmentation proposed by Lavker and Sun in skin can only be true in the limbal conjunctival epithelium if the process of pigmentation is similar. This process and its relationship to cell cycle times are investigated in the current study.

Any organ containing stem cells may be represented in terms of a hierarchy (Tseng 1989) (Fig 3). Cells of the proliferative compartment (stem and transient amplifying cells) are identifiable using nuclear labelling techniques as they are actively synthesising DNA for cell division. Under normal conditions stem cells are relatively quiescent and therefore more difficult to identify than transient amplifying cells with a single pulse label. Hence, studies using markers against proliferation have concentrated in identifying the latter (Kruse et al 1990; Lavker et al 1991). Other studies have attempted to identify stem cells directly (Chung et al 1992; Lauweryns et al 1993). Zieske et al (1992) developed and used a monoclonal antibody, 4G10.3, which they claimed bound presumptive corneal epithelial stem cells. They used this antibody in adult rat, rabbit and man and found a reciprocal staining pattern to that of AE5. They presumed that positive labelling of basal cells of the limbal conjunctival epithelium indicated that this antibody was a marker of poorly differentiated cells, and so concluded

that this antibody labelled stem cells. However, work published a year earlier by the same group demonstrated that this protein actually represented the glycolytic enzyme alpha-enolase (Zieske et al 1991), which also occurs in other cells including transient amplifying cells. Chung et.al.(1992) used the same antibody to investigate the staining pattern in developing rats. They found that during development, stem or stem-like cells were present throughout the basal layer of the corneal and limbal epithelium and as the cornea matured about the time of stratification, these cells were sequestrated to the limbus.

The corneal epithelium is unique in that it would appear to be the only tissue in the body whose stem cells are located away from the structure which they serve. Advocates of corneal epithelial stem cell theories have argued that the sequestration of these cells to the limbus is essential to maintain the clarity of the cornea, as dividing cells may hinder this property and as the cornea is normally avascular it would be unable to support these cells (Schermer et al. 1986; Tseng 1989). The only evidence which suggests that the corneal epithelium houses its own stem cells was presented by Lauweryns et.al. (1993) who described a "new" epithelial cell type in man. They found these cells were most numerous in the superior peripheral cornea, infrequent in the inferior and completely absent from the nasal and temporal positions. Like basal cells of the limbal conjunctival epithelium, these cells co-expresss cytokeratin 19 (CK19) and vimentin, as well as being reactive to AE1 a marker of 48KD keratin. Surrounding non-transitional cells in the cornea were negative for these markers. Co-expression of CK19 and vimentin is typical of epithelia which have a self regenerative capacity. These workers argued that the anatomy and immunohistochemical profile of these cells make them ideal candidates for

the corneal epithelial stem cells and their position in the peripheral cornea, close to the limbal vasculature guarantees ample nutrition as well as ensuring optical clarity along the visual axis. However, as transient amplifying cells as well as stem cells occupy the proliferative compartment, a proportion of the transitional cells described by these workers may actually represent the former.

Lauweryns and co-workers also found a mosaic suprabasal expression of CK19 in the peripheral corneal epithelium and occasionally in the central region. They suggested that this could be a marker for retained proliferative capacity by centrally migrating cells.

Like limbal basal cells, transitional cells described by these workers showed granular staining for the $\alpha_6\beta_4$ integrin, a marker for hemidesmosmes, the limbal density of which is lower than that in the cornea (Gipson 1989). They also both stained positively with AE1, strongly for metallothionein, but negatively for cytokeratin 3 (CK3) and transferrin receptor. These workers argued that this immunohistochemical profile is characteristic of cells residing in the proliferative compartment of a tissue.

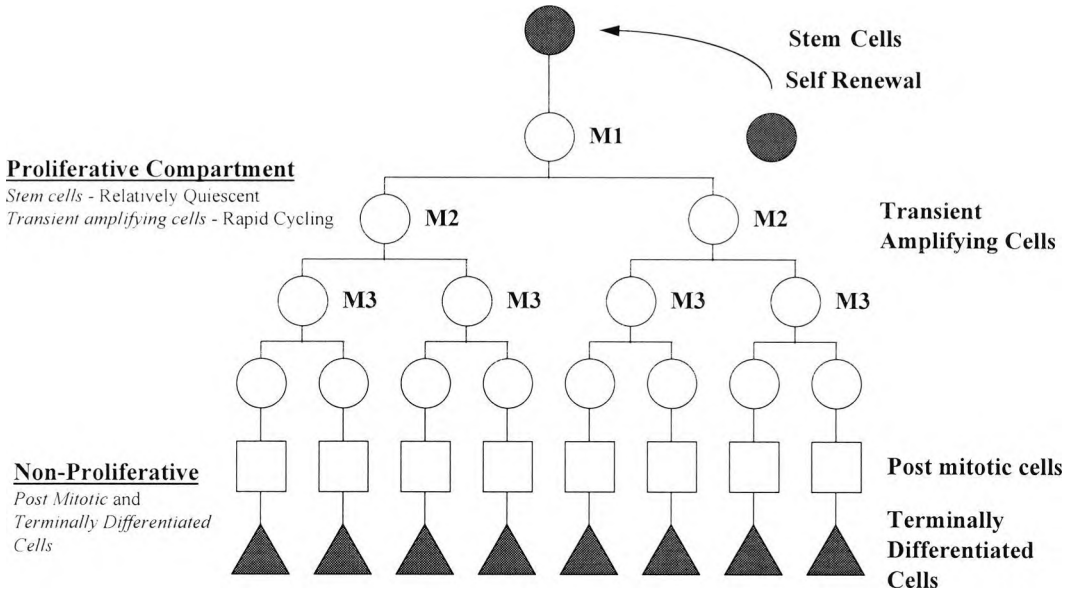


Figure 3. A schematic representation of cellular hierarchy in a tissue containing stem cells. Division of a stem cell produces two offspring, one which returns to the stem cell population by a process of self-renewal and another, the transient amplifying cell, that increase cell numbers by undergoing a few rounds of mitosis (indicated by M1, M2, M3). The offspring then mature (post mitotic cells) and differentiate (terminally differentiated cells). Stem cells and transient amplifying cells are found adjacent to the basal lamina whilst post mitotic and terminally differentiated cells are suprabasal. Modified from Tseng (1989).

The use of cell cycle proteins arguably represents the most interesting and newest development in the biochemical analysis of tissues containing stem cells. Zieske and Joyce (1995) examined the ocular surface with antibodies against cyclins and cyclin dependent kinases. These antibodies are specific to various stages of the cell cycle. Using in-situ hybridisation, they found that basal cells of the limbal conjunctival epithelium were held in a relative state of arrest between G_1-S phase of the cell cycle. Assuming these cells represented corneal epithelial stem cells these workers suggested that these cells are held in this relative state so that they can respond rapidly during times of need as they would not have to enter the cell cycle from the resting state (Go). Further work with cyclins, cyclin dependent kinases and other cell cycle proteins may

lead to a clear characterisation of stem cells and their offspring and the mechanisms involved in their regulation.

1.6. Centripetal Movement of Epithelial Cells From the Limbal Conjunctiva to the Cornea in Normal Eyes

Centripetal movement of epithelial cells is the fundamental process that must occur in a normal eye, if corneal epithelial stem cells are located in the limbal conjunctival epithelium. Its existence was recognised before the introduction of stem cell concepts, however most studies understood this to be in response to wound healing (Friedenwald 1951; Maumenee and Scholz 1948; Kuwabara et.al. 1976; Kaye 1980; Kinoshita et.al 1982). Davanger and Evensen (1971) observed the wedge shaped pattern of pigment slide into the peripheral cornea from the limbus in healthy Negroid eyes and suggested that centripetal movement was a normal feature. The observations of these workers acted as a stimulus for research into epithelial cell movement from limbus to cornea.

A few years later Bron (1973) noted similarities in vortex patterns in various corneal epitheliopathies. He hypothesised that the likeness in the patterns was due to the growth and subsequent centripetal movement of epithelial cells from the limbus to the cornea rather than any specific stimulus caused by the various disorders. He suggested the inferior displacement of the vortex was due to a predominantly downward cellular movement from the superior position. Further support for centripetal movement in normal eyes came from the work of Buck (1982) who found that hemidesmosomes in normal murine eyes had a radial arrangement along the basement membrane. These

observations suggested a centripetal path for cells migrating from the limbus to the cornea. Based on these and other experimental data on corneal epithelial wound healing Thoft and Friend (1983) proposed the "x, y, z," hypothesis of corneal epithelial cell maintenance. This theory suggested that corneal epithelial cell maintenance is dependent upon a continuous, centripetal migration of epithelial cells (y-component) as well as basal cell proliferation (x-component) and subsequent exfoliation (z-component) from the corneal surface. Although widely accepted, this theory was incomplete until the source of epithelial cells was defined.

Buck (1985) used a direct approach to test for centripetal migration. He marked the peripheral cornea of mice with a rotating needle containing a mixture of Indian ink and thorium dioxide. After seven days, he found that epithelial cells had migrated a median distance of 17μm per day towards the central cornea. Buck assumed that migration occurred in all layers but could only confirm it in the superficial and wing cell layers.

The process of centripetal movement acquired new status with the introduction of stem cell concepts. The x,y,z, hypothesis of Thoft and Friend was now complete, as the source of epithelial cells had now been identified as the limbal conjunctival epithelium. Schermer et al (1986) argued that transient amplifying cells migrate centripetally from the limbus, their site of origin, to become the basal layer of the cornea. Although this argument would appear to be widely accepted by advocates of stem cell theories, the mean by which these cells migrate still remains unclear. Current theories suggest that cell movement occurs along basal level (Tseng 1989), however, those cells generated furthest from the cornea would somehow need to bypass significant cellular traffic

before they reach their ultimate destination. Hence for these cells, current theories would seem impractical. The process by which epithelial cells reach the cornea from the limbus is considered in the current study.

Lemp and Mathers (1989) used corneal specular microscopy to investigate the driving force behind epithelial cell movement. Examining the central cornea, they suggested that preferential loss of surface cells by exfoliation from the apex secondary to shearing forces of the upper eyelid was the controlling factor behind this process. This argument was supported by Lavker et.al. (1991) who examined the relative proliferative rates of the limbal and corneal epithelium in SENCAR mice. Using tritiated thymidine labelling they suggested that population pressure per se could not be a major driving force for centripetal migration and hypothesised that centripetal migration may result from desquamation of central corneal epithelium thereby "drawing" cells from the limbus.

1.7. The Effects of Hydrogel Contact Lens Wear on the Limbal Conjunctiva

Most literature regarding the effects of hydrogel contact lenses on the ocular surface concentrates on the cornea, and perhaps somewhat surprisingly the limbus appears to be neglected. Arguably, the most evident response of the limbal conjunctiva to contact lenses is the filling of normally non-perfused fine calibre vessels and their sustained dilation. Although this may give a fuller picture of the limbal vasculature McMonies et al (1982) suggested that sustained dilation may be a precursor to new vessel growth ultimately leading to the vascularisation of the cornea. Although corneal vascularisation frequently occurs without any significant symptoms, it is a serious complication of lens

wear. Its presence reflects unsatisfactory tolerance and serves as an indication for the need to reassess contact lens fitting and management.

The aetiology of corneal vascularisation is thought to be multifactorial. Efron (1987) suggested the hypoxia induced by contact lenses leads to corneal oedema and stromal softening. Additionally, epithelial microtrauma from either the contact lens directly, or as a response to the toxicity of the care system leads to enzyme release which results in inflammatory cells being drawn to the site of damage. Release of vasostimulating agents by these cells induces blood vessel growth from dilated limbal capillaries. In order to minimise the risk of contact lens associated corneal vascularisation Silbert (1992) suggested fitting lenses with a high oxygen transmissibility, good movement and comfort together with using care systems that avoid preservatives such as benzalkonium chloride or thimersol which can be cytotoxic (Pfister and Burstein 1976; Mondino and Groden 1980).

Currently there is no literature regarding the effects of contact lenses on the corneal epithelial stem cells. A possible explanation may lie in the inability in identifying these cells directly. Kruse et al (1990) suggested that corneal epithelial stem cells may form a barrier preventing invasion of the cornea by the conjunctiva. Sustained mechanical irritation and hypoxia induced by hydrogel contact lenses together with the potential toxicity of contact lens care solutions may act to damage these cells leading to a breakdown of this barrier which may result in clinically evident signs. Therefore, it would be advantageous if an early indication of potential problems was available. The palisade pattern may offer this evidence, hence, the effects of contact lenses are

investigated in the current study. Previous workers have suggested that the palisades of Vogt may act as a repository for generating cells for the corneal epithelium (Davanger and Evensen 1971; Bron 1973). If this hypothesis is true then any alteration in the pattern may reflect elsewhere on the ocular surface, particularly on the cornea and conjunctiva.

CHAPTER 2

MATERIALS AND METHODS

2.1. Photographic Monitoring of the Limbal Conjunctiva

2.1.1. Description of Apparatus.

Due to the poor resolution and insufficient magnification of commercially available slit-lamp biomicroscope photographic systems, a dedicated anterior segment photographic system (Fig 4) was designed for this study. The system comprised a 4x light microscope objective lens (Olympus, U.K. Ltd), an optical tube and 10x eyepiece (Zeiss, Germany), which was connected to an Olympus OM10 SLR camera (Olympus, U.K.) by a specially manufactured adapter.

During use, the apparatus was mounted on a slit-lamp table and replaced the slit-lamp observation system. The eye under investigation was illuminated by fibre optics which were attached either side of the photographic system (Fig 5). The illumination system of the slit-lamp was substituted with a small flash gun which was raised by a platform. In order to reduce the intensity of the flash a 1.0 neutral density filter was placed over the aperture of the gun. The angle between the observation and illumination systems was always 45°. The subjects gaze was directed in the opposite direction to the area

being investigated (i.e. superiorly when the inferior limbal conjunctiva was to be photographed and inferiorly while the superior position was being investigated). Results were recorded on (Kodak) Ektachrome 200 ASA colour transparency.

2.1.2 Control Group

The control group for this study consisted of 20 subjects, comprising 11 females and 9 males, with a mean age of 21.05 years (range:19-24 yrs, SD: 1.54). All subjects were non-contact lens wearers who had no desire to wear them for the duration of the study (one year). Each individual was chosen on the basis that they had no previous history of ocular pathology and had passed a full examination of both the anterior and posterior segments and most importantly because they displayed prominent limbal palisades.

2.1.3. Monitoring of Control Group.

Photographs of the palisades of Vogt in the inferior and when visible, superior positions were taken at baseline (0) and at then at 3 monthly intervals (3, 6 and 12 months from baseline). The nature of conjunctival vessels or the palisade pattern served as a guide, ensuring the same region was photographed each time. Due to the precise depth of focus of the photographic system used, several photographs were taken of the area of interest. The most consistent transparencies were used for analysis.

Figure 4. Photographic system used to monitor the palisade pattern. The system consists of a light microscope objective lens x4 (0), an optical tube (T), a specially adapter (containing a light microscope eyepiece x 10), SLR camera (C) and flash gun (F). The palisade pattern is viewed through the camera.

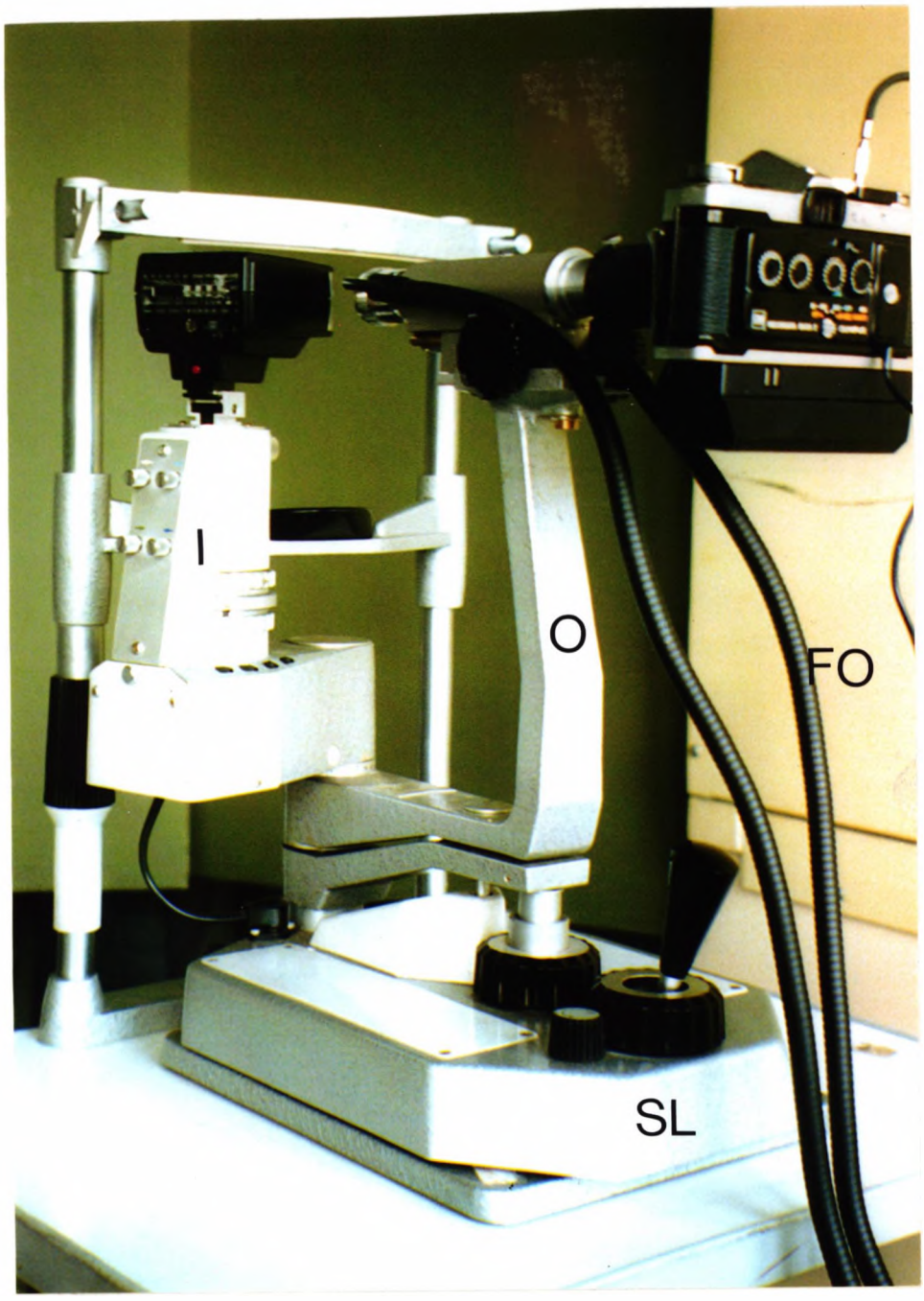


Figure 5. The apparatus mounted on a slit-lamp microscope (SL). The photographic portion is housed where the observation system would normally be (0). The flash gun which freezes saccadic eye movements is mounted where the illumination system would sit. The area to be photographed is illuminated by focal illumination from a fibre optic source (F0) which is set at the lowest intensity.

2.1.4 Experimental Group

The experimental group for this study consisted of 21 subjects, 11 females and 10 males, with an average age of 22.57 years (range:18-33 yrs, SD: 3.25). All subjects were first time lens wearers and had no previous history of any anterior segment pathology. Subjects chosen for this aspect of the study displayed prominent palisades of Vogt and had passed a suitability test for hydrogel contact lens wear. The latter involved a full record of general health and medical history (general and ocular), refraction, keratometry, full slit-lamp examination of the ocular surface and careful fundoscopy. Further subject information is given in the appendix.

2.1.5. Contact Lens Fitting of Experimental Group.

All subjects in the experimental group were fitted with hydrogel lenses kindly donated by Bausch & Lomb (B&L) U.K. ltd (B&L 70 lenses, B&L, Middlesex, U.K.). All hydrogel lenses were 70% water content, 14.3mm in total diameter and were lenticulated with a back optic zone diameter of 11mm ensuring a constant peripheral edge thickness (of 0.14mm). Lenticulation ensured the same oxygen transmission to the limbal conjunctiva irrespective of lens power. The centre thickness of the lenses varied slightly with power (range 0.14 to 0.16mm - manufacturers data).

Subjects were fitted within the guidelines supplied by the manufacturers. They were taught to clean their lenses daily using a three step cleaning regime(10:10 starter-pack, Ciba, High Wycombe, U.K.); surfactant (Miraflow) for at least 30 seconds followed by rinsing in normal saline, peroxide disinfectant (10:10 step I) overnight and neutralising

(10:10 step II) the following morning for at least 30 minutes. Guidelines of the College of Optometrists (UK) regarding contact lens practice were followed and subjects were instructed to avoid "over-wearing" their lenses and never to sleep with their contact lenses on.

2.1.6. Monitoring of Experimental Group.

Prior to contact lens fitting, the palisades of Vogt in the inferior position and when visible the superior position, were photographed to provide baseline (0) information. After contact lens fitting photographs of the same areas (with contact lenses removed) were taken at regular intervals (1, 3, 6, and 12 into lens wear). An aftercare examination was performed 2 weeks into contact lens wear to ensure no adverse reactions to hydrogel lens wear, however photographs were not taken at this visit. In order to investigate any changes in palisade parameters during the early stages of adaptation measurements were recorded 1 month after lens fitting.

Subjects underwent full aftercare examination at every appointment prior to photography. Any changes in the integrity of the cornea or conjunctiva were noted and acted upon if necessary. Subjects were further advised on care of their contact lenses and any queries were answered.

2.2. Histology

Two whole human anterior eye segments (HW23 and HW28) and selected segments from a number of other eyes (see appendix for further details) were sectioned at various orientations for the purpose of histological examination.

Several anterior eye segments of rhesus and cynomolgus monkey were sectioned at various orientations for the purpose of the primate comparative anatomy study (see appendix for information of all specimens examined).

2.3. Preparation of Tissues for Histological Examination (Light and Electron Microscopy)

Anterior halves of cadaver eyes were orientated by noting, where possible, the positions of extraocular muscle insertions; the extent of the conjunctival overlap of the peripheral cornea and in man, the presence and shape of the palisades of Vogt. These were then cut into approximately 12 equal radial segments from which most of the sclera and uvea was dissected away.

All tissues for histology were fixed by immersing in a solution of 2% glutaraldehyde and 3% paraformaldehyde which was buffered to pH 7.4 with sodium cacodylate. Tissues were stored in fixative until they were to be embedded. Prior to embedding tissues were transferred to a buffered sucrose solution overnight, then washed with distilled water the following morning and post fixed in 1% osmium tetroxide for one

hour. They were then dehydrated through graded ethanol's (50%, 70%, 90%) for 20 minutes in each concentration and then in absolute ethanol for an hour with one change of solution. Tissue segments were then cleared in xylene for an hour (during which time they were agitated and the solution changed once), then transferred to a solution consisting of an equal volume of xylene and Araldite for 30 minutes before immersion in Araldite overnight at room temperature (whilst being constantly rotated) The following morning tissues were removed and embedded in Araldite-filled trays or capsules and then incubated at 60°C for 48 hours. After two days, embedded tissues were removed from the incubator and allowed to cool to room temperature before being processed for microscopy.

Semi-thin sections (0.75-1.00μm) for light microscopy were cut with glass knives on a Reichert-Jung Ultracut E microtome. Sections were collected, mounted on glass slides, dried and then stained with a solution of equal volumes of 1% toluidine blue in 2.5% sodium carbonate. Interrupted and sometimes full serial sections were collected. Sections were cut predominantly transversely, parallel to the limbus, although some segments were sectioned tangentially and radially.

Ultrathin sections (75-100nm) for transmission electron microscopy were cut with a diamond knife in areas of interest. Sections were collected on copper grids and stained with a saturated solution of uranyl acetate in 70% ethanol for 20 minutes. The grids were then washed three times in distilled water and then stained in a 0.4% lead citrate in 0.1M sodium hydroxide solution for a further 20 minutes. The grids were washed in a

similar manner as before then allowed to air dry overnight before storing for examination.

Sections were examined using a JEOL JEM-100B electron microscope.

2.4. Conjunctival Biopsies for Immunohistochemistry

Three millimetre circular biopsies of full thickness epithelium and some underlying anterior stomal tissue were taken by surgeons at Moorfields Eye Hospital from patients undergoing routine cataract extraction. All biopsies were taken from the superior position from an area bridging the peripheral cornea, limbus and some bulbar conjunctiva. The area to be sampled was marked with a corneal biopsy trephine and the tissue was carefully dissected out before the eye was sectioned for removal of a cataract.

2.4.1. Primary Antibodies for Immunohistochemistry

Bromodeoxyuridine (BrdU) is a thymidine analogue which is incorporated into DNA during S-phase of the cell cycle (Hall 1993) Hence, immunolabelling with the mouse monoclonal antibody, anti-BrdU is specific to cells that are well committed to proliferation and are at any stage of S-phase of division. In vitro incorporation of BrdU is not exclusive to dividing cells; there is evidence that positive expression occurs in cells that are participating in DNA repair (DAKO data sheets). In contrast, Ki-67 is expressed throughout the cell cycle. firstly being expressed in mid G_1 and increases in

level through S and G_2 and peaking in M phase (Gerdes 1984). Furthermore, Ki-67 is not expressed during the DNA repair process (Hall 1993). Norton et al (1995) tested a panel of antibodies in their study, including Ki-67, and found significantly increased positive staining following treatment using a novel heat mediated antigen retrival technique (see later) on wax sections. Hence BrdU and Ki-67 were used as detectors of proliferation in the present study.

2.4.2. Incorporation of Labelling Agent Bromodeoxyuridine (BrdU)

Tissue biopsies for immunohistochemistry were transferred immediately upon removal to Dulbecco's minimum essential (tissue culture) medium (DMEM Sigma Chemical Co, Poole, UK.) to allow transportation. Biopsies were then incubated for 4 hours in a 1:1000 solution of BrdU (Amersham Int., Bucks, UK.)/DMEM at 37°C, after which specimens were fixed in chilled acetone containing protease inhibitors (20mM iodoacetamide and 2mM phenylmethyl sulfonyl fluoride) at -20°C overnight.

2.4.3. Processing of Biopsies to Glycolmethacrylate (GMA) Resin

Tissue biopsies were processed to GMA resin using the technique described by Britten et.al. (1993). Following incubation in acetone plus protease inhibitors at -20°C overnight, specimens were transferred into acetone and then methylbenzoate at room temperature for 15 minutes each prior to processing. Tissues were then immersed in a solution of GMA monomer (JB4 Solution A, JB4 Kit, TAAB, Aldermaston, UK)

containing 5% methylbenzoate. Infiltration was carried out at 4°C over a 7 hour period with three changes of the solution.

GMA embedding resin was prepared by mixing 10ml of GMA monomer (JB4 Kit, Solution A) with 250µl of N,N-dimethylaniline in PEG 400 (JB4 Kit, Solution B, accelerator) and 45mg benzoyl peroxide (JB4 Kit, dry catalyst C). Specimens were embedded in freshly prepared resin solution in flat bottomed polythene capsules (TAAB). Capsules were filled to the rim and sealed to exclude air during polymerisation. Polymerisation was carried out at 4°C for 16 hours (overnight). Blocks were then moved to a -20°C freezer where they were stored in an air-tight container with silica granules (to absorb any moisture) until they were to be sectioned.

2.4.4. Ultra-Microtome Sectioning of GMA Embedded Tissues.

GMA embedded blocks were orientated, and excess resin was trimmed away with a razor-blade before 1.00µm sections were cut with a glass knife using a microtome (Reichert-Jung Ultracut E). Sections cut transversely, parallel to the limbus, were floated onto a 0.2% ammonia solution (prepared in double distilled water) for 30 to 60 seconds, allowing sections enough time to stretch and then picked up onto slides coated with APES (3-amino propyl tri-ethoxy silane). Slides were left to dry at room temperature for 1 to 6 hours before being stored, wrapped in foil, at -20°C until they were to be used for immunocytochemical staining.

2.4.5. Immunohistochemical Staining of GMA Embedded Sections

Neither removal of resin or trypsinisation are necessary for GMA embedded sections (Britten et.al. 1993). The area of the slide containing the sections was outlined with a DAKO pen. Slides were immersed in a solution of 0.3% hydrogen peroxide in 0.1% sodium azide for 30 minutes at room temperature in-order to inhibit endogenous peroxidase activity (Li et.al. 1987), followed by two washes in 0.05M tris buffered saline (TBS, pH 7.6) for five minutes each.

The slides were drained and tissue culture medium (RPMI, GIBCO BRL, Paisley UK) containing 20% fetal calf serum and 1% bovine serum albumin was applied (in order to block non-specific background staining) for 30 minutes. Slides were again drained and the mouse monoclonal primary antibody, Anti-Bromodeoxyuridine (DAKO, High Wycombe, UK.), diluted to 1:20 in TBS was applied over the sections. Slides were then incubated in a humidity chamber overnight (16-20 hours).

The following morning, slides were washed twice for five minutes each in TBS, drained and incubated with biotinylated $F(ab')_2$ fragment of rabbit anti-mouse immunoglobulin (DAKO) for two hours at a dilution of 1:200 in TBS. Slides were then washed twice in TBS for five minutes each, drained and incubated with streptavidin biotin-peroxidase complexes (DAKO prepared as per manufacturers instructions) for two hours. The slides were washed twice in TBS for five minutes each before the antigenic sites were demonstrated by incubating in aminoetyhlcarbazole (AEC, Sigma) for 30 minutes (AEC substrate was prepared by mixing 0.6ml AEC in dimethyl formamide with 2ml 0.1N

acetic acid and 8.0ml 0.1N sodium citrate. The resultant solution was filtered and 50µl of 30% hydrogen peroxide was added prior to use). Slides were then washed once in TBS, followed by a 5 minute wash in cold tap water. The sections were lightly counterstained in eosin and mounted in glycergel mounting medium (DAKO) and left overnight at 4°C to set. Sections without primary antibody served as negative controls.

Due to the erratic nature of staining, (presumably due to GMA either damaging or irretrievably masking the antigenic sites that this study was attempting to demonstrate) this technique was abandoned and a wax/heat mediated antigen retrieval (HMAR) protocol was adopted (see later). The latter method is reputed to improve the specificity of the primary antibody used (Ki-67) resulting in no need for serum blocking for non-specific background staining (Norton et.al. 1994).

2.4.6. Processing of Biopsies to Paraffin Wax

Following incubation in 10% formal saline for between 24 and 48 hours, biopsies were washed in distilled water and then dehydrated through graded ethanol's - 50%, 70%, 90% for 30 minutes in each concentration and then in absolute ethanol for an hour with one change of solution. Biopsies were then cleared in xylene for one hour (with one change), before infiltrating with molten wax at 60°C over a 3 hour period; with hourly changes of the solution. The tissues were finally embedded in moulds filled with molten wax and left at room temperature allowing the wax to solidify.

2.4.7. Ultra-Microtome Sectioning of Wax Embedded Tissues

Wax blocks were orientated so that sections were cut transversely, parallel to the limbus. Excess wax was trimmed away with a razor-blade and the remainder of the block was mounted upon a piece of wooden dowel which was then clamped to the microtome chuck. Sections (3.00μm) were cut with a glass knife, using a (Reichert-Jung Ultracut E) microtome, collected and allowed to stretch for a few seconds in a 10% methanol solution prior to transferring to a water bath at 70°C. After a few minutes sections were picked up onto APES (3-amino propyl tri-ethoxy silane) coated slides. Slides were left to dry at room temperature for 1 to 6 hours before transferring them to an oven at 60°C overnight prior to antigen retrieval for immunostaining.

2.4.8. Antigen Retrieval Using a Novel Heat Mediated Antigen Retrieval (HMAR) Technique

Following removal of wax with xylene for a total of 15minutes (3 changes), sections were re-hydrated through graded ethanol's -absolute, 90%, 70%, 50%, 5minutes in each concentration then distilled water. Sections were then treated using the novel heat mediated technique for antigen retrieval (HMAR) described by Norton et al (1995). In order to prevent drying of sections, slides (up to 12) were placed in a steel staining rack and immersed in distilled water. The rack was then transferred to boiling citrate buffer held within a stainless steel pressure cooker and treated under "superheat" conditions for 4 minutes. The staining rack was removed once the buffer had cooled and slides were

washed and kept immersed in tap water in order to prevent drying out of sections prior to immunostaining.

2.4.9. Immunohistochemical Staining of Wax Embedded Tissue

Sections treated using the HMAR technique were subjected to immunocytochemical staining. Slides were transferred to a 3% solution of hydrogen peroxide in methanol in order to block endogenous peroxidase activity for 30 minutes at room temperature, followed by two 5 minute washes in Phosphate buffered saline (PBS, pH 7.4).

Serum blocking for non-specific background staining was not necessary for sections pre-treated using the HMAR technique (Norton et.al. 1995). The area of the slide containing the section was drained and outlined with a DAKO pen and the mouse polyclonal primary antibody, Ki67 (DAKO, High Wycombe, UK.), diluted 1:100 in PBS/BSA/Azide (1%BSA, 0.1%Azide in PBS pH 7.4) was applied over the sections; slides were then incubated in a humidity chamber at room temperature for one hour. Slides were then washed twice for five minutes each in PBS, drained and incubated with biotinylated $F(ab')_2$ fragment of rabbit anti-mouse immunoglobulin (DAKO) for one hour at a dilution of 1:100 in PBS. Slides were then washed twice in PBS for five minutes each, drained and incubated with streptavidin biotin-peroxidase complexes (DAKO, prepared as per manufacturers instructions) for one hour. Subsequently, slides were washed twice in TBS for five minutes each before the antigenic sites were demonstrated by incubating in Diaminobenzamide (DAB, Sigma U.K.) for 5 minutes (DAB was prepared by adding one DAB "tablet", Sigma U.K. to 15ml PBS. The

resultant solution was filtered and activated prior to use by adding 12μl of 30% hydrogen peroxide). Slides were then washed once in PBS, followed by a 5 minute wash in cold tap water.

The sections were then counterstained with haematoxylin, dehydrated through graded alcohols and cleared in xylene before mounting (using conventional techniques) and left overnight at room temperature to set. Sections of human tonsil germinal centres served as positive controls whilst sections of limbal conjunctiva without primary antibody were negative controls.

CHAPTER 3

RESULTS

CLINICAL OBSERVATIONS OF THE HUMAN LIMBAL CONJUNCTIVA

3.1. Qualitative Analysis

3.1.1. Control Group

The palisades of Vogt occur principally in narrow crescent shaped bands at the upper and lower limbus approximately 0.25 to 0.50mm distal to the forward most limbal loops (Fig 6). They were most prominent in the vertical meridian where the conjunctival/scleral overlap of the cornea is greatest. A variety of palisade forms existed; those located inferiorly were generally linear, whereas superiorly when present, they were less regular and often consisted of many interspersed papillae (Figs 7a&b). Furthermore, the degree of associated pigment was also variable, Figure 8 illustrates the linear variety from the inferior position in an Asian eye that are bracketed by pigment, giving a typical appearance of a pair of parallel pigment lines. By contrast the slit-lamp photograph in Figures 7a&b from a Negroid eye, illustrate the common dense, diffuse pigmentation of the interpalisade zone. Figure 9 illustrates the difficulty in visualising palisades in Caucasian eyes, where generally there is no associated pigment. This figure

illustrates how palisades often act as conduits for recurrent conjunctival vessels that move forward in a radial manner towards the cornea. As these vessels approach the cornea they frequently give rise to fine branches which form the limbal vascular arcades.

The most common form of palisade encountered in the current study was linear, although interruptions in palisades resulted in "island features" frequently being encountered (Fig 10). Interconnections between adjacent palisades resulted in other more "complex" shapes existing alongside the commoner forms (Fig 11). Generally, there was symmetry between the palisade pattern of the two eyes of an individual although this was not always exact. In one subject (KP), who had previously worn a "poorly fitting" hydrogel contact lens in his right eye only, there was very little symmetry between the palisade pattern of each eye (Fig 12).

Over the duration of this study, palisade morphology and the pattern of associated pigment appeared to remain unchanged in all the subjects examined (Figs.13, 14). The stability of the palisade pattern in this group is investigated quantitatively later in this chapter.

3.1.2. Experimental Group

Arguably the most evident change in the limbal conjunctiva to contact lens wear, particularly during the early stages of adaptation, was the dilation of vessels and perfusion of previously empty capillaries resulting in a fuller picture of the limbal vasculature being visible. The apparent increase in vessel calibre did not appear to

affect palisade morphology Palisade morphology and their associated pigmentation pattern appeared to be unaltered in all individuals of this group over the duration of the study (Figs 15, 16, 17). Their length, width and the distance between adjacent palisades are quantified in the following section.

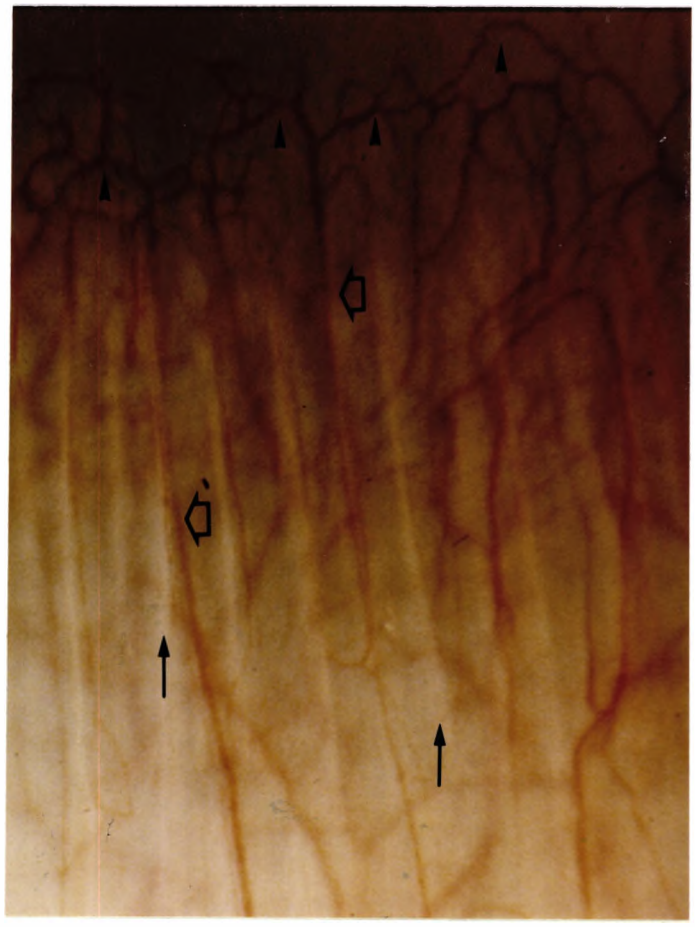


Figure 6. A fuller picture of the limbal vasculature is apparent when the eye is congested. The palisade zones are located 0.25 to 0.50mm distal to the anterior corneal arcades. In this slit-lamp photograph of the inferior position in a Caucasian eye (subject DB), radial vessels (empty arrows) move forward centripetally through palisades (arrows) and branch, forming the anterior corneal arcades or "limbal loops" (arrowheads). (x70)

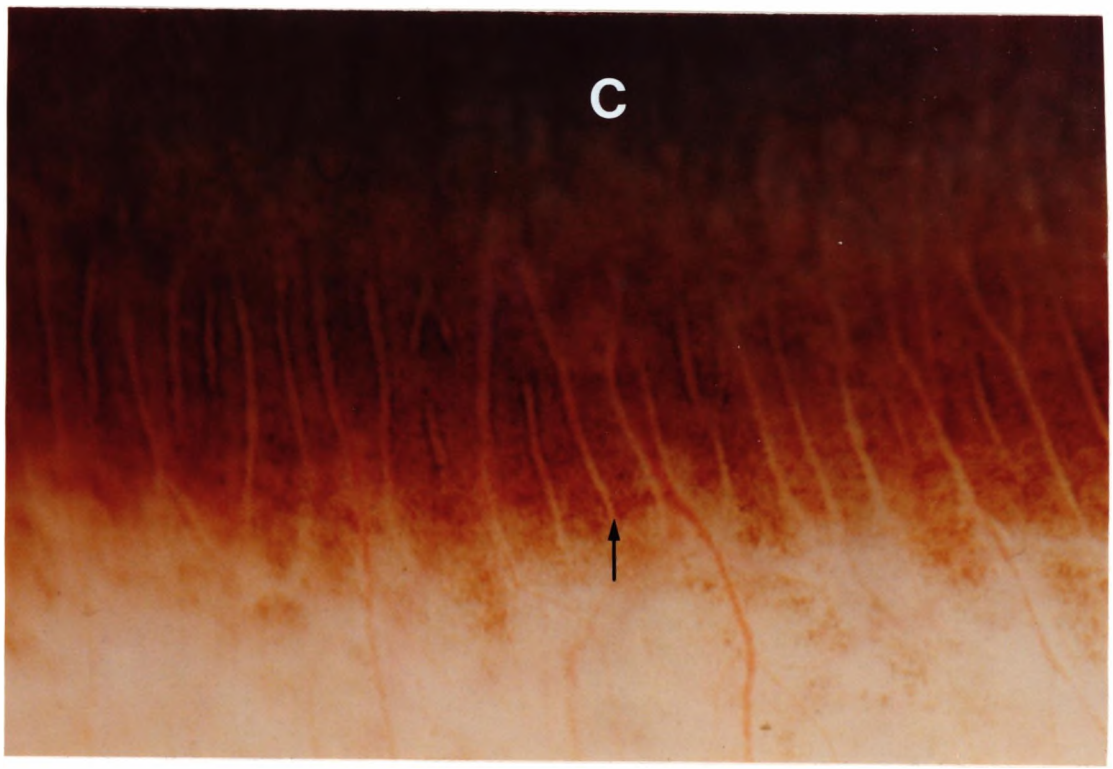


Figure 7a. The palisades of Vogt in the inferior position in a Negroid eye. The crests of palisades are represented by the narrow clear interval (arrow). The wider diffusely pigmented areas represent the interpalisade zone. C=Cornea. (x76).

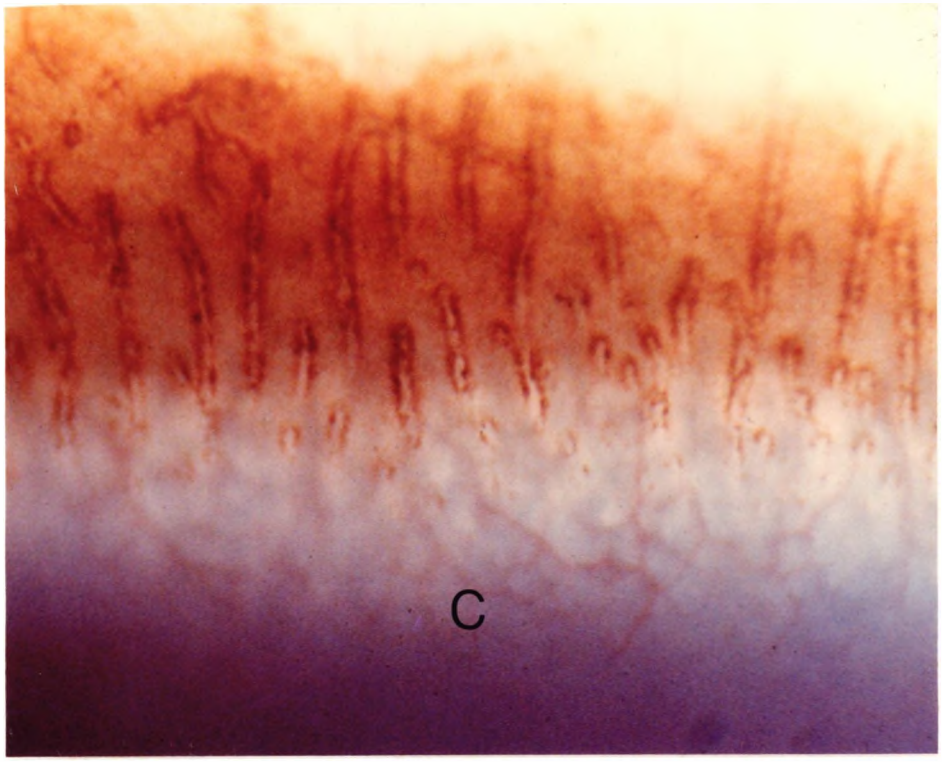


Figure 7b. The palisades of Vogt in the superior position in a Negroid eye. This example shows an irregular palisade pattern displaying numerous papillae in addition to the linear form. Each palisade is outlined by dense pigment. C=Cornea. (x76).

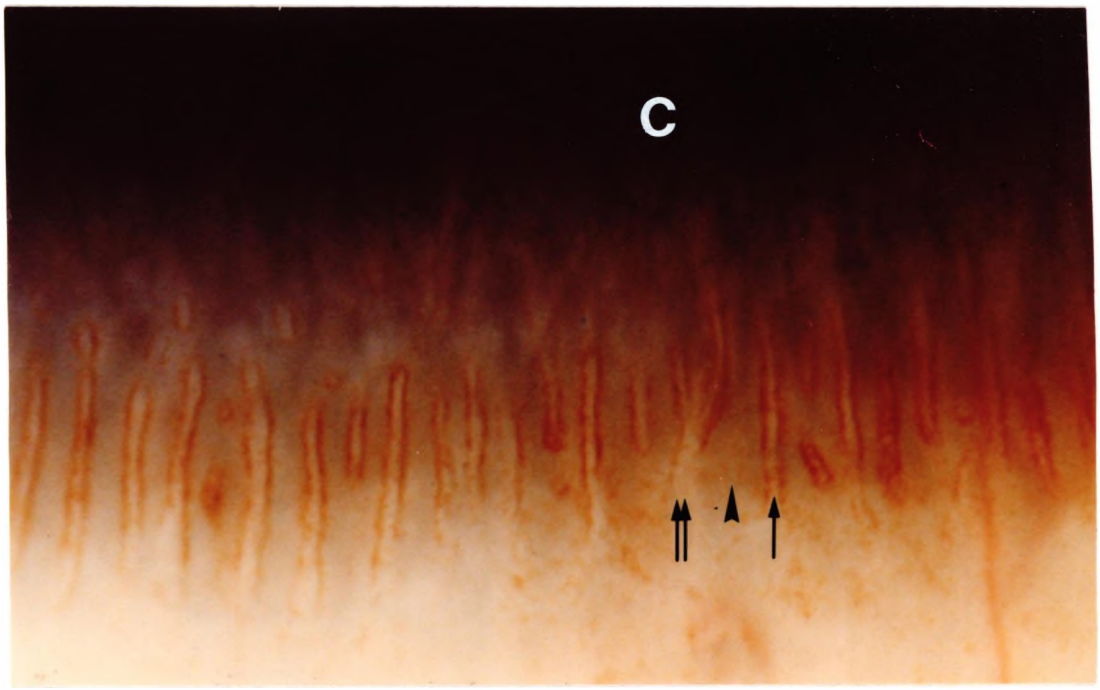


Figure 8. The palisades of Vogt in the inferior position in an Asian eye. Each palisade is outlined by dense pigment giving the appearance of pigment bracketing. Variation in palisade form is illustrated. A Y-shaped palisade (double arrow) is seen adjacent to a linear form (single arrow). In this example the interpalisade zone (arrowhead) is only lightly pigmented. C=Cornea. (x70).

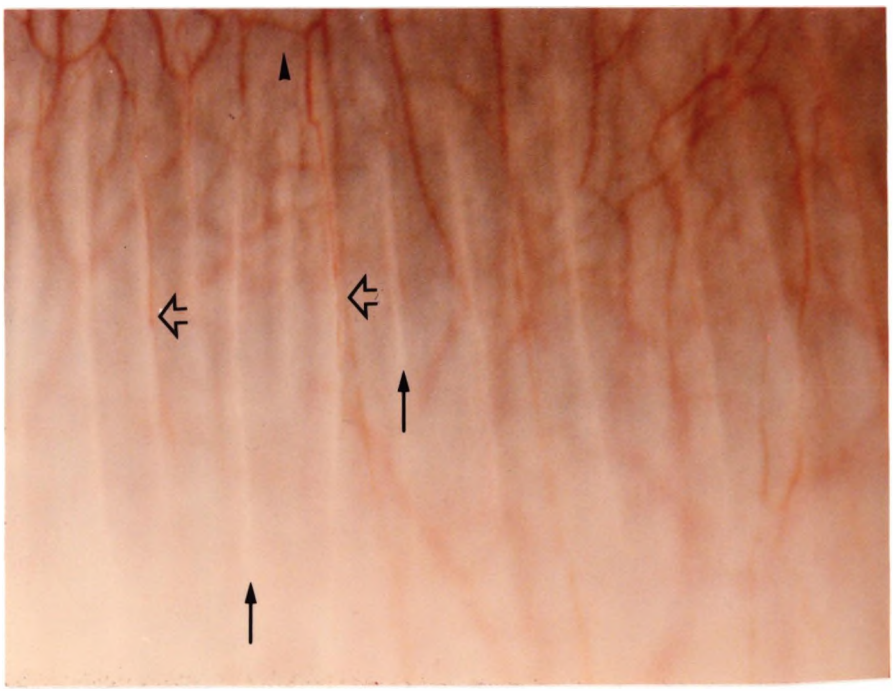


Figure 9. A slit-lamp photograph of the palisades of Vogt in the inferior position in a Caucasian. The connective tissue palisades (arrows) are difficult to visualise against the white sclera. Palisades often act as conduits for radial vessels (empty arrows) that are frequently the source of the limbal loops (arrowhead). (x77).

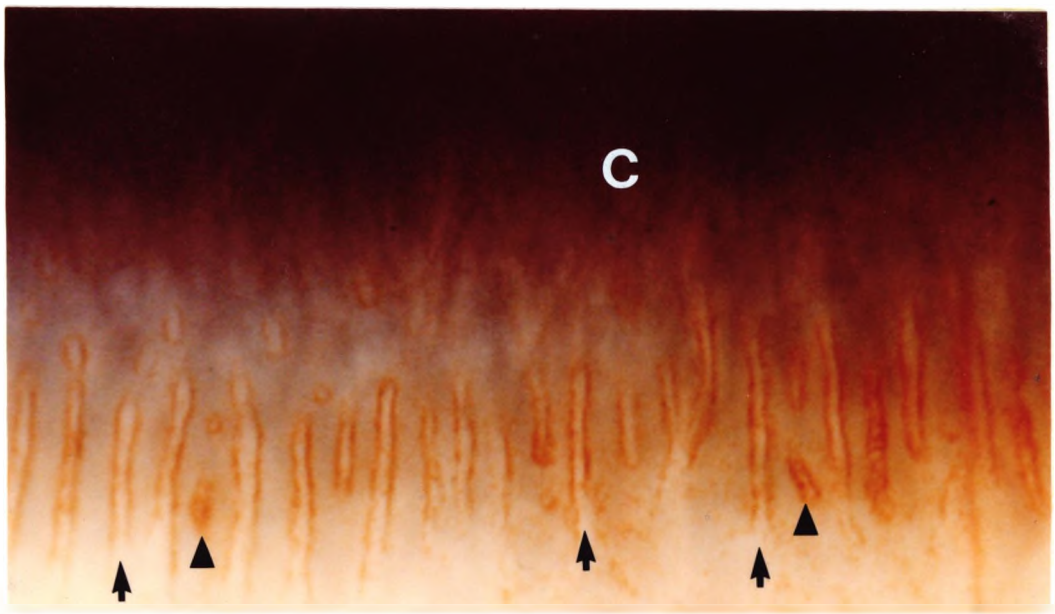


Figure 10. The palisades of Vogt in the inferior position in an Asian eye. Pigment bracketed palisades are present. Linear palisades are the most common form (arrows), however, where a palisade is interrupted "island" features (arrowheads) are clearly visible. C=Cornea. (x72).

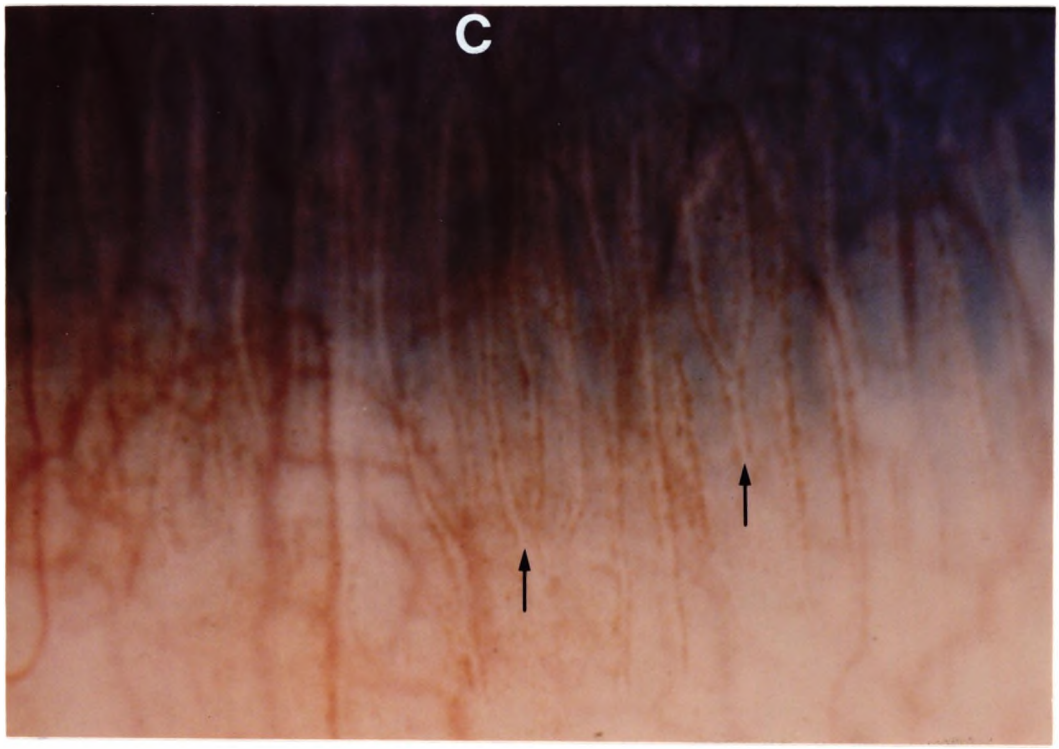


Figure 11. The palisades of Vogt in the inferior position in an Asian eye. In this example the palisades are lightly outlined with pigment. Interconnections between adjacent palisades results in "complex" shapes being visible. Two Y-shaped palisades are indicated (arrows). (x78).

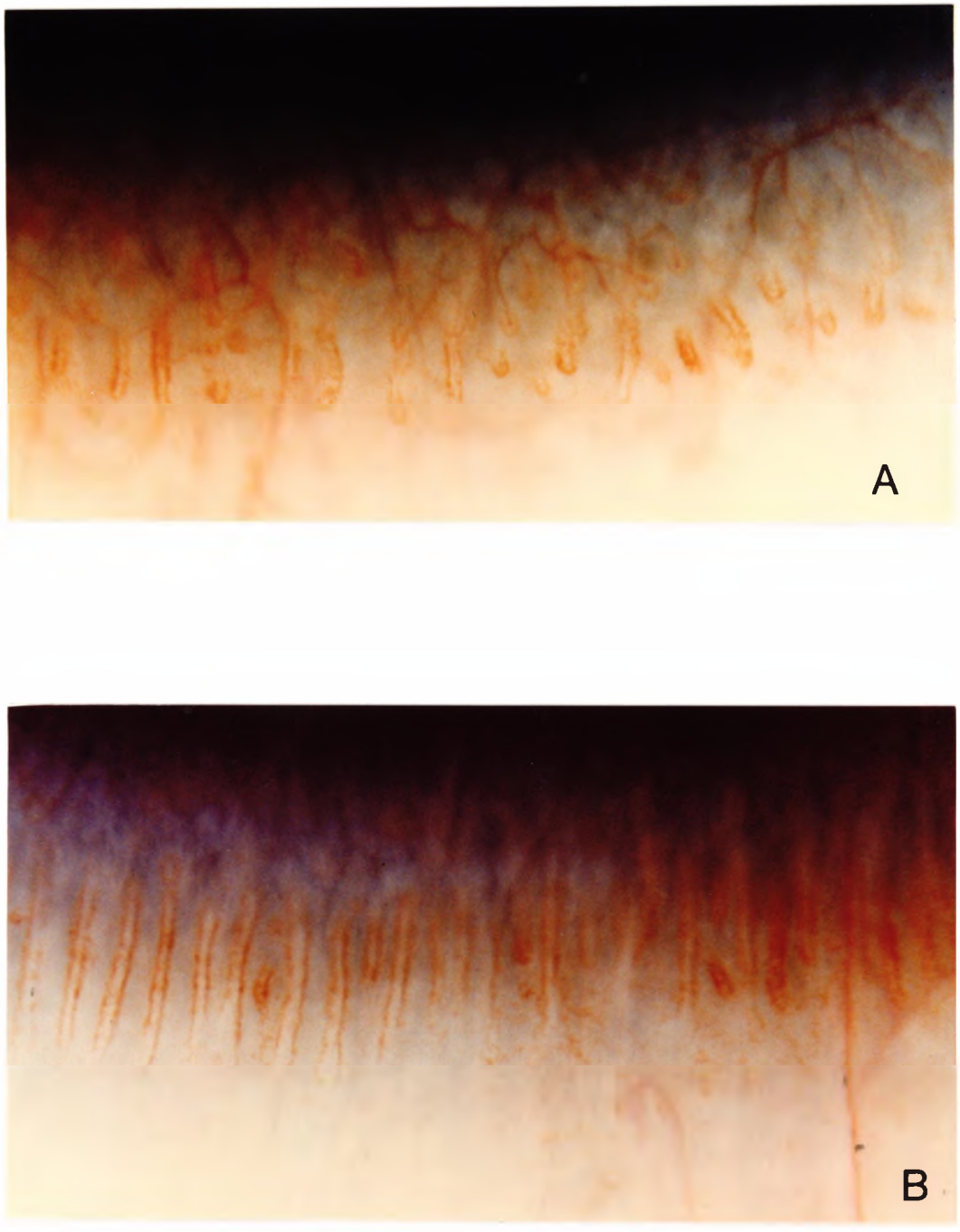


Figure 12. Generally, there is symmetry between the palisade pattern of the two eyes of an individual. However, the palisade pattern in the inferior position of subject KP who had previously worn a poorly fitting hydrogel contact lens in his right eye only, (photograph A) bears no similarity to its counterpart in the left eye (photograph B). (x68).

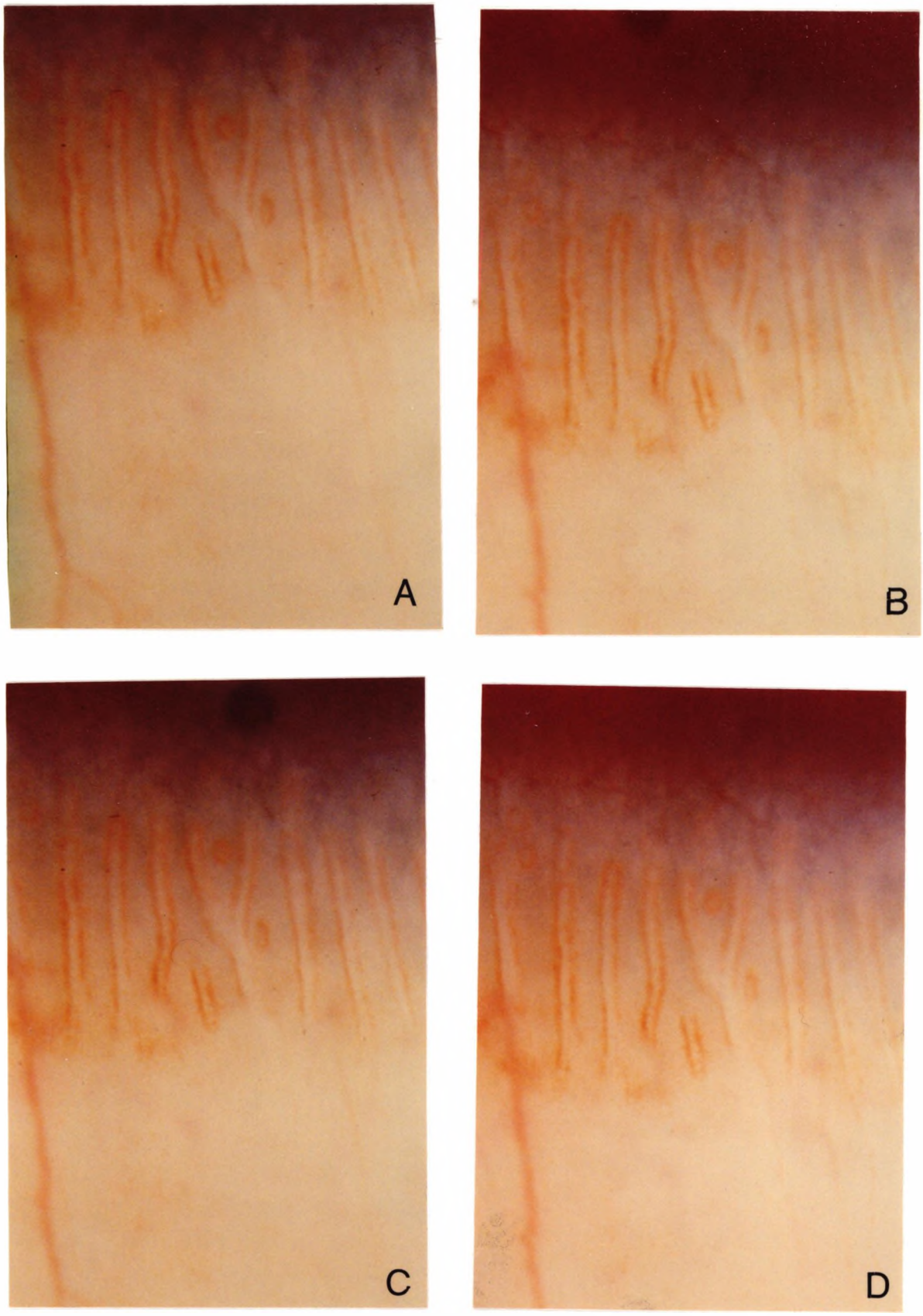


Figure 13. Slit-lamp photographs illustrating the stability of the palisade pattern over a duration of one year. The palisade pattern and the pattern of associated pigment of subject SB of the control group, appears unaltered over the duration of the study. Photographs were taken at baseline (A), 3 months later (B), 6 and 12 months from baseline (C and D respectively). (x68).

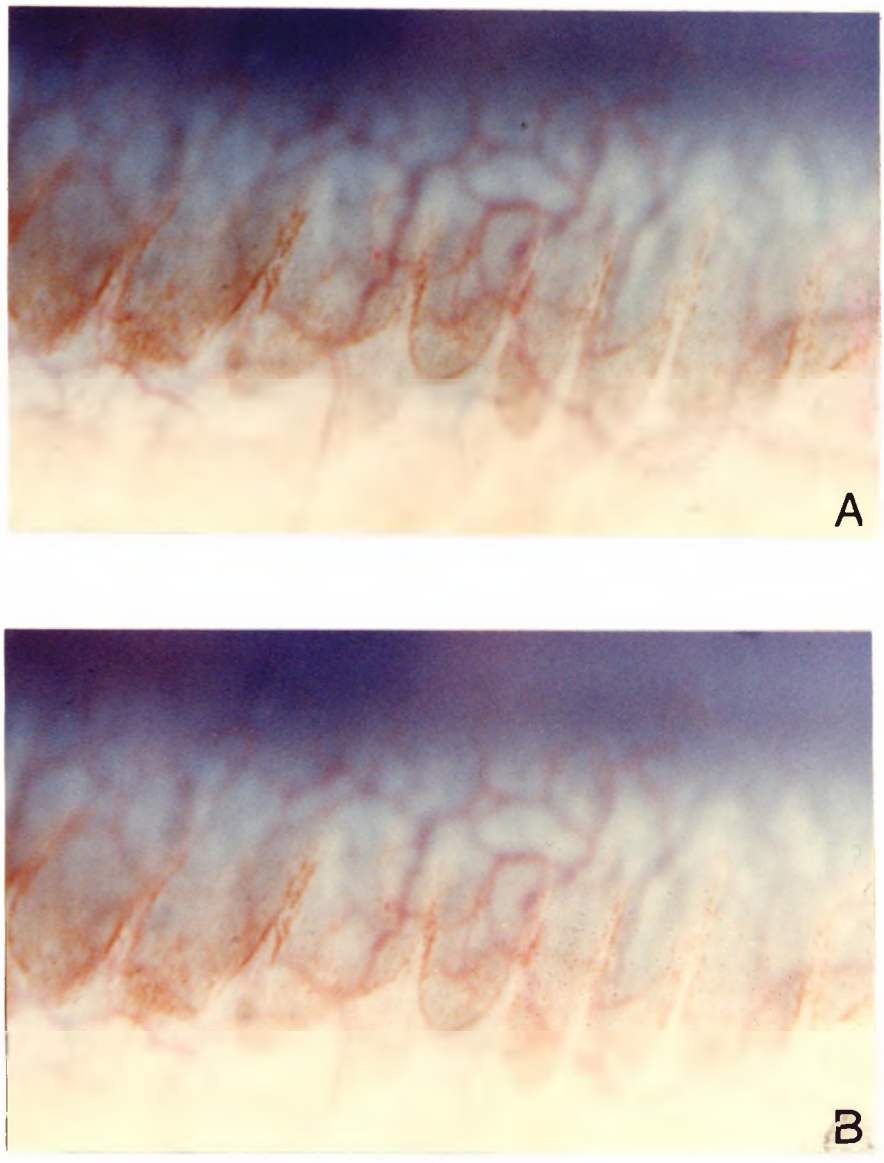


Figure 14. The stability of the palisade pattern of subject BD of the control group over a duration of one year is illustrated. The "dog-tooth" palisades are bracketed by pigment and there is some diffusion of pigment into the interpalisade zones. Limbal loops are visible anterior to the palisades. Photographs were taken at baseline (A) and at 3 monthly intervals after. Photograph B illustrates the palisade pattern 3 months after baseline.

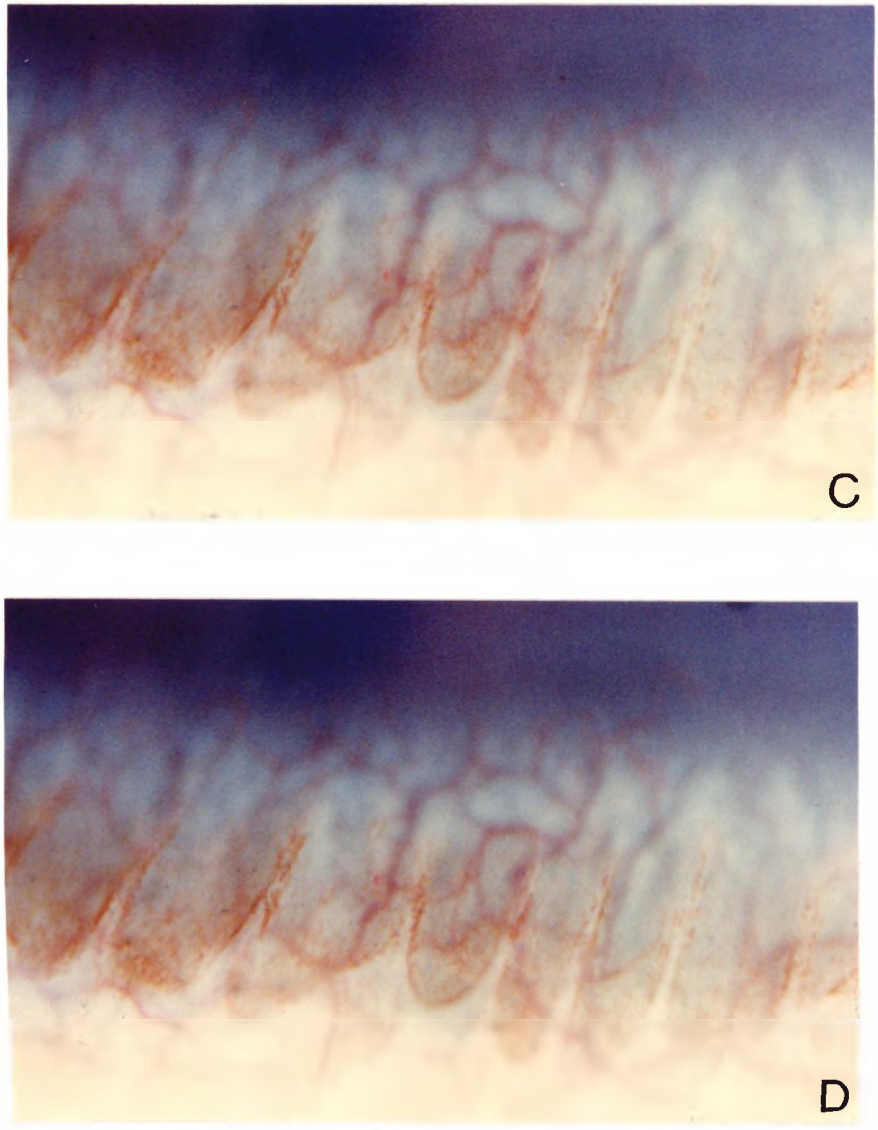


Figure 14 (contd.). Slit-lamp photographs illustrating the palisade pattern of subject BD of the control group after 6 months (C) and one year (D) from baseline. No alteration of the palisade pattern or its associated pigment pattern was observed over the course of this study. (x72)

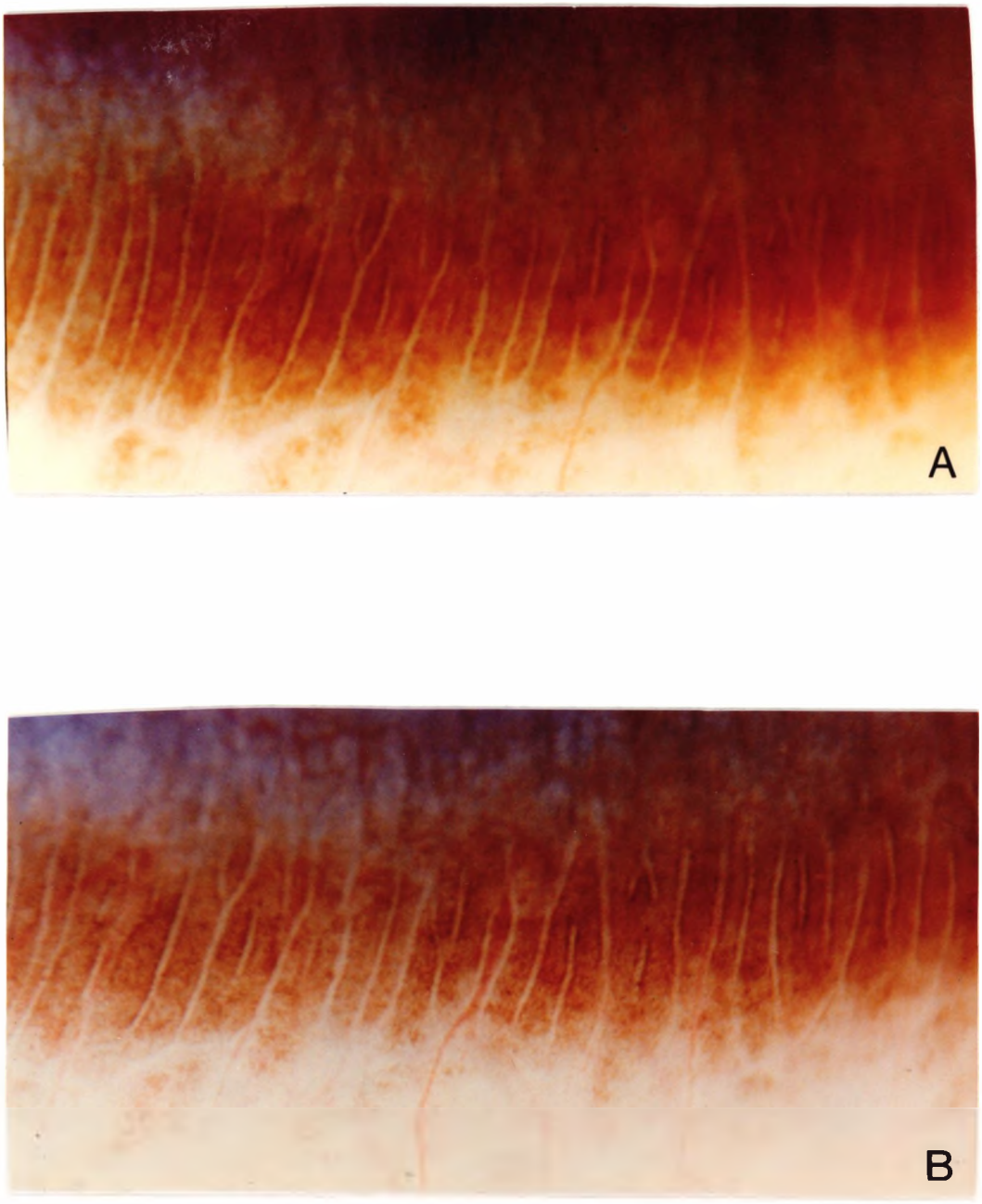
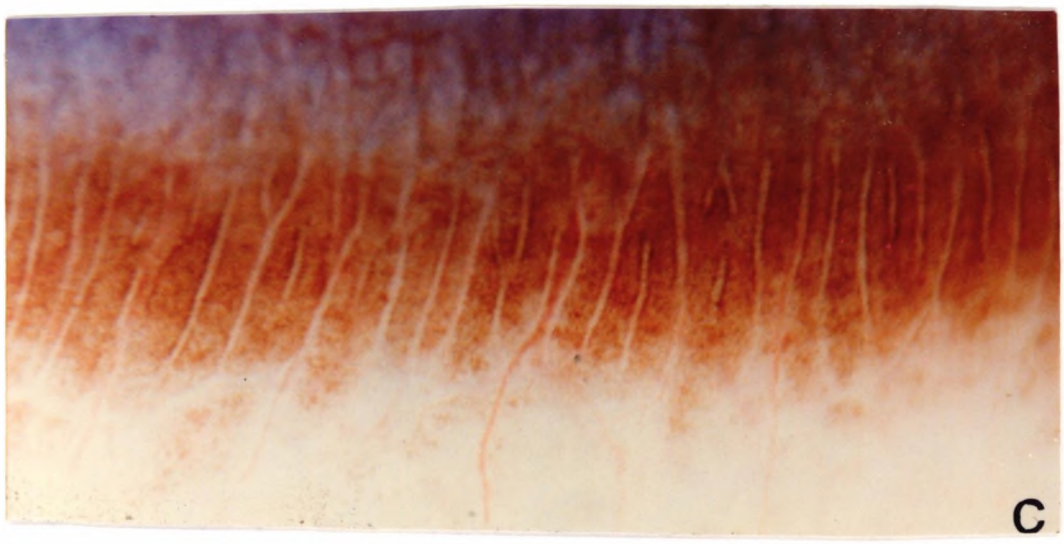
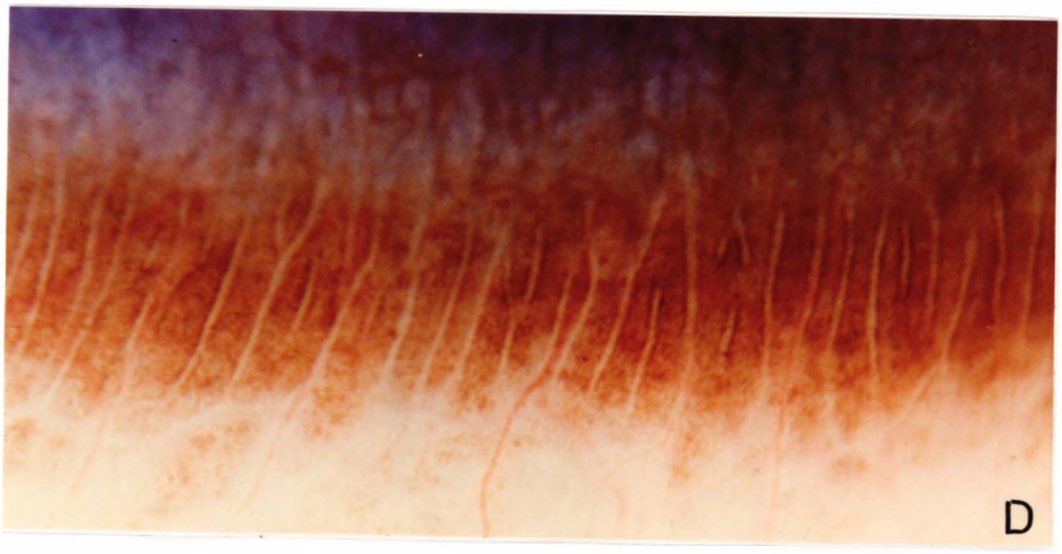


Figure 15. The palisade pattern of subject NP of the experimental group is illustrated over a duration of one year. The crests of the palisades are seen as fine white lines within the diffuse pigment. Photograph A was taken prior to hydrogel contact lens fitting. Photograph B illustrates the palisade pattern 1 month into lens wear.





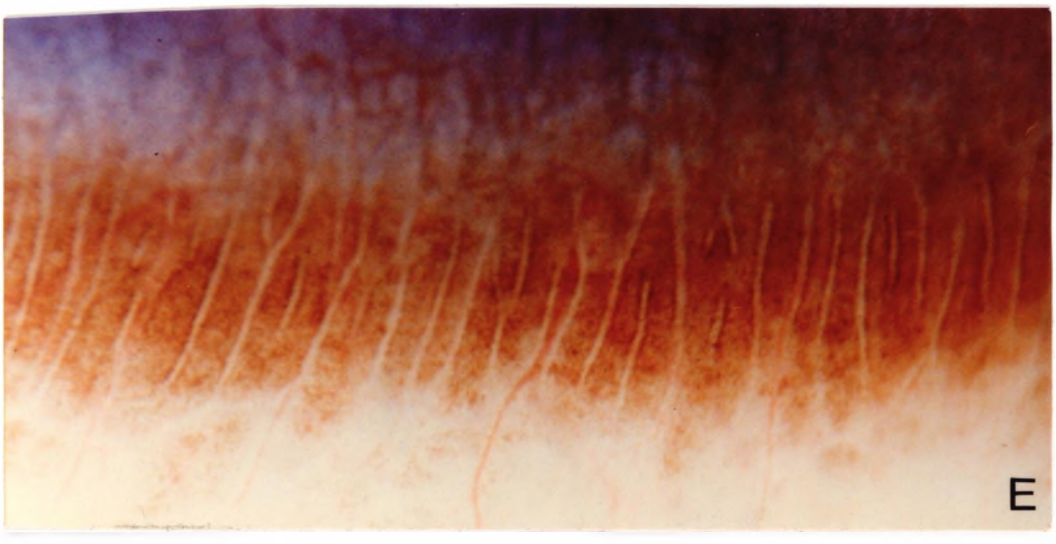


Figure 15 (contd.). The palisade pattern of subject NP after 3 months of hydrogel wear (photograph C); 6 months into lens wear (photograph D) and 12 months (photograph E). The contact lens was removed at each aftercare visit in order to check the integrity of the ocular surface as well as photograph the palisade zones. (x71)

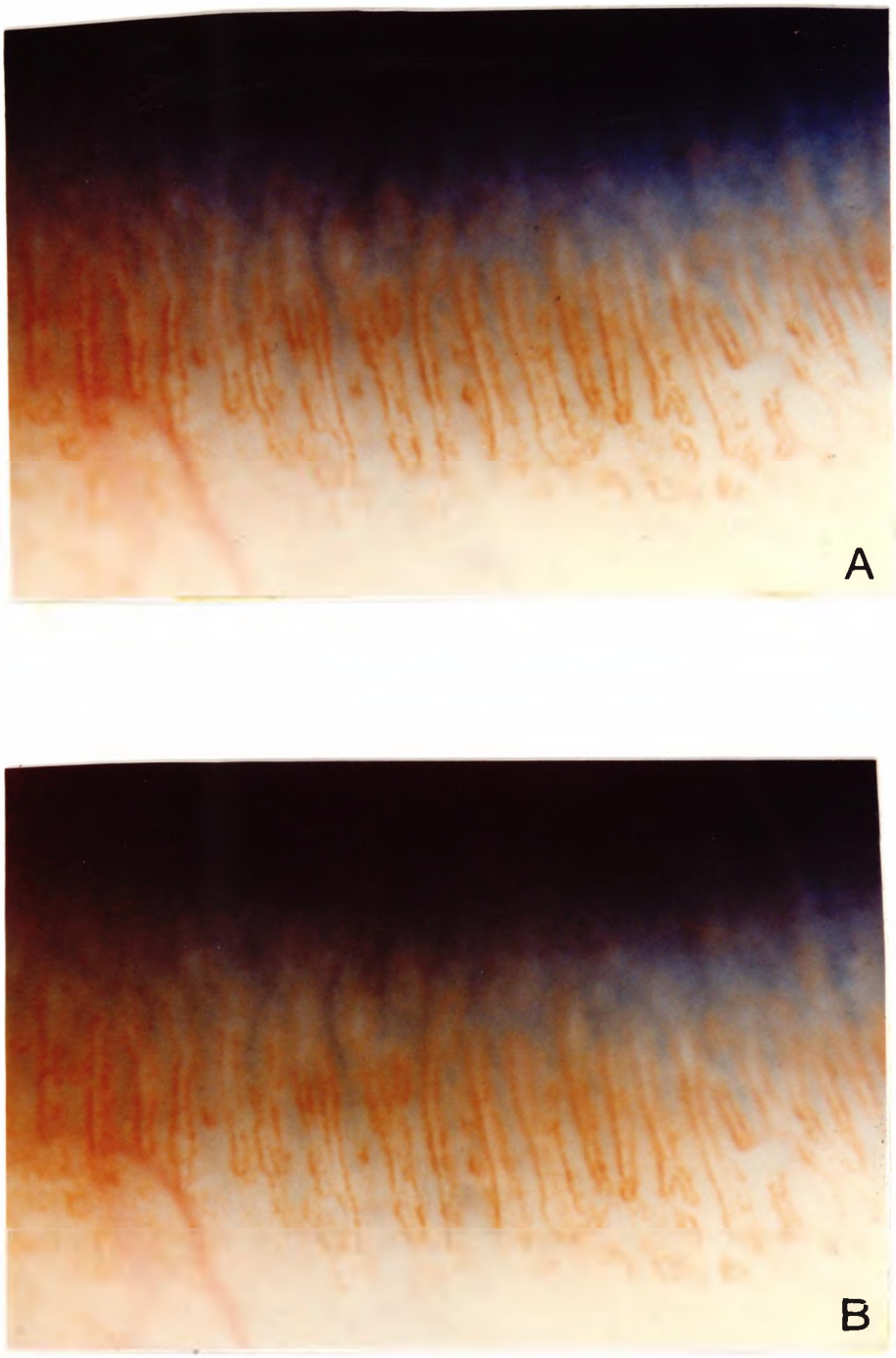


Figure 16. The palisade pattern of subject NA of the experimental group is illustrated. There appears to be no change in the palisade pattern over the duration of the study. Photograph A= baseline, B=1month into soft contact lens wear.

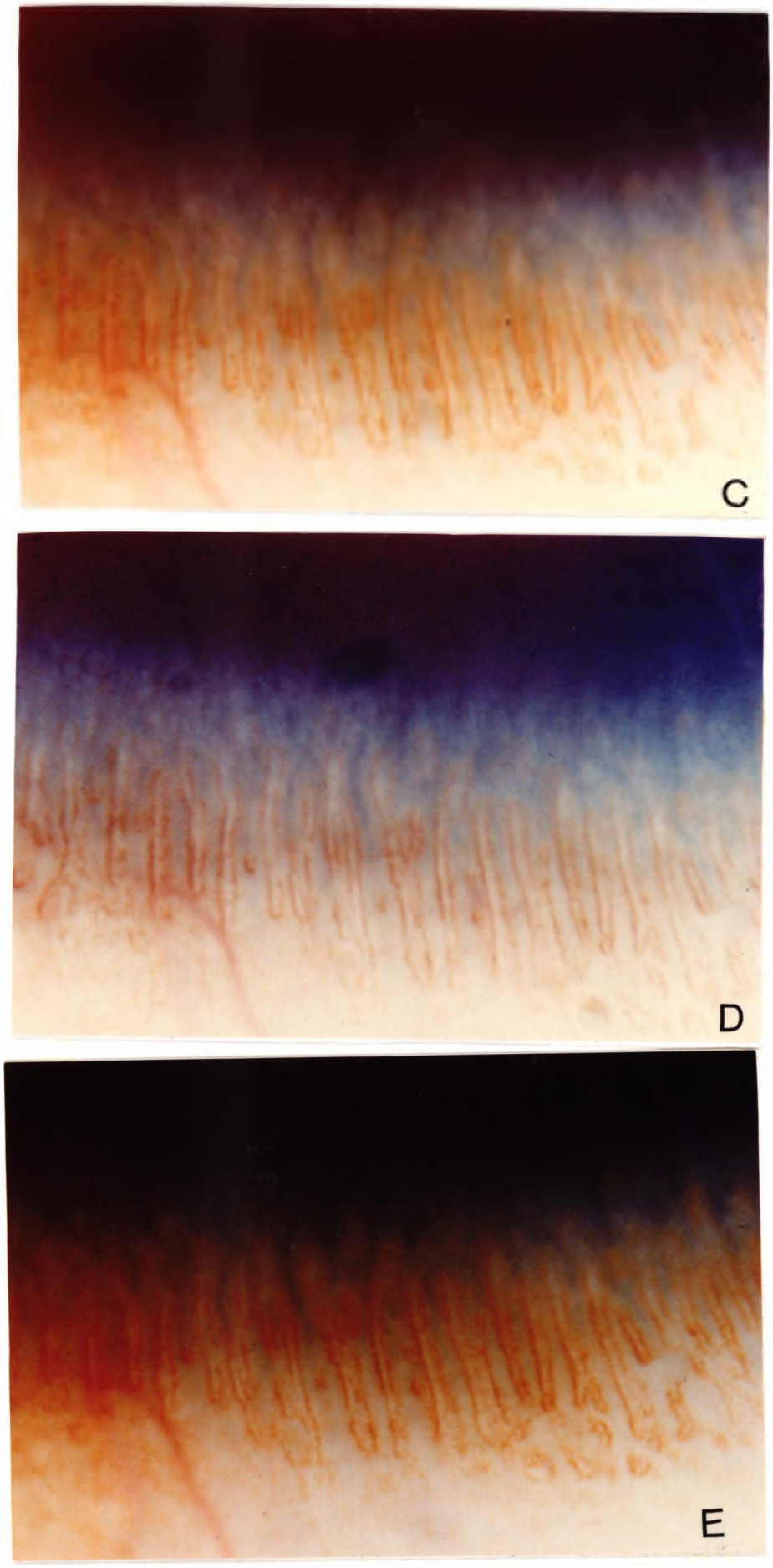


Figure 16 (contd.). Subject NA of the experimental group C=3months; D=6months and E=12 months into hydrogel lens wear. (x68).

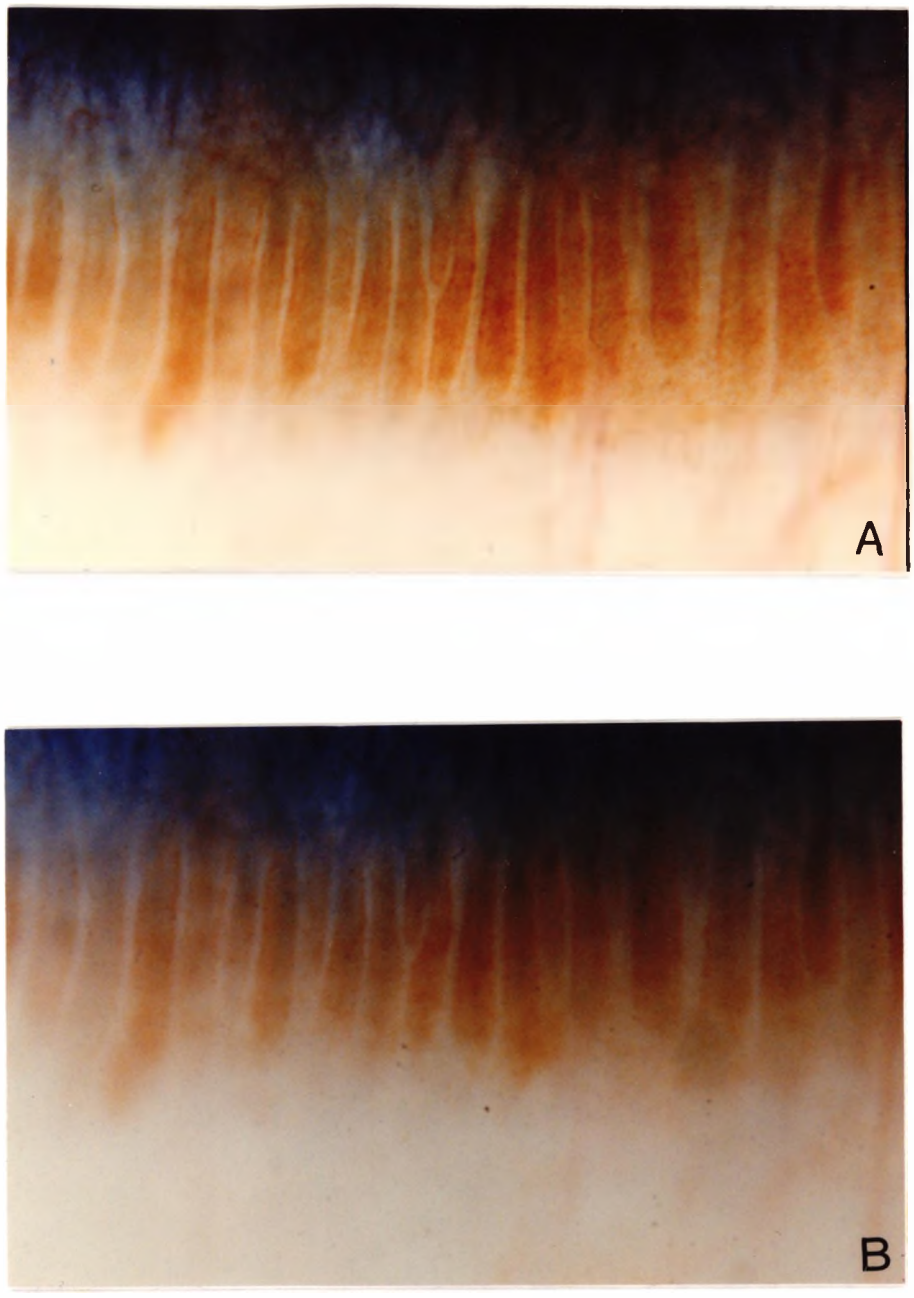


Figure 17. The palisade pattern of subject SN of the experimental group is illustrated. There appears to be no change in the palisade pattern over the duration of the study. Photograph A= baseline, B=1 month into soft contact lens wear.

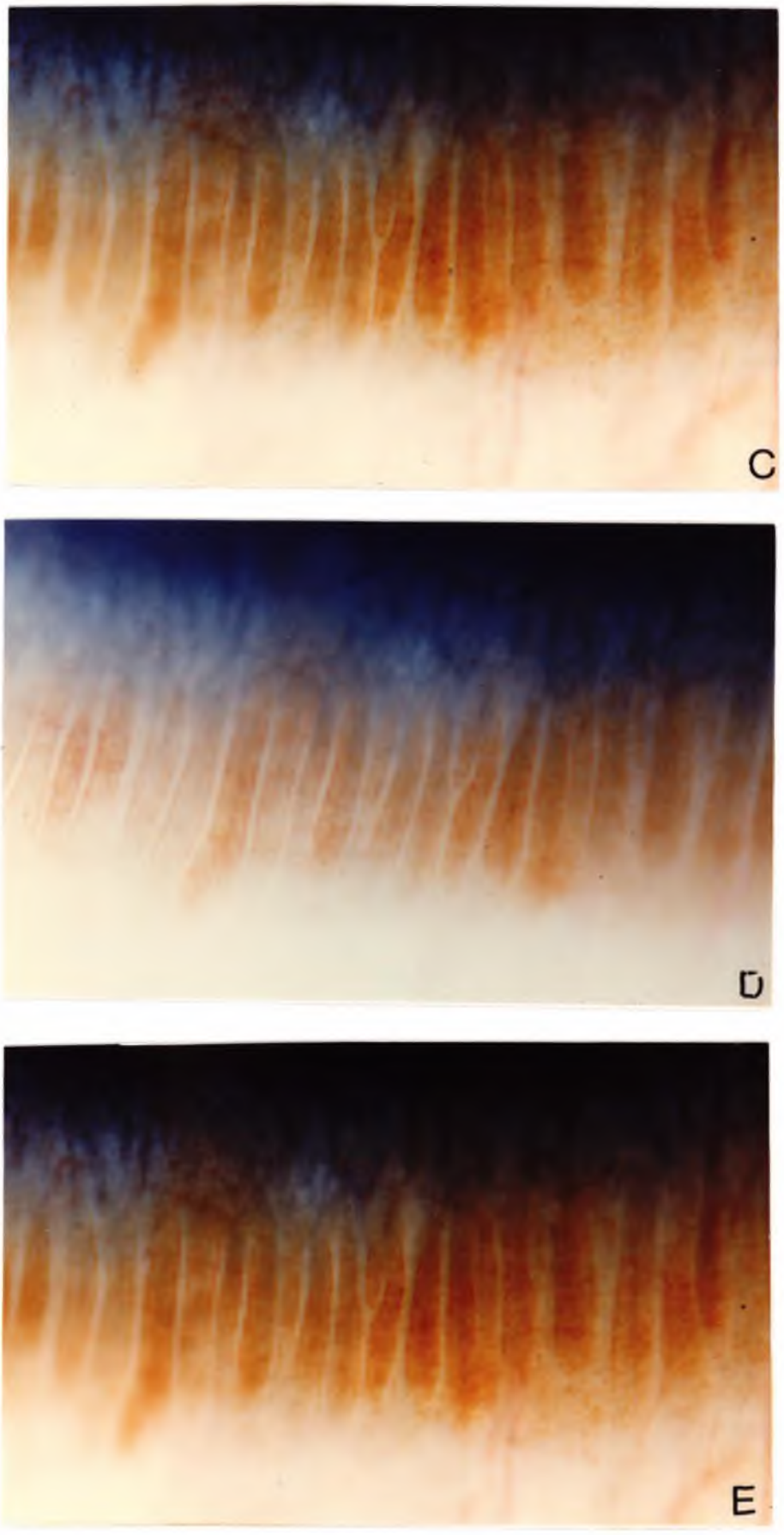


Figure 17 (contd.). Subject SN of the experimental group. C=3months; D=6months and E=12 months into lens wear. (x67).

3.2. Quantitative Analysis

3.2.1. Control Group

In this group of twenty subjects the average length of palisades was measured as 0.579mm (range 0.318-1.15mm), the mean width 0.028mm (range 0.018 to 0.037) and the average distance between adjacent palisades was 0.09mm (range 0.0057 to 0.106mm). There were between 10 and 13 palisades per mm and the average distance from the forward-most limbal loops was 0.3 to 0.5mm from the palisade crest.

Qualitative results strongly suggested that the palisade pattern was stable over a period of one year (the duration of the study). This was investigated quantitatively. Mean values of palisade width (Plot 1), distance between adjacent palisades (Plot 2) and length (Plot 3) were monitored over a duration of one year (Figure 18).

The near linear plots for each subject suggested the measured parameter remains stable with time. A one way ANOVA was used to confirm these observations. The results of the analysis indicated that any changes were highly insignificant ($p>0.05$).

3.2.2. Experimental Group

In this group average length of palisades was measured as 0.517mm (range 0.318-1.16mm), the mean width 0.027mm (range 0.019 to 0.045) and the average distance between adjacent palisades was 0.08mm (range 0.0058 to 0.12mm). As in the control

group, there were between 10 and 13 palisades per mm and the average distance from the forward-most limbal loops was 0.3 to 0.5mm from the palisade crest.

Mean values for palisade width (Plot 4), distance between adjacent palisades (Plot 5) and length (Plot 6) were recorded and re-measured 1, 3, 6, and 12 months after fitting hydrogel lenses (Figure 19). Once again, similar to the control group, the near-linear plots for each subject under investigation, suggested no significant change of the parameter under investigation with time. This was confirmed in each case by a one way ANOVA, the results of which confirmed the initial hypothesis ($p>0.05$).

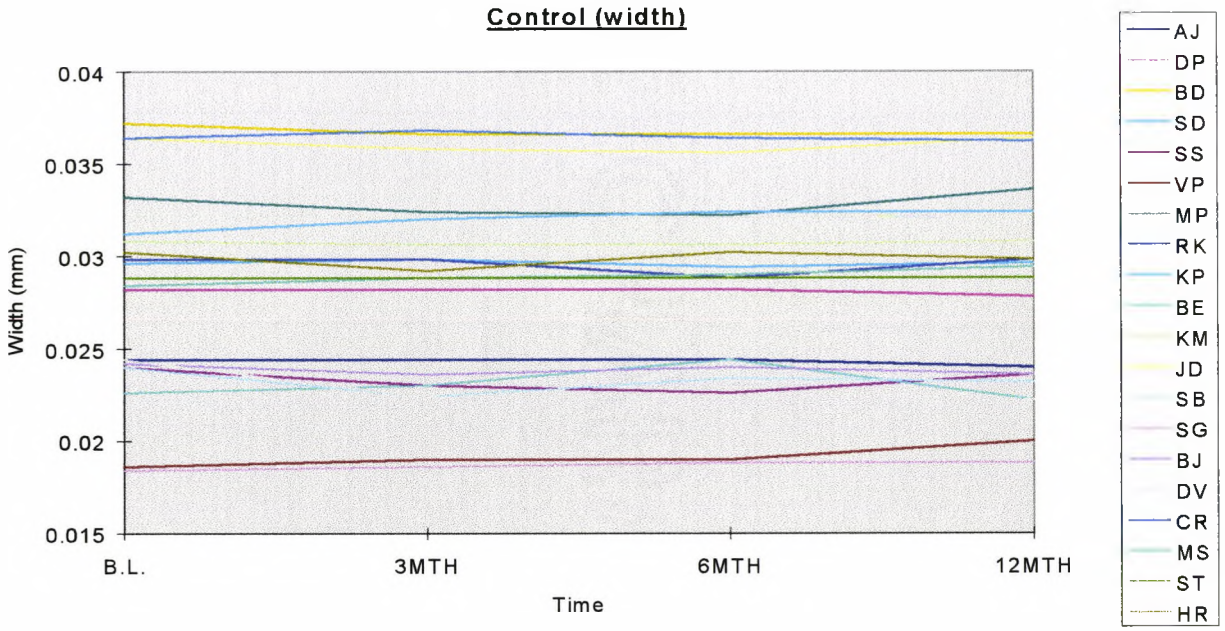


Figure 18. (Plot 1) Graphical representation of the width of palisades over a duration of one year. Measurements were taken at baseline, 3, 6 and 12 months. Each line on the graph represents one subject. The key on the right identifies the individual. The near linear relationship in each case corroborates the qualitative findings

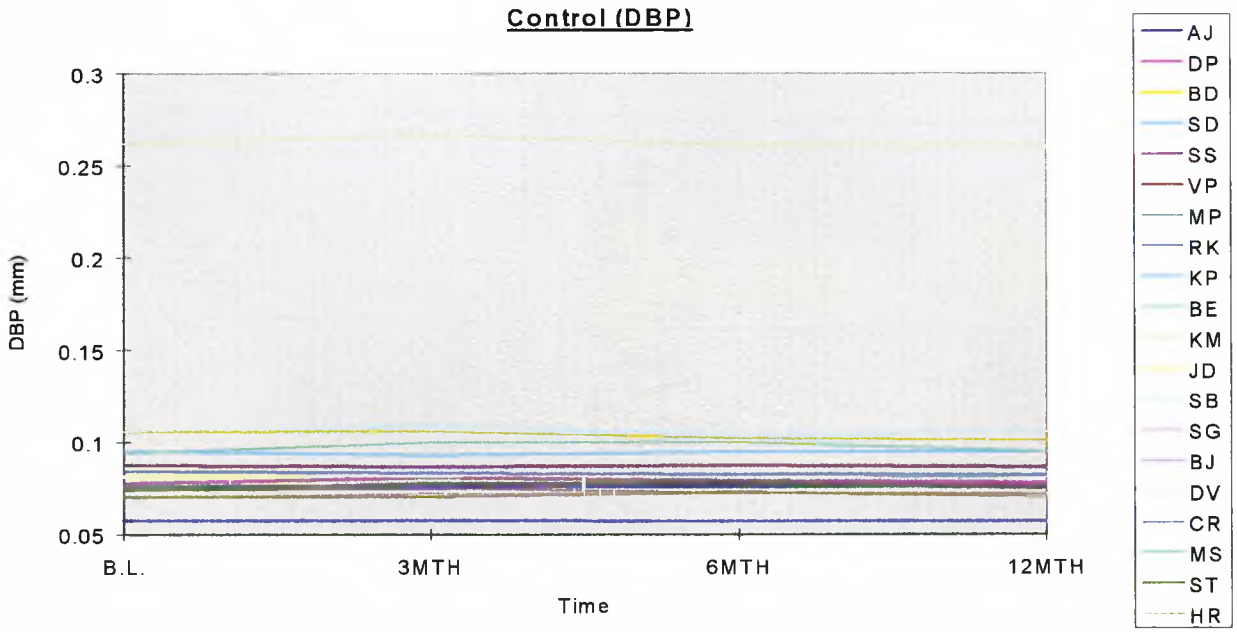


Figure 18. (Plot2). Graphical representation of the distance between adjacent palisades over a duration of one year.

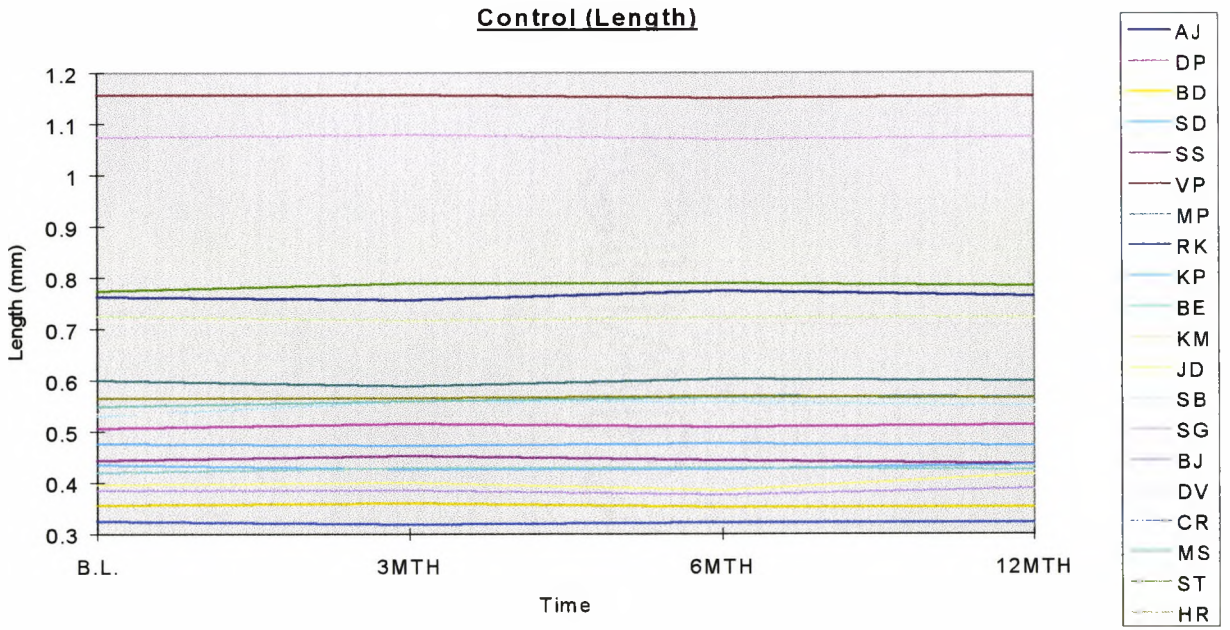


Figure 18. (Plot 3). Palisade width over a duration of one year.

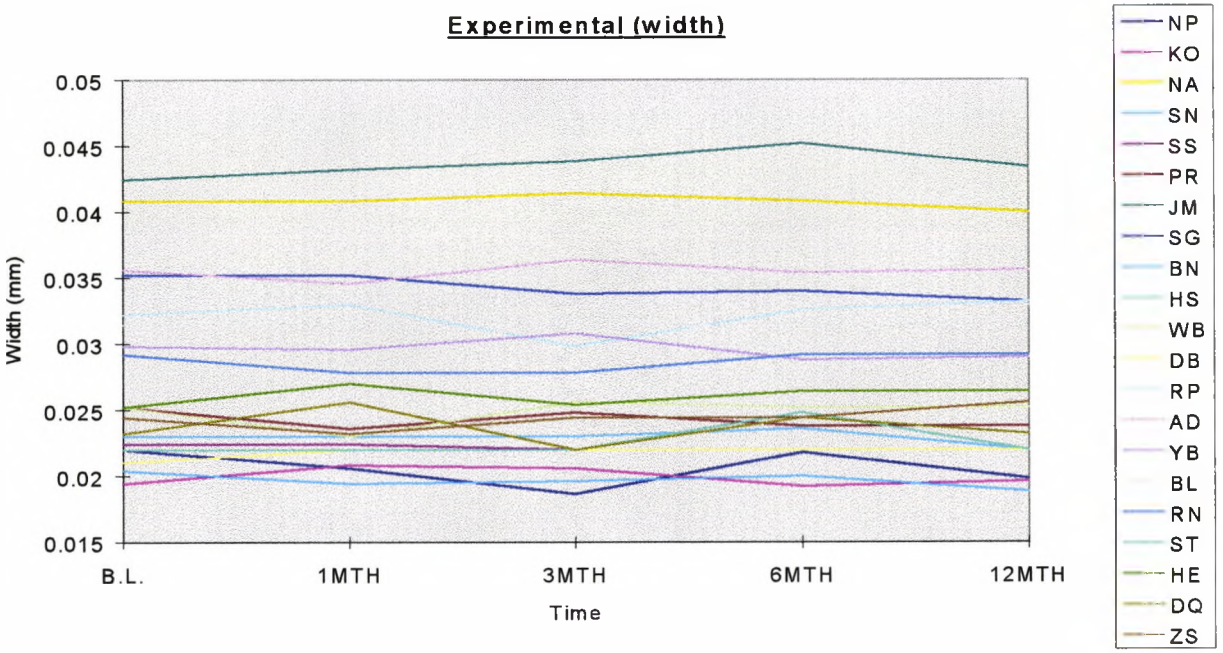


Figure 19. (Plot4). Palisade width over a duration of one year in the experimental group.

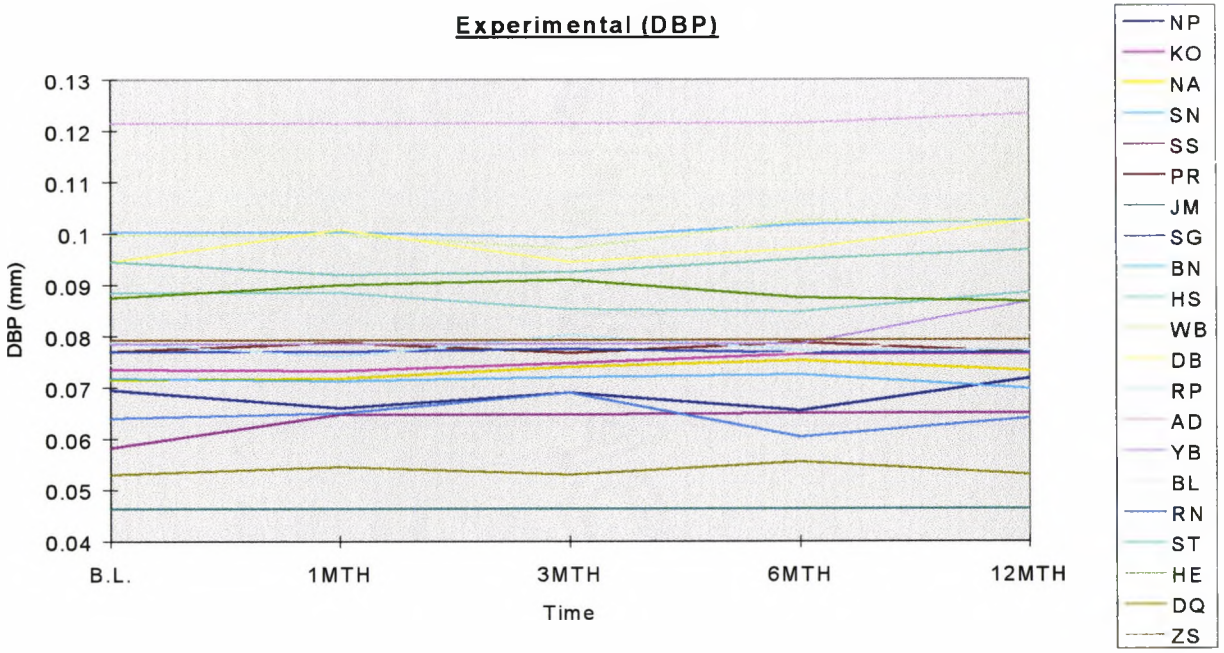


Figure 19. (Plot 5). Distance between adjacent palisades over a duration of one year

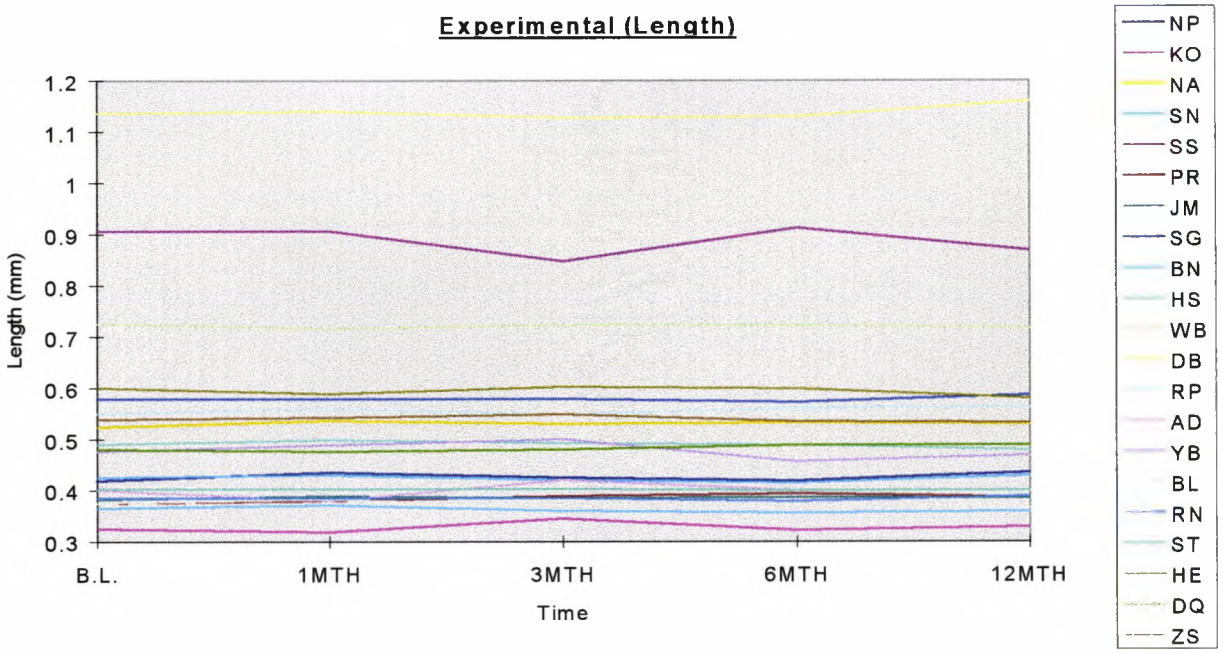


Figure 19. (Plot 6). Palisade length over a duration of one year.

HISTOLOGICAL OBSERVATIONS

3.3. Human Limbal Conjunctiva

3.3.1. Light Microscopic Observations

Most of the tissue for this aspect of the study was sectioned transversely, parallel to the limbus. The histological appearance of the palisade zones resembled sections taken through the rete-ridges of skin and both areas shared many common features. Palisades were identified in section from the vertical positions as a series of broad, finger-like stromal elevations that displaced the overlying epithelium into which they project. Segments from horizontal positions generally lacked such modifications.

Palisades frequently acted as a conduit for blood vessels and lymphatics; in addition, they frequently accommodated complex nerve endings (Fig 20). Palisade capillaries formed a complex vascular network running through and between the stromal elevations. The vessels were derived from larger conjunctival and episcleral vessels that were located deeper in the stroma. When followed anteriorly, in serial section, many of these recurrent vessels branched to become the limbal vascular arcades. Like blood capillaries, palisade lymphatic vessels were derived from larger channels, located in the stroma. Serial sections demonstrated that these vessels, unlike blood capillaries, did not loop but terminated blindly in the palisade zones. Lymphatics were differentiated from blood vessels by their characteristic thin walls and the complete absence of erythrocytes

along the whole length of the channel. Complex corpuscular nerve endings were often seen associated with palisades (Fig 21), however, these structures were not exclusive to the stromal elevations and were also present in the interpalisade zones. Corpuscular nerve endings were located close to epithelium, rarely more than 25μm from the epithelium and were typically found within 5-10μm from the basal cell layer. They were round to oval in shape, and varied in size from 20-60μm in diameter. These structures were typically encapsulated and served by a myelinated axon which was derived from deeper lying nerve bundles.

A flat conjunctival surface was achieved by epithelial cells covering the stromal elevations and filling the troughs between adjacent palisades. The epithelial layer of the palisade zones like the remainder of the limbal conjunctiva is non-keratinised, stratified, and surface cells are squamous. Cell thickness varied from 4-5 cells at palisade crests to 12-15 cells in between palisades (Figs 20-23). In the horizontal positions where palisades were absent, the epithelium thickened from 5-6 cells at the cornea to a maximum of 8-10 at the limbus. Arguably, the most interesting feature of the palisade epithelium was the existence of a dichotomy of the native cells of the basal layer(Figs 22, 23). The majority of basal cells were small and cuboidal and had a large, densely staining central nucleus that occupied most of the perikaryon. These cells were arranged in sequence adjacent to the basal lamina, occasionally, however, the regular array of these cells was interrupted by a second, larger variant. The latter was more columnar and its nucleus which occupied less of the cytoplasm compared to that of small cells was less densely staining (Figs 24, 25). Large basal cells existed individually or in small groups of no greater than three cells (Figs 22-25). Following its discovery in the

palisade epithelium, basal cell dichotomy was investigated (and confirmed) throughout the remainder of the limbal conjunctival epithelium. The ratio of small cells to large was approximately 10:1 in the palisade epithelium but decreased closer to the cornea, until the entire basal layer of the corneal epithelium consisted of large cells.

In pigmented conjunctivae, melanin was mostly confined to the basal layer of the epithelium (Fig 26). It was particularly concentrated in cells along the slopes of a palisade and less prominent in those at the crest (Fig 27). Suprabasal pigmentation was infrequent but when present, was confined only to a few cells above the basal layer. Another variation in the morphology of the two basal cells types was observed in pigmented conjunctivae. Small cells consistently contained more melanosomes in their perikaryon than their large counterparts, interestingly, the latter frequently remained free of pigment (see later).

3.3.2. Electron Microscopic Observations

Surface cells of the palisade zones displayed slender microvilli and microplicae along their outermost face. A fine, branching glycocalyx network was attached to these processes. Surface cells stained darker than other cells of the epithelium at EM level. The cytoplasm of these cells contained few organelles and cytokeratin filaments. Adjacent cells were joined by tight junctions (zonula occludentes) along their apical surface, desmosomes along the rest of the lateral surface (Fig 28) and membrane interdigitations and desmosomes along their inferior surface. Adjacent cells of the intermediate layers were joined by membrane interdigitations and desmosomes. Cells of

this layer had prominent central circular nuclei and their perikaryon contained more organelles and cytokeratin filaments compared to surface cells. Basal cells were joined to the basal lamina by hemidesmosmes and to adjacent cells by membrane interdigitations and desmosomes. Small basal cells had a large, darkly staining central nucleus that occupied the majority of the perikaryon leaving little room for other organelles and cytokeratin filaments. By contrast the cytoplasm of large cells contained many more organelles and cytokeratin bundles. In pigmented conjunctivae, mature melanosomes were arranged in clusters, perinuclearly. Electron microscopy validated the observations of pigment heterogeneity at light microscopic level and confirmed that small cells accumulated more pigment than their larger neighbours (Fig 29).

Occasionally non-native cells were seen in the epithelial layer. These cells were present mainly adjacent to the basal lamina, although sometimes macrophages were present suprabasally. Non-native cells formed no attachments with other cells or the basal lamina. Conjunctival Langerhans cells exhibited broad dendritic processes. Their large, darkly staining central nucleus occupied most of the cell leaving little room for other cell organelles. These cells lacked the Birbeck granules which are characteristic in counterparts in the epidermis. Lymphocytes and macrophages were sparsely present in the tissues examined and appeared no different morphologically to those described elsewhere in other epithelia. Lymphocytes had a large, intensely staining oval nucleus, a prominent Golgi complex and little endoplasmic reticulum but many free ribosomes. The cytoplasm of macrophages by contrast was richly populated and contained many characteristic phagocytic vesicles and secondary lysosomes.

Melanocytes were abundant in pigmented conjunctivae and were found adjacent to the basal lamina. The perikaryon of these cells stained lightly than other non-native cells. These cells had a large central nucleus, and much few er densely staining mature melanosomes in their perikaryon compared with adjacent pigmented native cells (Fig 30). Processes packed with mature melanosomes were often seen extending from these cells along the basal lamina towards native basal epithelial cells, suggesting interaction between these cells. Pale staining areas, similar in density to the perikaryon of melanocytes, were ocassionally encountered within the cytoplasm of native basal cells. These areas contained many mature melanosomes and further supported the suggestion of interaction between melanocytes and epithelial cells (Figs 31, 32).

The palisade stroma consisted largely of collagenous tissue and fibrocytes. Like the remainder of the limbus, fibrocytes in the palisade zones had a large, deeply-staining ovoid nuclei that occupied most of the cell, leaving little room for other organelles. Fibrocyte processes often acted as septa, partitioning areas of collagen. Palisade vessels that were buried within the collagen matrix were non-fenestrated and their walls were formed by two or more endothelial cells (Fig 33). Adjacent vascular endothelial cells were linked by tight junctions. Occasionally, pericytes provided an extra layer and when they were present an associated basal lamina was discerned. In contrast lymphatic vessels displayed thinner endothelial cell walls and were differentiated from blood vessels at ultrastructural level by the absence of associated pericytes. Lymphatic vessels also displayed characteristic anchoring filamnets that were absent in blood vessels.

Ultrastructurally, corpuscular nerve endings displayed features similar to those described previously and to those elsewhere in the body (Fig 34). The supplying axon having shed its perineural sheath, loses its myelin sheath before entering the corpuscle and then divided several times to form a variable number of axon terminals. These consisted of a series of varicosities. In transverse section they contained neurofilaments, scattered microtubules, vesicles and aggregates of mitochondria. The whole structure was encased in a capsule formed by fibrocyte-like cells. Capsular fibrocytes lacked a basal lamina and contained organelles similar to stromal fibrocytes.

3.3.3. Immunohistochemical Staining of the Limbal Conjunctival Epithelium

In skin, where pigment donation is known to be in operation, Lavker and Sun (1983) have shown that the quantity of pigment within basal epidermal cells reflects their cycling time. They demonstrated that lightly pigmented cells were faster cycling than their more densely pigmented neighbours. Ultrastructural examination of the palisade epithelium suggests that pigment donation may be operating in the limbal conjunctiva. Large cells were found to be consistently less pigmented than their smaller neighbours. Immunohistochemical techniques were employed to investigate the relationship between density of pigmentation and cell cycle times in the limbal conjunctival epithelium.

Positively labelled cells were distinguished by the presence of discrete nuclear accumulation of D.A.B. chromogen. Positive staining was present in all layers of the limbal conjunctiva (Fig 35). In the basal layer, it was chiefly confined to large cells, small basal cells rarely stained (Fig 36), hence the ratio of stained to non-staining cells

was approximately 1 to 10-15. A similar ratio of labelled versus non-labelled cells was present in the suprabasal layers.

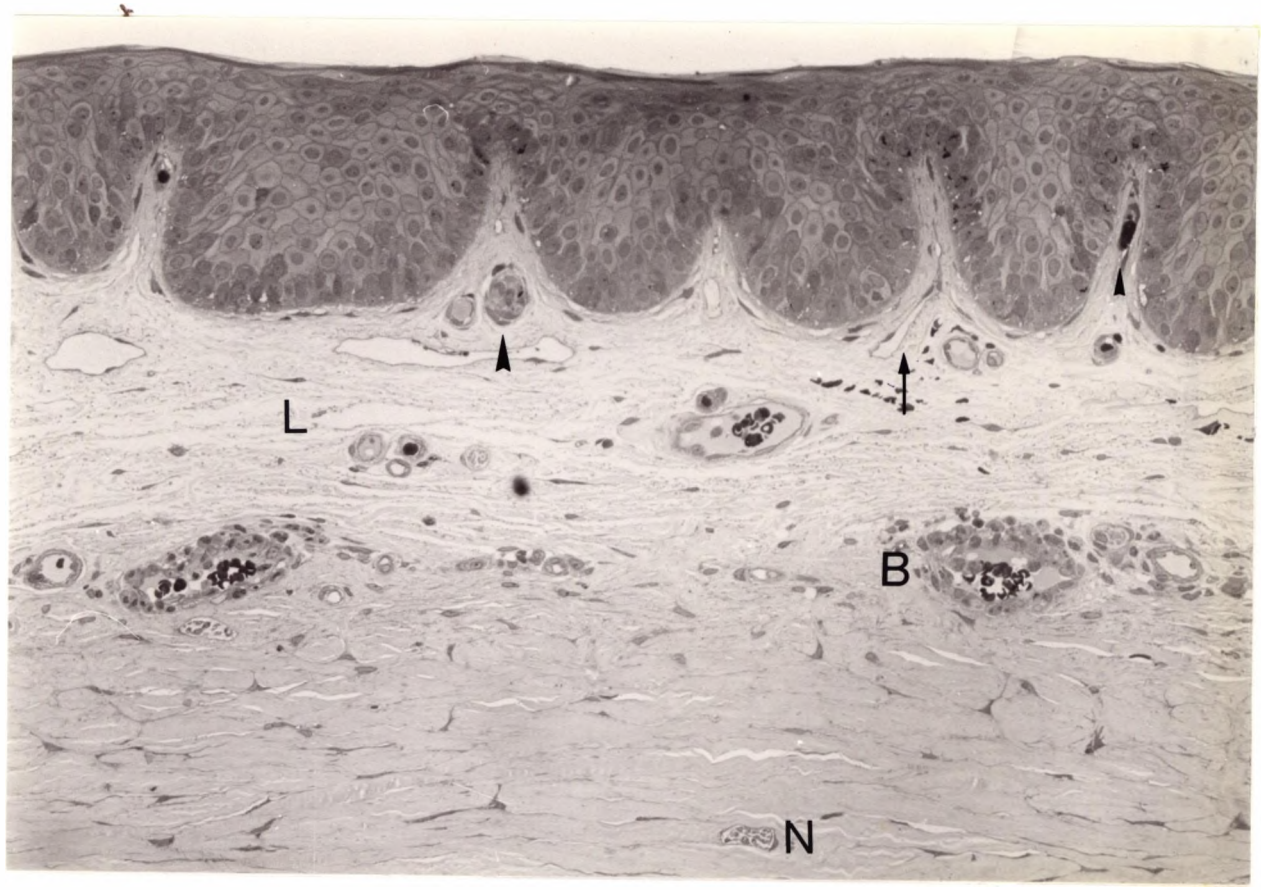


Figure 20. Transverse section (cut parallel to the limbus) through a segment from the inferior position illustrating the characteristic appearance of the palisade zones in histological section. Palisades often act as conduits for recurrent capillaries (small arrowhead) and fine lymphatic vessels (arrow). In addition, they frequently accommodate and terminal complex nerve endings (large arrowhead). Palisade capillaries and lymphatics are derived from larger vessels located in the stroma. These are illustrated in this example: B= a large blood vessel that is surrounded by extravascular inflammatory cells; L= a lymphatic vessel (L). A nerve bundle (N) is also highlighted, this may supply the myelinated axon which serves the corpuscular nerve ending. (x297).

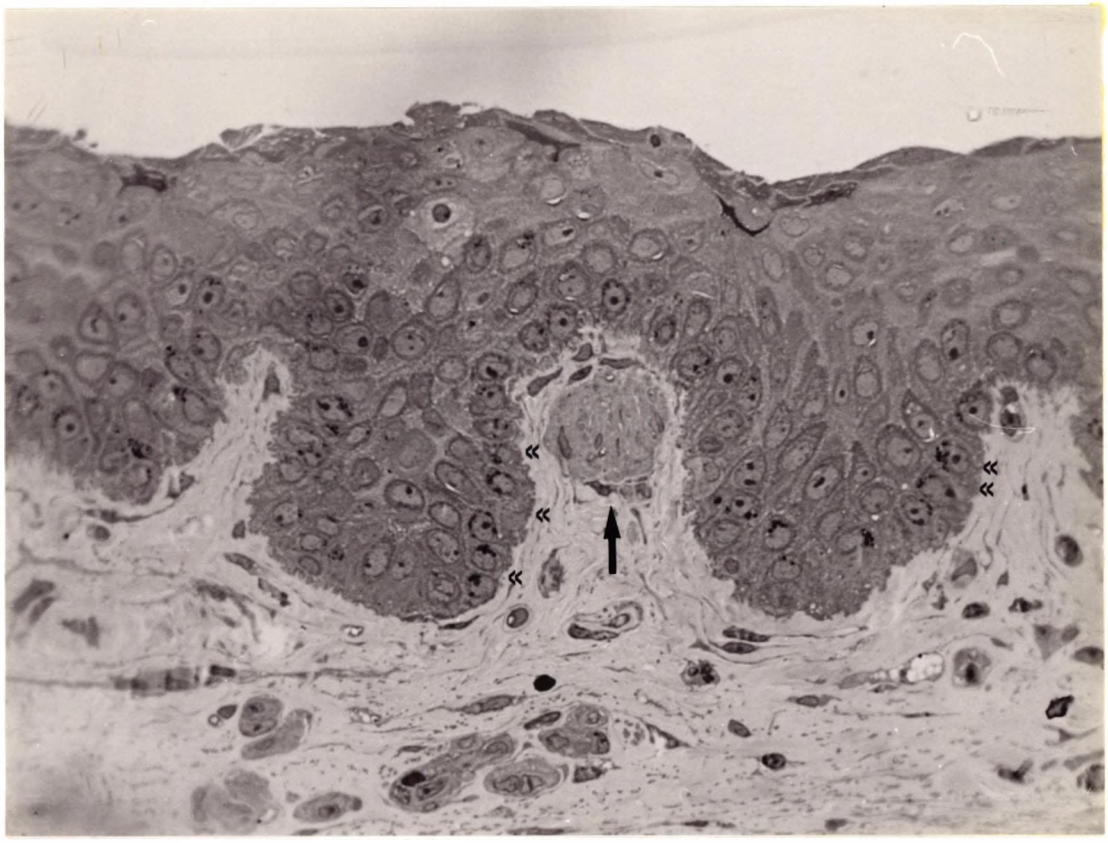


Figure 21. A corpuscular nerve ending (arrow) is located towards the apex of a palisade close to the basal layer of the epithelium. Epithelial cells forming the slopes of the palisade are lightly pigmented (<<), whereas cells at the apex are not. This distribution of pigment would result in the clinical appearance of a pair of parallel pigment lines, typically seen in Asian eyes. (x413).

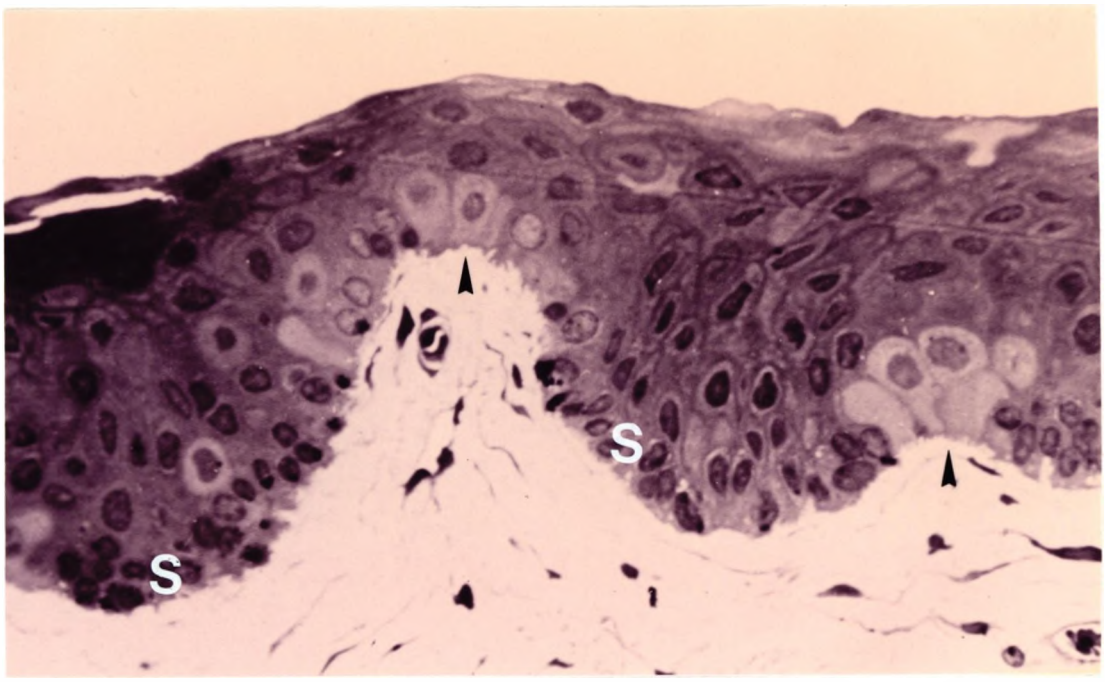


Figure 22. Transverse section (cut parallel to the limbus) through the palisade zone. A characteristic stromal elevation is evident. The epithelium at the crest of the palisade is 4-5 cells thick and 12-15 cells thick at the interpalisade zones. Basal cell dichotomy is illustrated. The regular array of small cuboidal cells (S) is interrupted by small groups of large columnar cells (arrowheads). (x654).

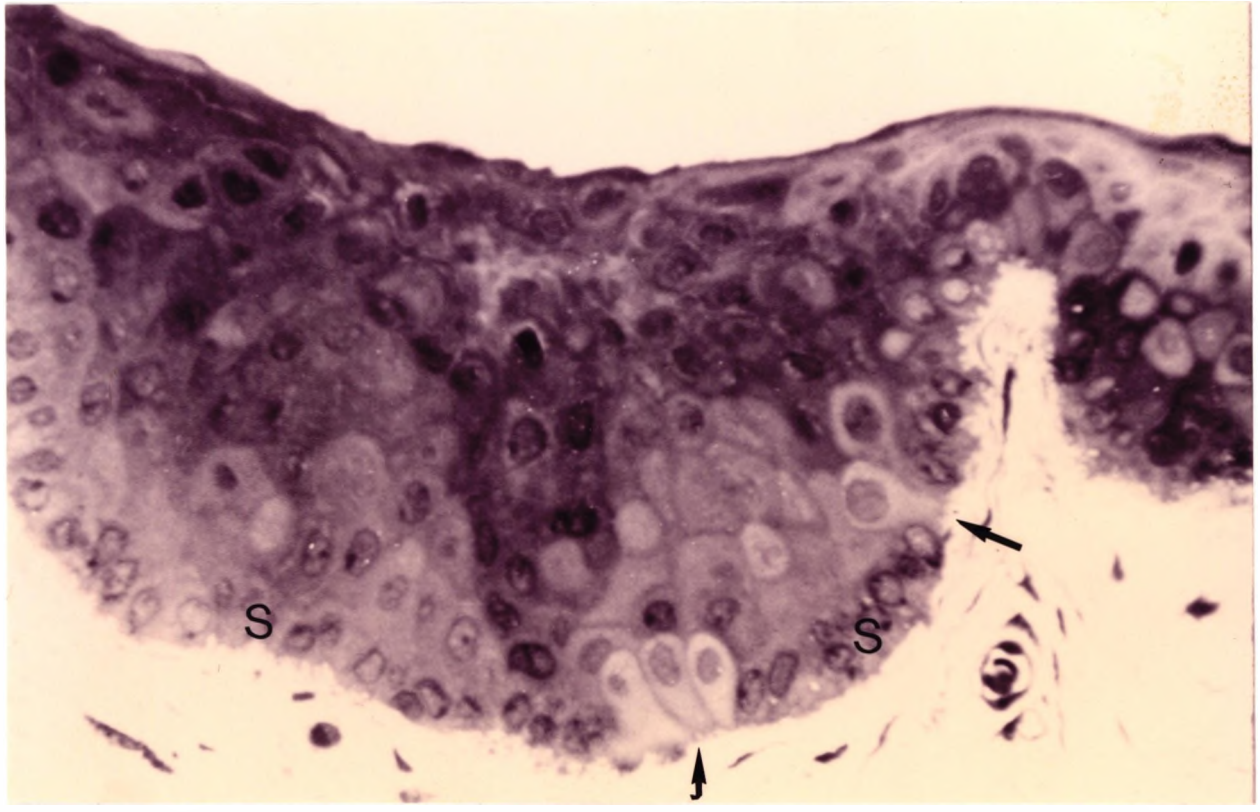


Figure 23. Light micrograph illustrating basal cell dichotomy. Small cells (S) are interrupted by small groups of large cells (arrows). (x680).

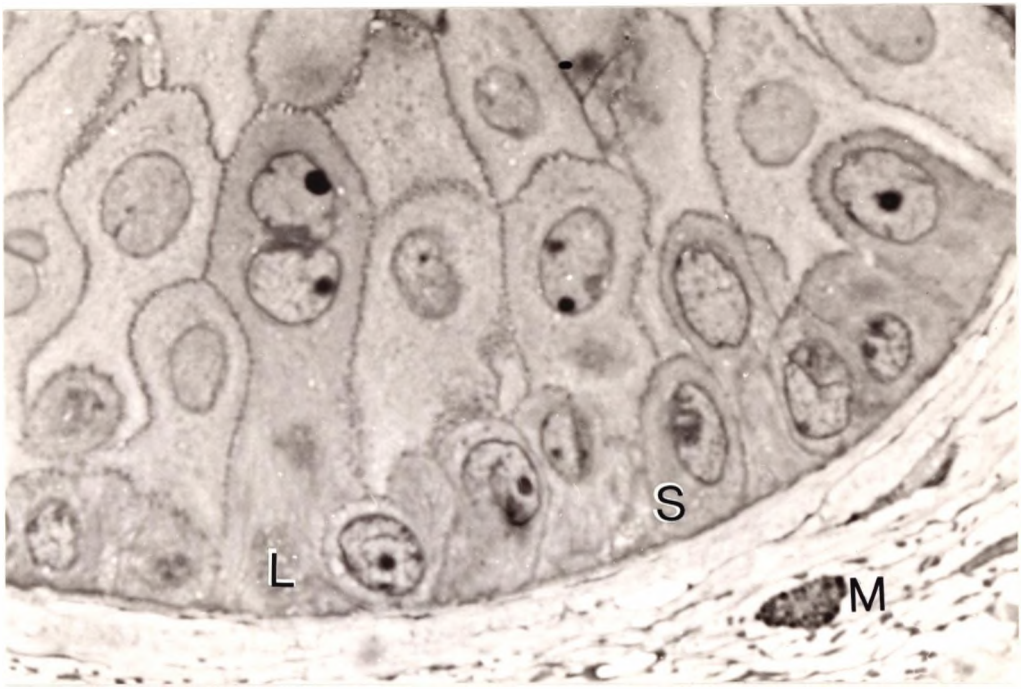


Figure 24. High magnification light micrograph illustrating the basal layer of the palisade epithelium. Basal cell dichotomy is apparent. The sequence of small cells (S) is interrupted by a single large cell (L). Also visible is an extravascular mast cell (M). (x1078)

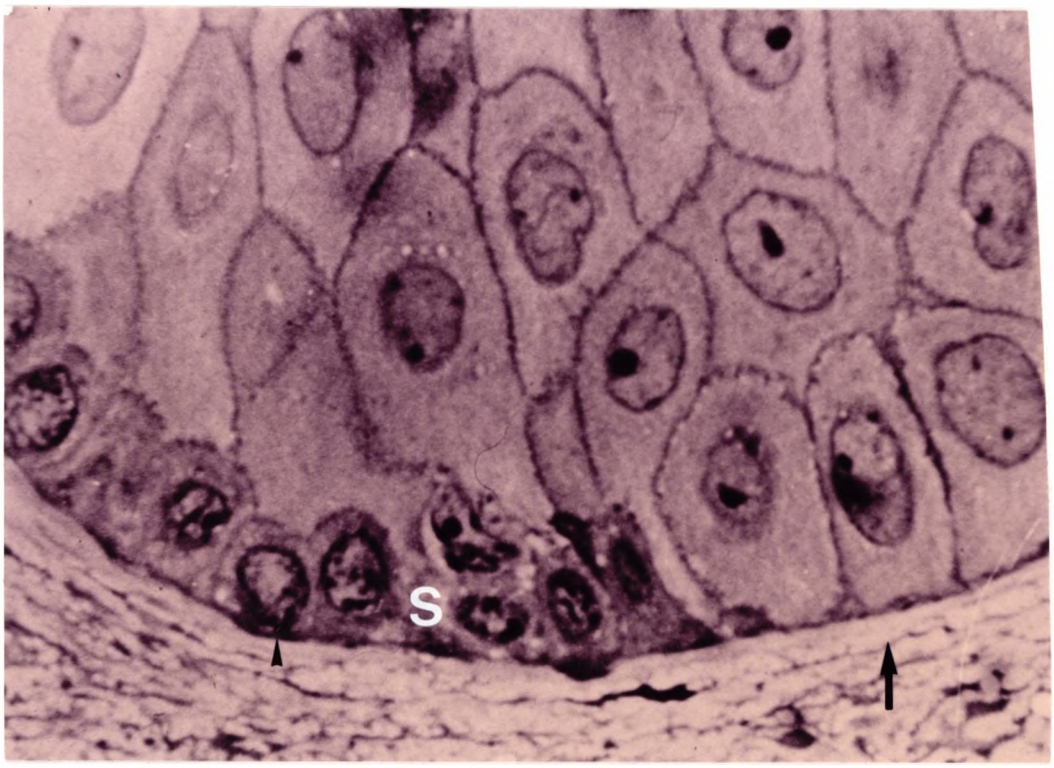


Figure 25. High magnification light micrograph illustrating basal cell dichotomy in the palisade epithelium. The large, densely staining nucleus of the small cells occupies more of the cytoplasm than that of large cells. In this example the basal lamina is indicated (arrowhead), the sequence of small cells (S) is interrupted by three large cells (arrow). (x1086).

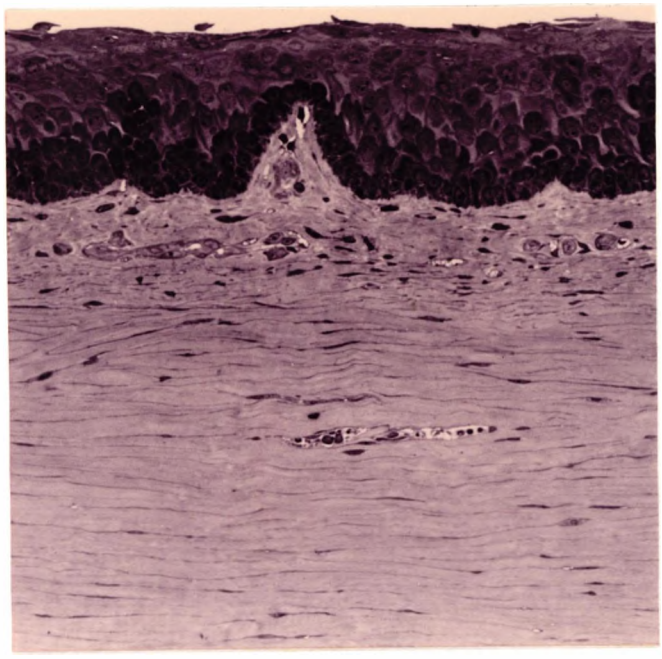


Figure 26. Light micrograph through the palisade zones in a heavily pigmented eye. Melanin is concentrated in the basal cells which stain much denser than the remainder of the epithelium. (x267).

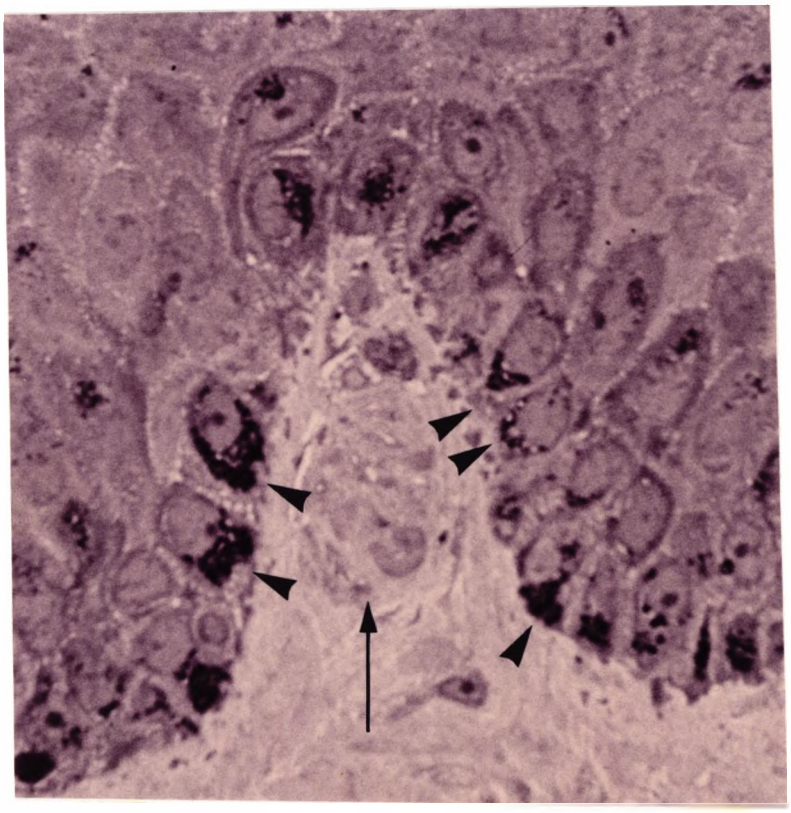


Figure 27. The palisade appearance of a pair of lines pigment (pigment bracketed variety) is due to the concentration of melanin within basal cells at the slope of a palisade (arrowheads). In this example cells at the crest of the palisade appear less densely pigmented, although frequently these cells are void of any melanin. A complex nerve ending is situated in the apex of the palisade (arrow). (x963).

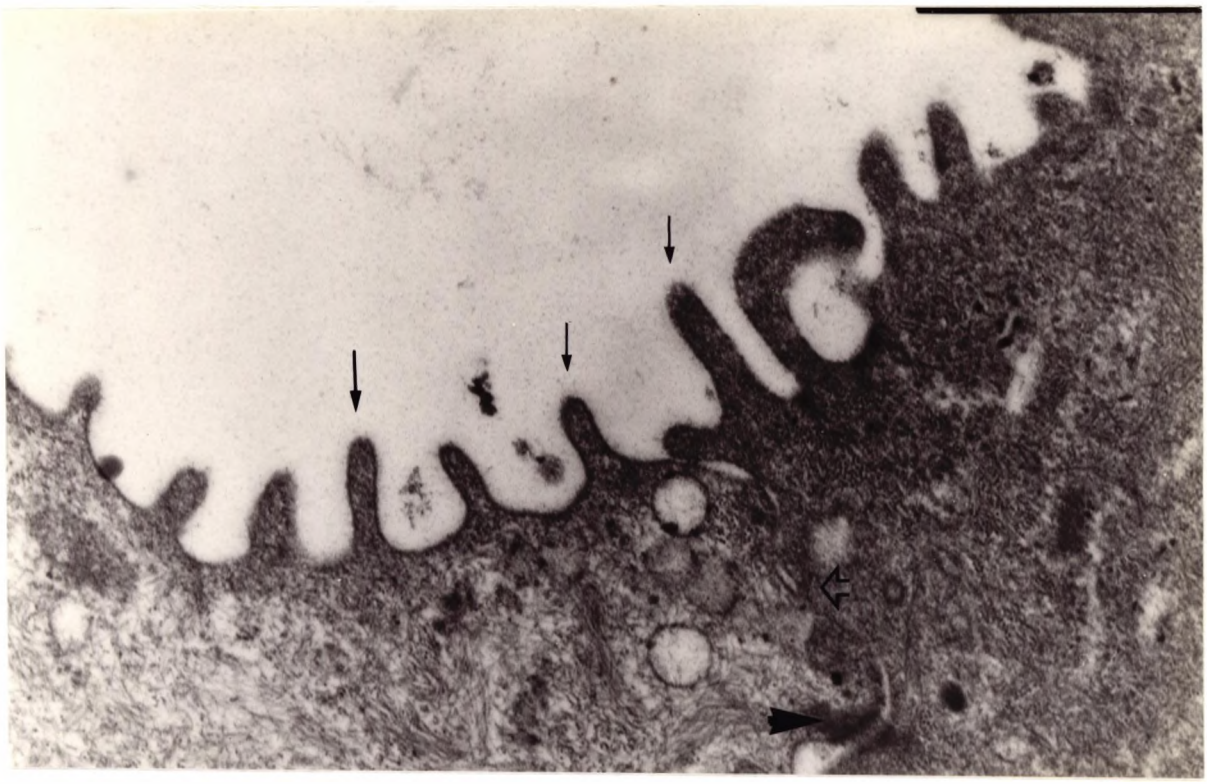


Figure 28. As elsewhere on the ocular surface, the outermost face of surface cells of the palisade zones are characterised by the presence of numerous microvillae (arrows) and microplicae. The remnants of the glycocalyx network is barely discernible on these processes. In order to prevent the passage of fluid and ions, tight junctions (open arrow) are present close to the apical surface of these cells. Elsewhere these cells are attached strongly to their neighbours by membrane interdigitaions and desmosomes (arrowhead). (x25 900).

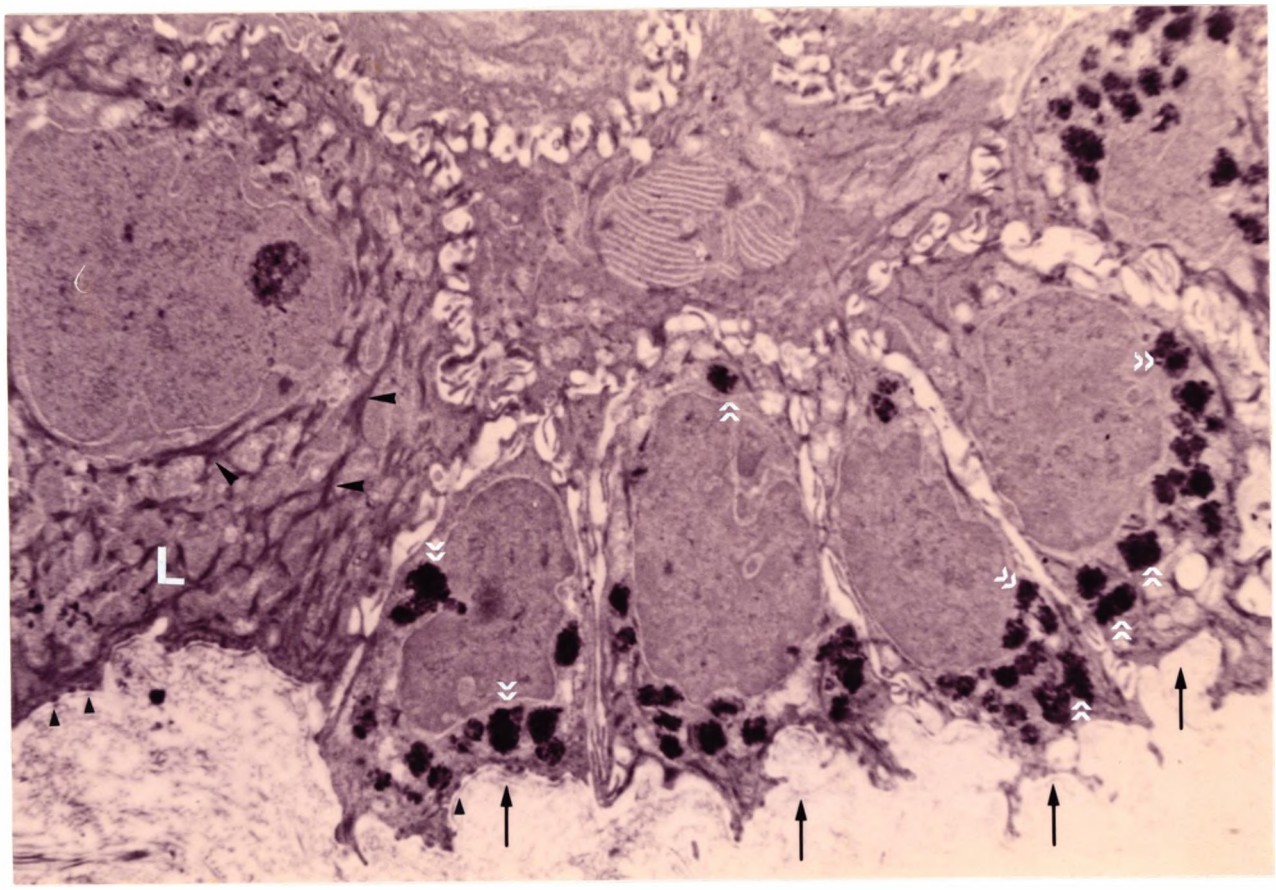


Figure 29. Electron micrograph illustrating basal cell dichotomy. Four small cells are present (arrows) alongside a single large cell (L). Pigment heterogeneity is also evident in this example. Clusters of perinuclear melanosomes (<<) occupy the little space left in the perikaryon of the small cells. By contrast the large cell is free of pigment. The nucleus of the large cell occupies less space compared with that of small cells, which leaves ample space within its perikaryon for other organelles and cytokeratin filaments (arrowhead). Basal cells are attached to the basal lamina by hemidesmosomes. (x6568).

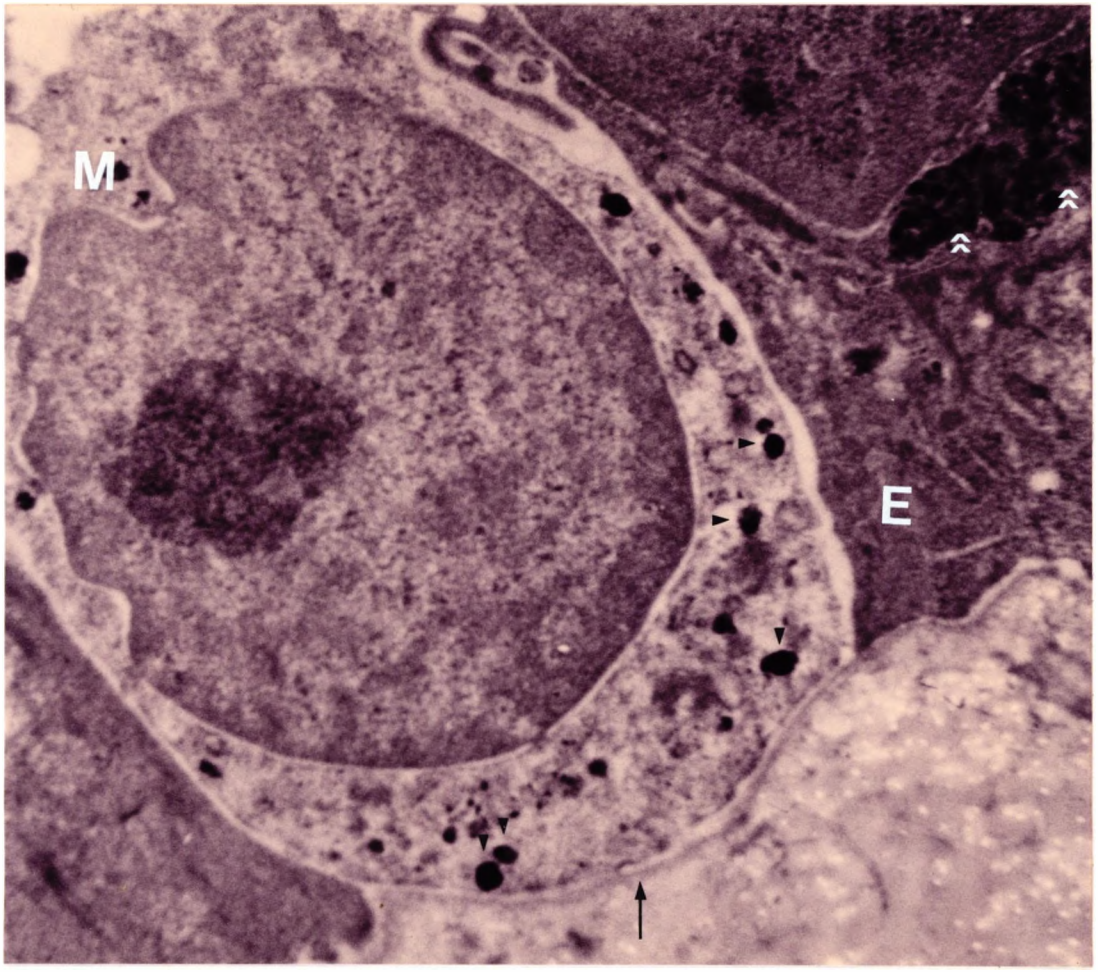


Figure 30. Melanocyte (M) in the basal layer of the palisade epithelium. The perikaryon of this cell contains few melanosomes (arrowheads) compared with that of the adjacent native epithelial cell (E). Clusters of mature melanosomes are packed perinuclearly in the native cell (<<). The basal lamina is also indicated (arrow). (x 19 700).

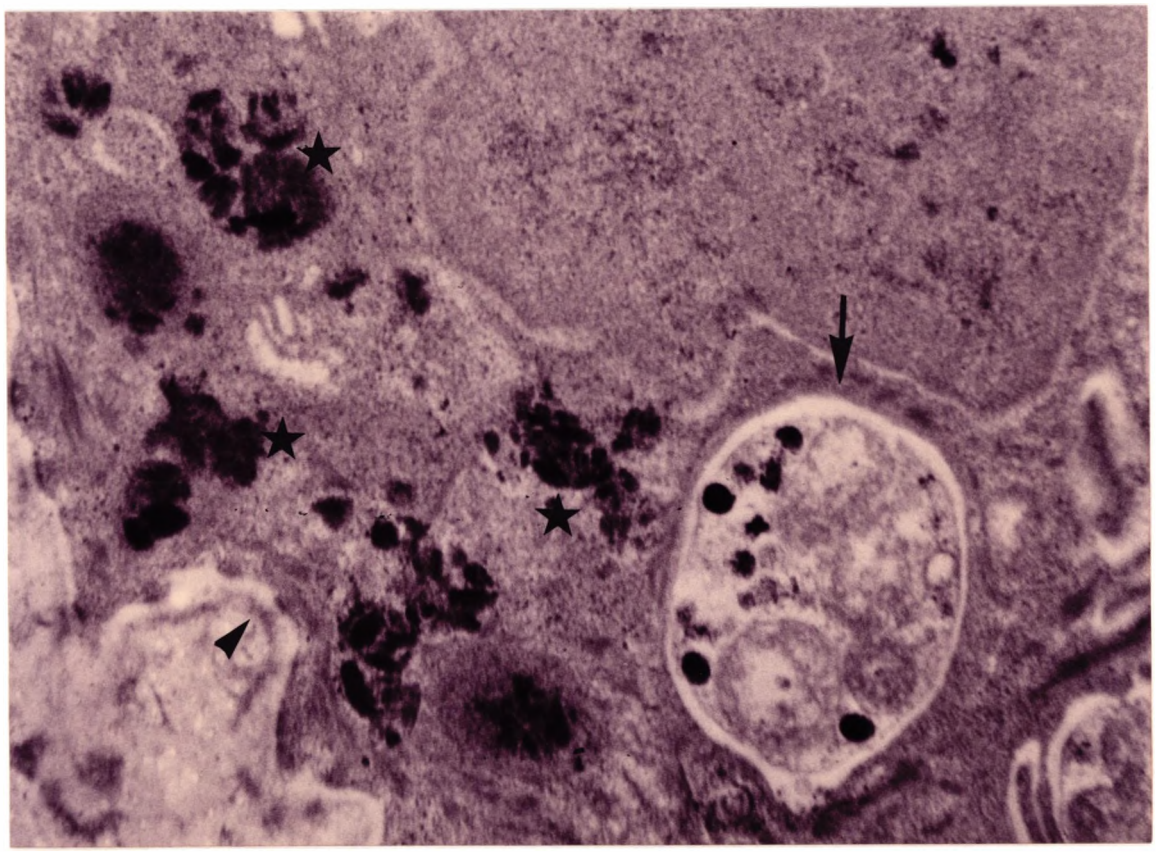


Figure 31. Vesicle bound melanosomes within the cytoplasm of a native basal epithelial cell (arrow). The vesicle stains differently to the cytoplasm of the palisade epithelial cell but is similar to the perikaryon of the melanosome illustrated in the preceding figure. The presence of such figures support the existence of pigment donation in the palisade epithelium. Clusters of non-vesicle bound melanosomes are present in the cytoplasm of the native epithelial cell (stars). The basal lamina is also indicated (arrowhead). (x26 509).

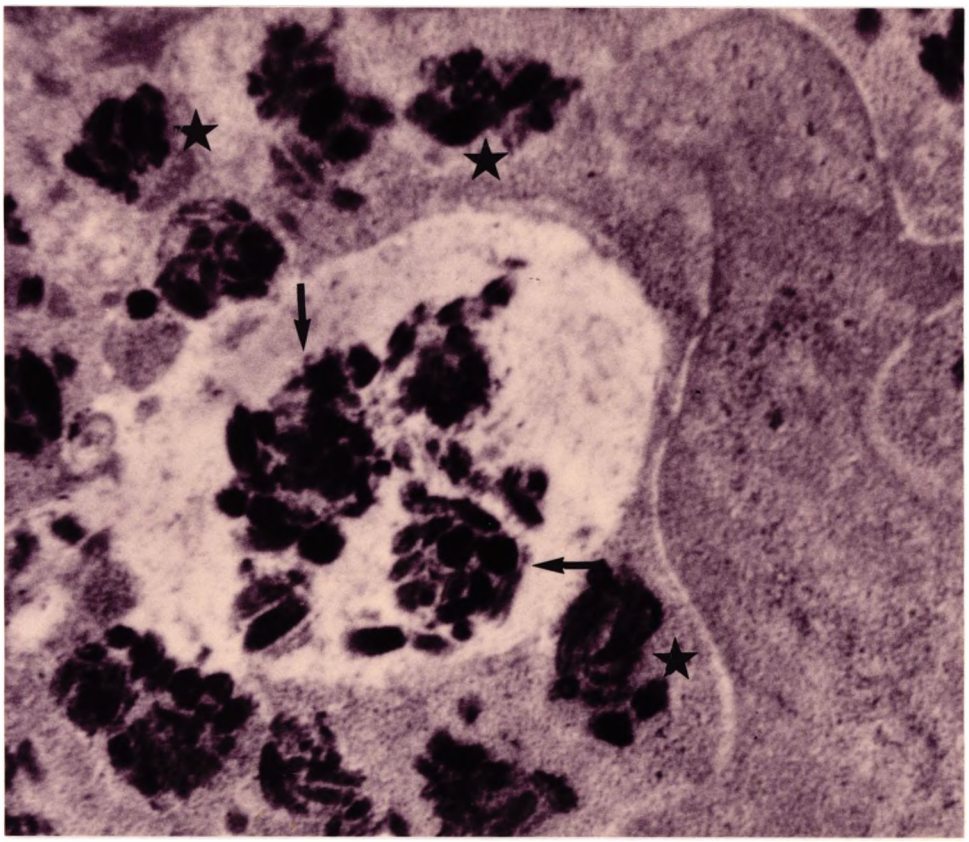


Figure 32. In the latter stages following pigment donation, the membrane of the vesicle is lost, yet the clusters of electron dense melanosomes (arrows) still remain distinct to those in the perikaryon of the epithelial cell (stars). (x29 161).

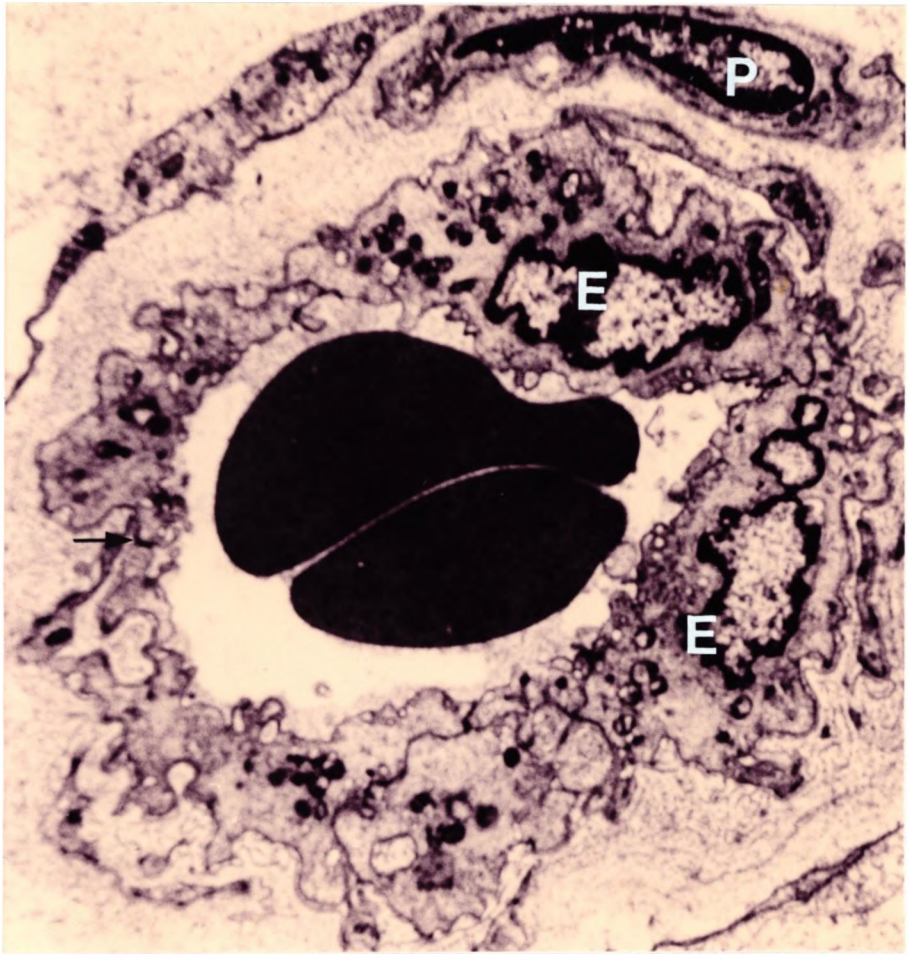


Figure 33. Palisade vessels are formed when adjacent endothelial cells (E) are bound by tight junctions (arrow). These vessels are non-fenestrated and pericytes (P) characteristically provide an extra coat. Two erythrocytes can be discerned in the lumina of this vessel. Lymphatic vessels (not illustrated) are similar in morphology but are thin walled and lack pericytes and red blood cells. (x14 087).

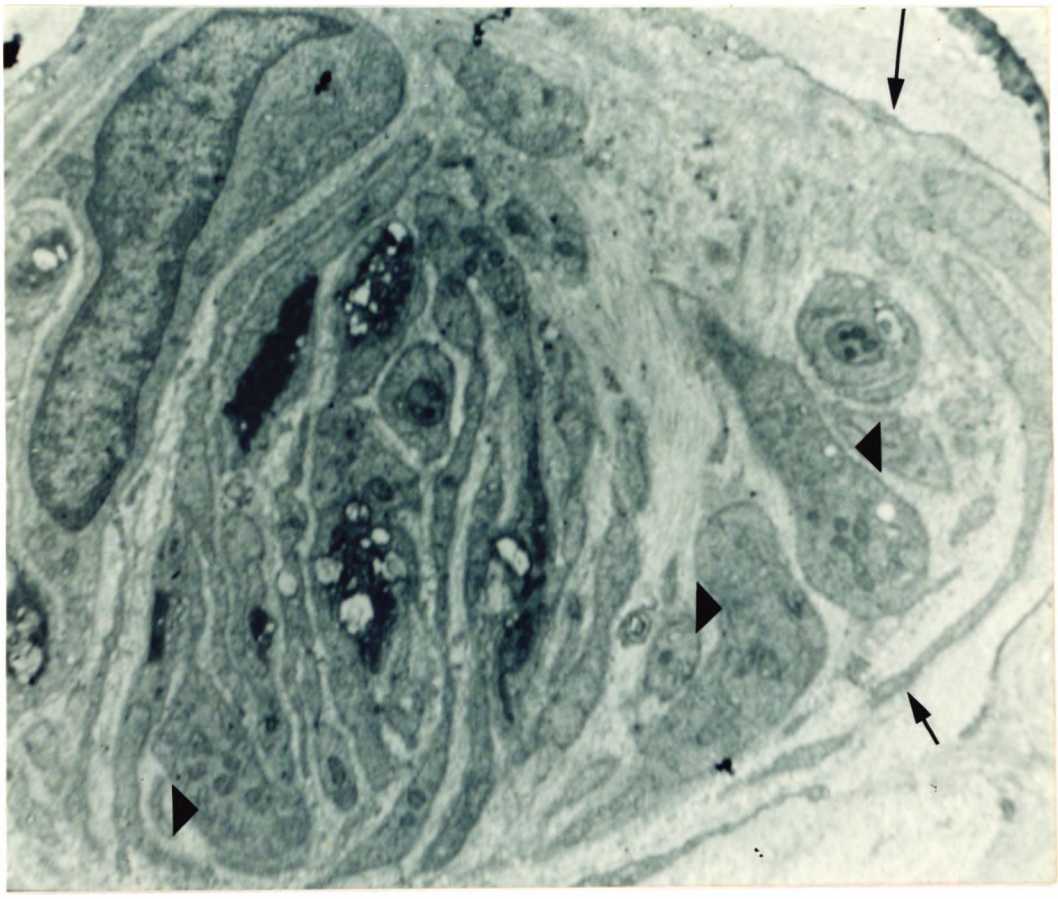


Figure 34. A corpuscular nerve ending in the palisade zone. The capsule is just visible (arrows). Axon terminals are also indicated (arrowheads). (x5015).

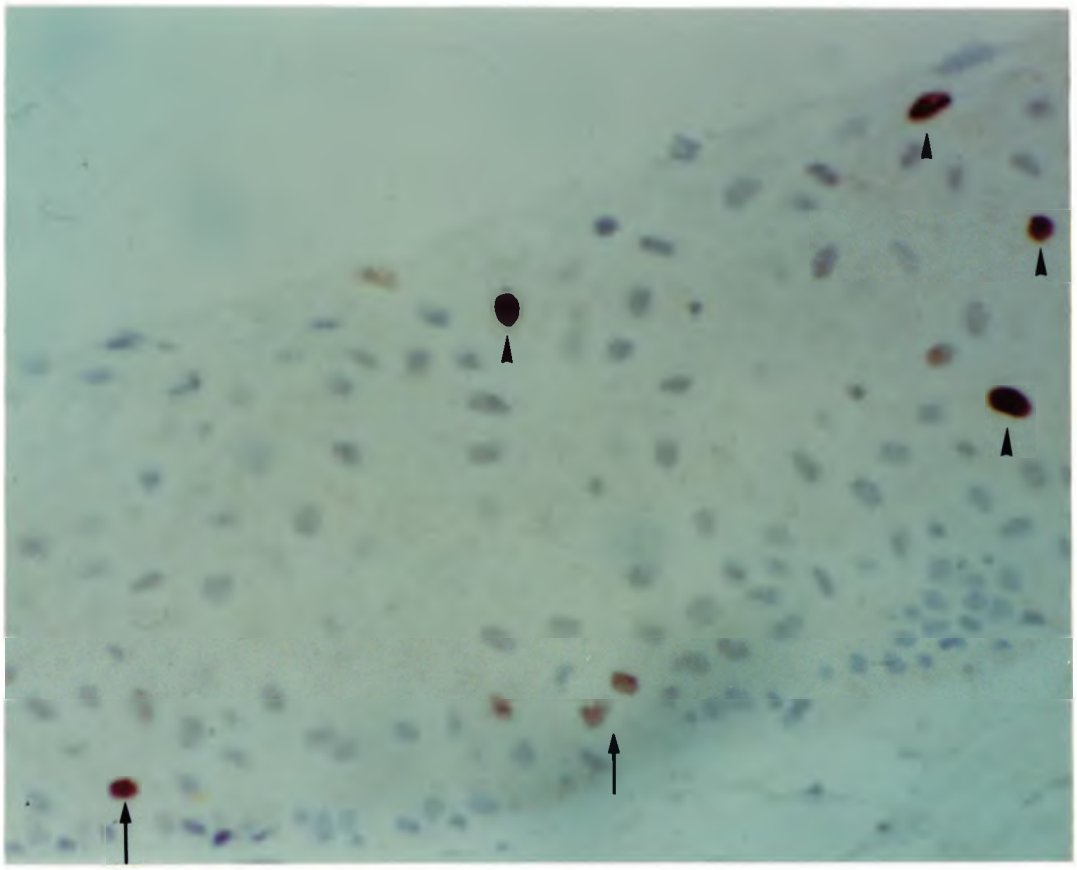


Figure 35. Section through the limbal conjunctival epithelium showing positive immunostaining in all layers. Positively stained cells are illustrated. This predominantly transverse section shows basal (arrows) and suprabasal (arrowheads) staining is present in similar ratios. The section is lightly counter-stained with haemotoxylin enabling the nuclei of other cells to be visible. (x267).

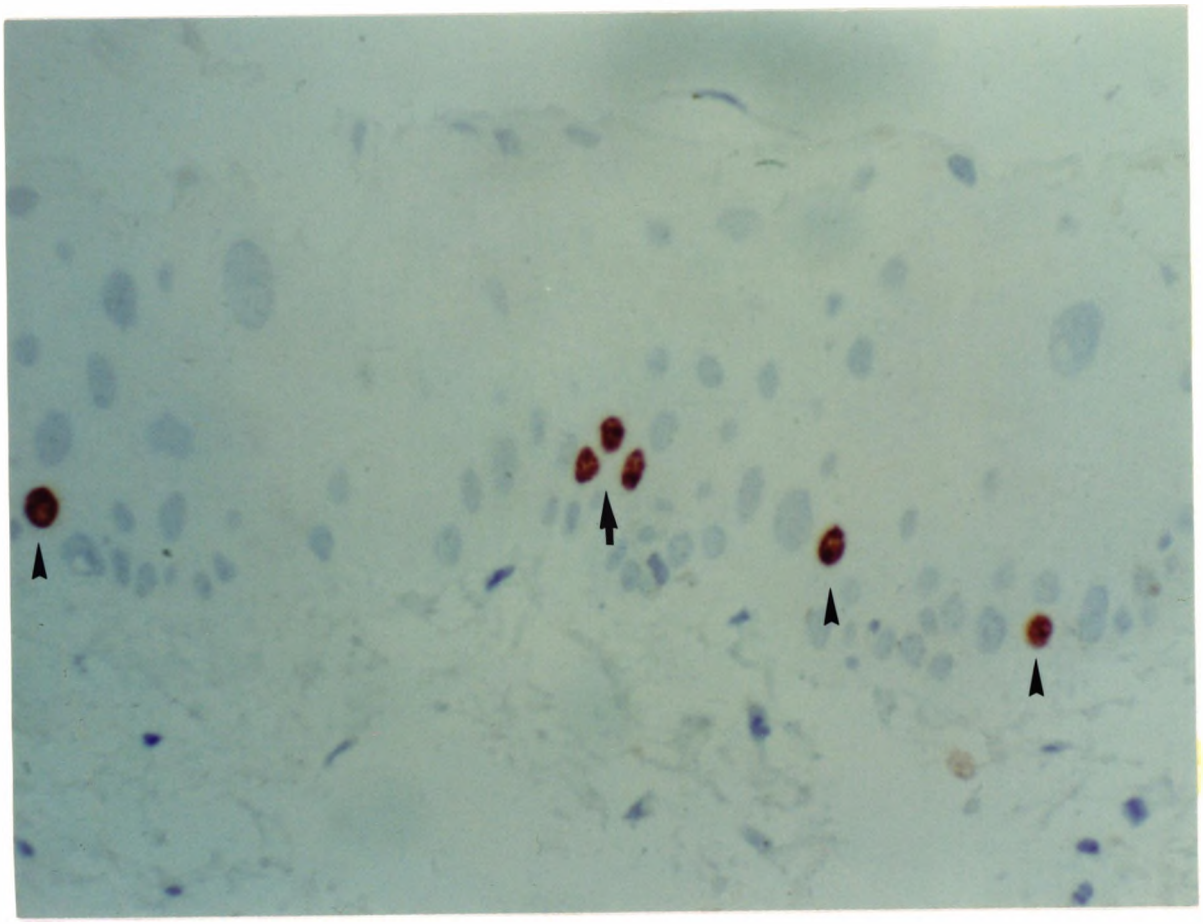


Figure 36. Positive immunostaining in the basal layer is confined to large cells. In this oblique section a single (arrowhead) and a cluster of three (arrow) positively stained large cells are illustrated. The light counter-staining with haematoxylin enables the nuclei of other basal cells to be visualised. The ratio of positively stained cells to non-staining cells throughout the basal layer is consistent with the ratio of small cells to large cells. (x310).

3.4. Primate Comparative Anatomy

3.4.1. Light Microscopic Observations: Cynomolgus Monkey

The limbal conjunctiva of the cynomolgus monkey showed no features equivalent to the palisades of Vogt in man. After the termination of Bowman's layer there was a gradual transition to bulbar conjunctiva. Like man, blood vessels close to the cornea were looped, hence in section they appeared paired. Larger blood vessels were present deeper in the stroma and episclera alongside large thin walled lymphatic vessels and myelinated nerve bundles. Corpuscular nerve endings, present in the limbal conjunctiva of man were absent in the cynomolgus monkey.

The limbal conjunctival epithelium is stratified and surface cells were squamous and non-keratinised. The epithelial layer thickened gradually from 5-6 cells at the cornea to 12-15 at the limbus before reducing to 2-4 cells in the bulbar conjunctiva. The surface cell layer was 1-2 cells thick, intermediate layer was 10-12 cells thick and the basal layer one cell thick. Two morphologically distinct native cell were present in the intermediate layer. The first was cuboidal with a central circular nucleus. The second varied greatly in its form but was characterised by the presence of long, broad, insinuating processes, which resulted in these cells resembling a star (Figs 37). The processes of these "Star" cells were seen to extend to other levels and some extended from suprabasal level to make contact with the basal lamina (Fig 38). They were found in all positions of a 2mm wide annular band bordering the cornea. Flat sections indicated the full dendritic span of "Star" cells. These cells constituted up to 50% of cells in the intermediate layers.

Due to their large dendritic span they often appeared larger than neighbouring cells. Their processes were of variable size, randomly orientated and flat sections confirmed that they extended to all levels of the epithelium except the surface. Where they attached to the basal lamina, the nuclei were located more superficially than that of other basal cells, giving the appearance of a "second-tier" of basal cells . "Star" cell processes frequently contacted each other which sometimes gave the appearance of enveloping neighbouring cells (Fig 37). The regularity, present in the basal layer of the human limbal conjunctival epithelium was absent in the cynomolgus monkey. In the latter, cells varied greatly in size and both large and small cells were freely interposed with no particular order.

In contrast to man, melanin was present in all layers of the limbal conjunctival epithelium. However, like man its density decreased from the basal layer which was the most densely pigmented. At LM levels melanin granules in basal cells appeared black, however in more superficial layers was rustic brown. The amount of pigment contained within individual cells of the same layer varied ranging from dense pigmentation through to cells containing little or no pigment. The most interesting presentation of this heterogeneous distribution was in the intermediate layer, where star cells consistently accumulated less pigment than their neighbours without processes. This characteristic was consistent in star cells that contacted the basal lamina.

3.4.2. Electron Microscopy (Cynomolgus Monkey)

As indicated by light microscopy, the limbal conjunctival epithelium of the cynomolgus monkey shared many features common to man. Surface cells of the epithelium displayed slender microvilli and microplicae and a fine branching glycocalyx network attached to these processes. Squamous cells displayed a large central nucleus and contained very few organelles and cytokeratin filaments. Adjacent cells of this layer were joined along their apical surfaces by tight junctions and elsewhere along their membranes by interdigitations and desmosomes. However, in contrast to man, surface cells were occasionally pigmented. The few melanosomes in these cells were coarse in comparison to that found in the basal cells of man and unlike in the latter, were arranged freely around the nucleus and not in clusters. These features of pigmentation were found in all the layers of the epithelium.

Cells of the intermediate layer showed the greatest variation from those of man. The diversity in Star cell morphology was confirmed by electron microscopy. Those star cells communicating with the basal lamina often displayed a solitary process whereas the majority displayed numerous long, broad processes. Star cells characteristically had a large central nucleus and contained few mature melanosomes within their cytoplasm. By contrast, cells of the same layer without processes were always heavily pigmented (Fig 39, 40).

Basal cells varied greatly in size, but those attached directly to the basal lamina not via a cytoplasmic stalk, invariably remained columnar in shape. Adjacent cells were attached

to each other by membrane interdigitations and desmosomes and to the basal lamina by hemidesmosomes. A variation in the density of pigment was also present in the basal layer, however, unlike man, there was little regularity. With the exception of suprabasal star cells that attached to the basal lamina via a cytoplasmic stalk, there was little or no tendency for larger cells to be less pigmented than their small counterparts. Light microscopic observations suggest that melanin at basal level varies from that present suprabasally. However, melanosomes in basal cells appeared no different morphologically to those in the suprabasal and surface cells at EM level.

Non-native cells in the epithelium formed no intracellular complexes with neighbouring cells or the basement membrane and were found predominantly adjacent to the basal lamina. A similar complement of these cells were present in the epithelium of the cynomolgus monkey as in human tissue. Langerhans cells, lymphocytes and polymorphs all displayed features similar to those previously described in man. Macrophages displayed similar features to those present in man and were predominantly found at the basal level although sometimes they were present in suprabasal levels. However, a species variation existed between the melanocytes of man and monkey. Although morphologically similar, the cytoplasm of these cells in the cynomolgus monkey, tended to contain significantly more mature melanosomes compared to man.

The greater number of melanocytes in the limbal conjunctival epithelium of the cynomolgus monkey compared to man, presented an ideal opportunity to study the process of primate epithelial cell pigmentation. Flat sections best indicated the full dendritic span of these cells (Fig 41). Melanocytes and their processes did not extend

beyond basal level. Frequently, a melanocyte process packed with mature melanosomes was seen to extend towards an epithelial cell. Occasionally, the latter would engulf this process by a procedure akin to phagocytosis - the epithelial cell appeared to "nip-off" the process resulting in a package being taken into the perikaryon of the recipient epithelial cell (Fig 42). The giving and receiving of melanosomes appeared to be an active process involving both donor and recipient and was encountered at basal level only.

With the exception of the absence of corpuscular nerve endings, the limbal conjunctival stroma of the cynomolgus monkey was similar in content to man. Therefore the morphology of stromal fibrocytes blood vessels, lymphatics, mast cells and nerve fibres were similar to those previously described.

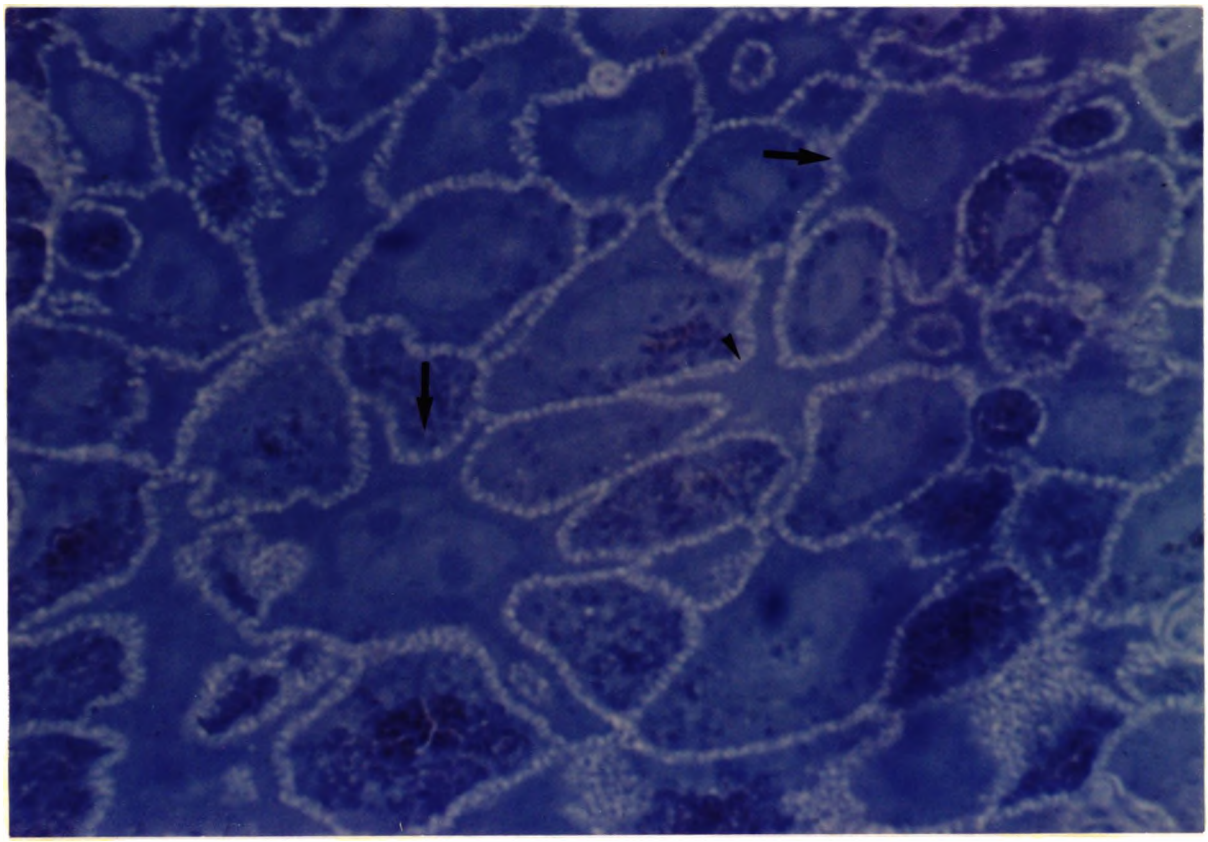


Figure 37. Flat section through the intermediate layers of the limbal conjunctival epithelium of the cynomolgus monkey. "Star" cells are illustrated. The processes of these cells extend to other layers (arrowhead) of the epithelium and frequently envelope neighbouring cells. These cells are characterised by the scarcity of pigment within their cytoplasm compared to adjacent cells. (x1117).

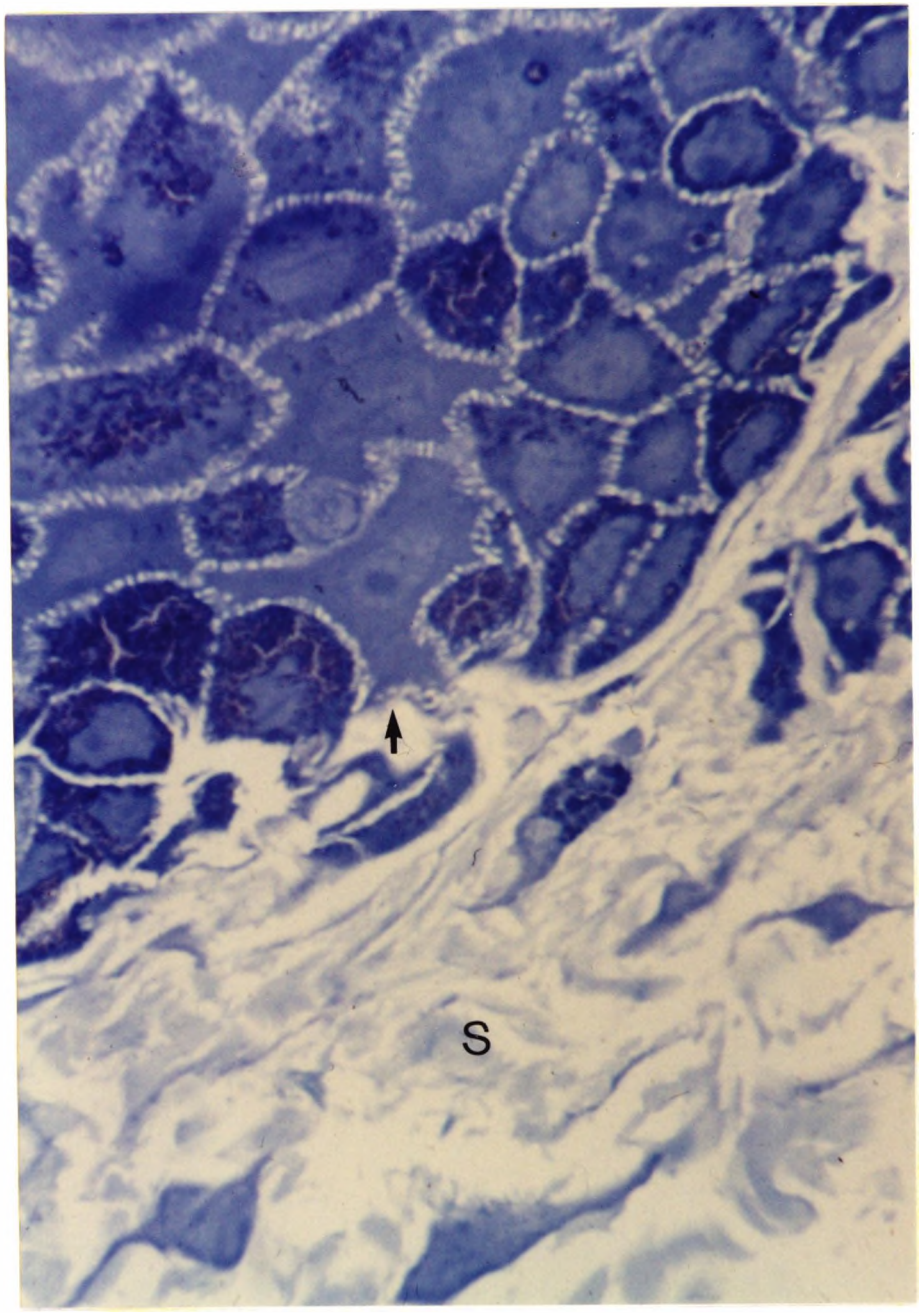


Figure 38. Star cell processes extend to other layers of the epithelium. In this tangential section, a "cytoplasmic -stalk" is seen to extend form a star cell (arrow) to the basal lamina, thus giving the appearance of a second-tier of basal cells. Cells without processes, adjacent to the Star cell are densely pigmented. S=conjunctival stroma. (x1089).

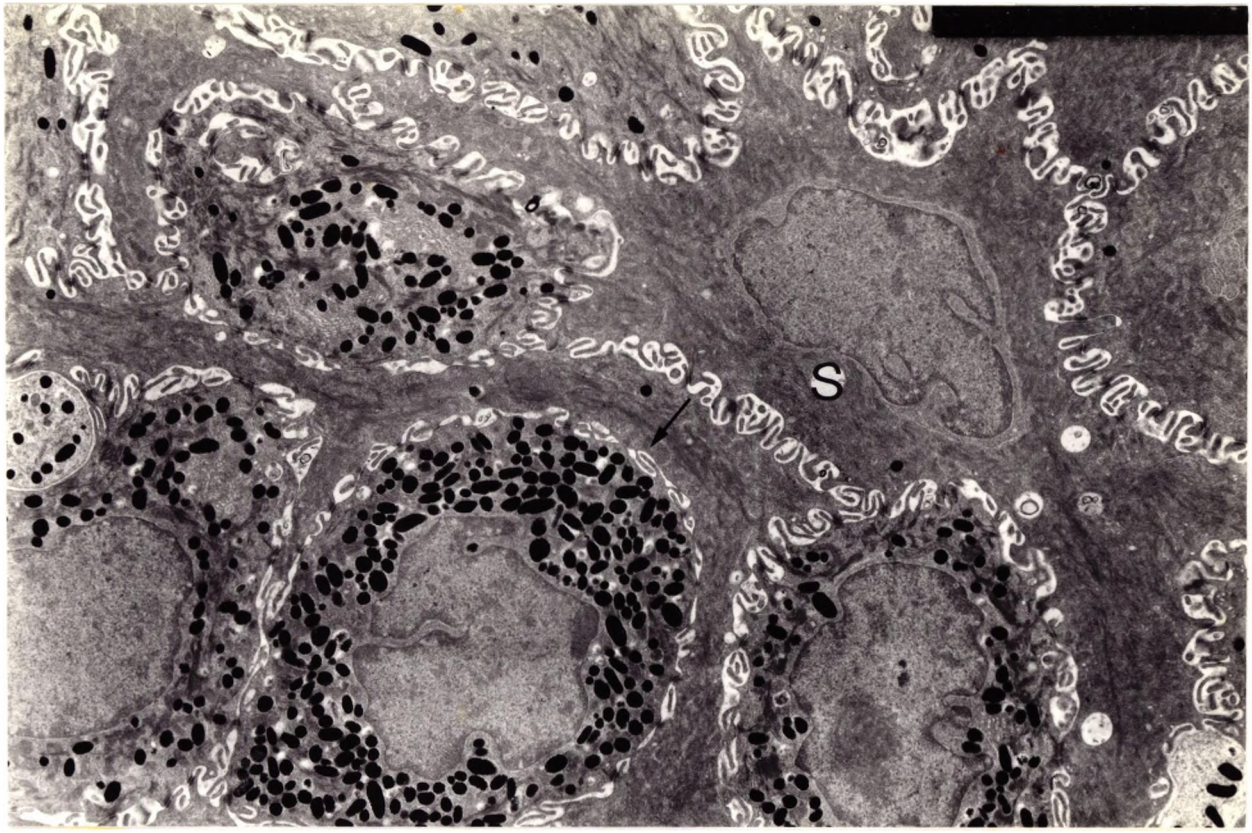


Figure 39. A Star cell (S), with numerous processes is illustrated in this electron micrograph. The perikaryon of this cell is void of melanosomes. By contrast the cytoplasm of adjacent cells without processes (arrow) are packed with pigment granules. (x7966).

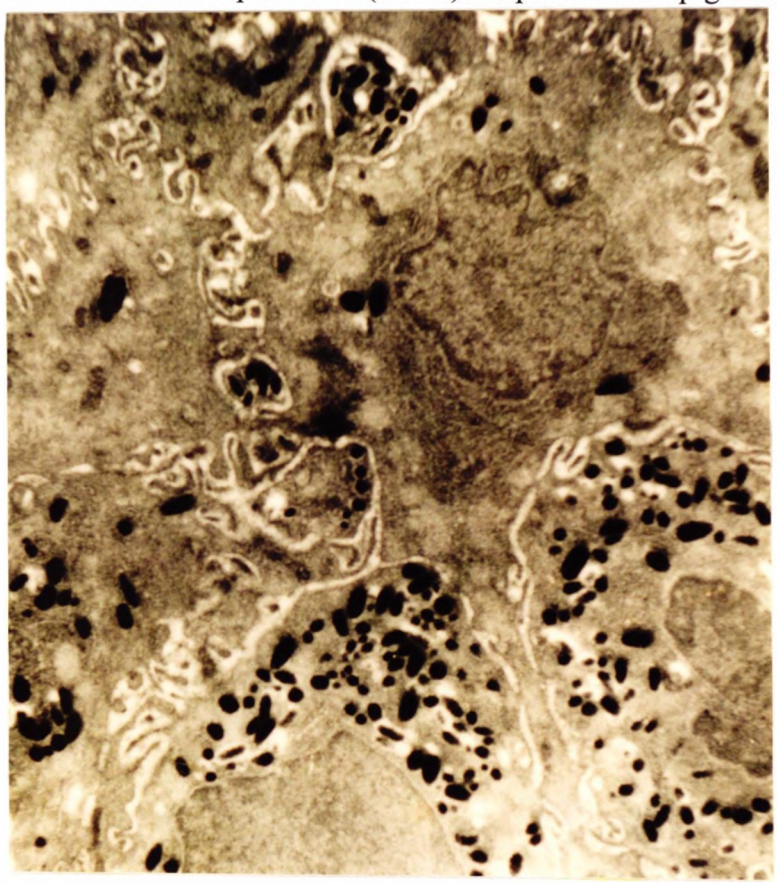


Figure 40. Electron micrograph of another star cell illustrating variety in these cells morphology. (x8047).

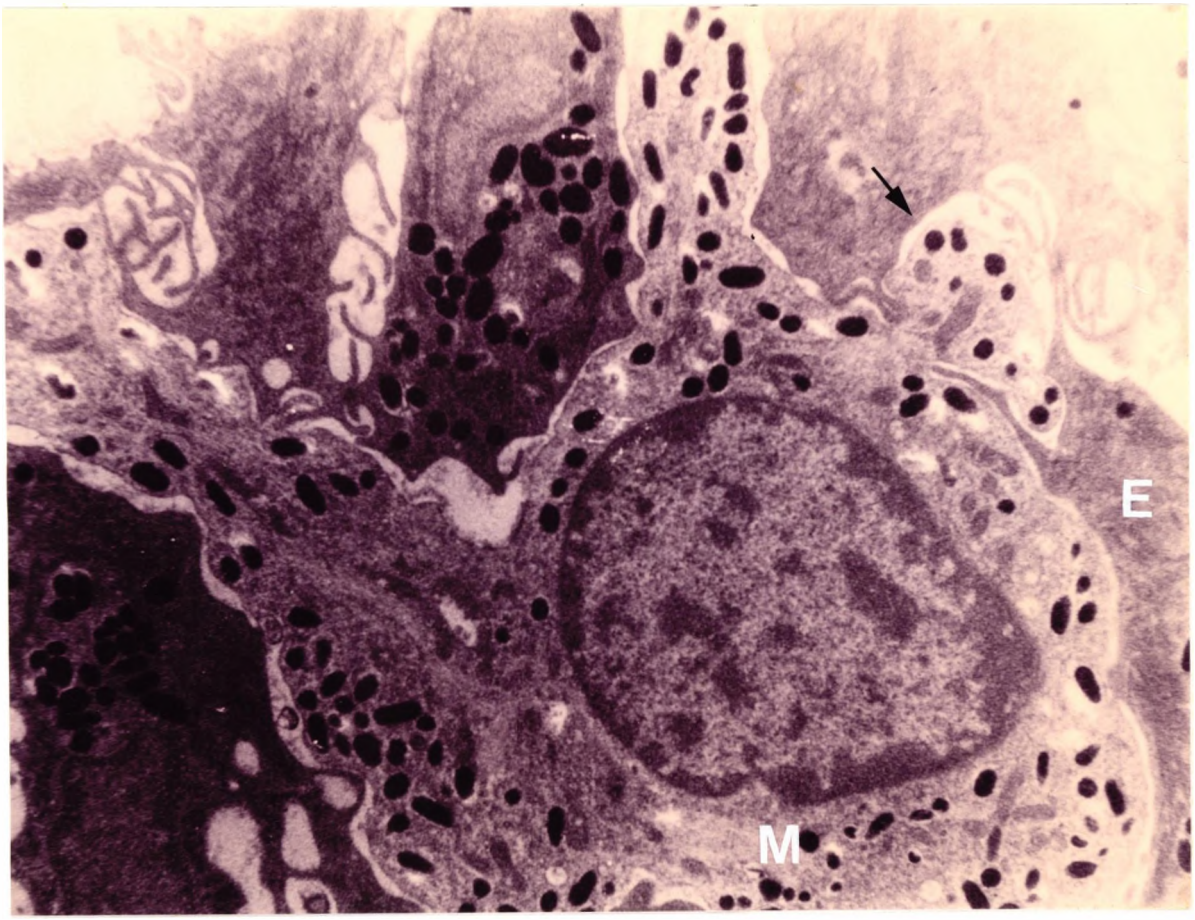


Figure 41. A fuller picture of the dendritic span of a melanocyte is illustrated in this tangential section. The perikaryon of melanocytes (M) in the limbal conjunctival epithelium of the cynomolgus monkey are packed with mature melanosomes. The processes of these cells actively extend towards other native basal cells. Pigment donation is illustrated in this electron micrograph as a melanocyte process (arrow), containing mature melanosomes is "engulfed" by an adjacent epithelial cell (E). (x20 618).

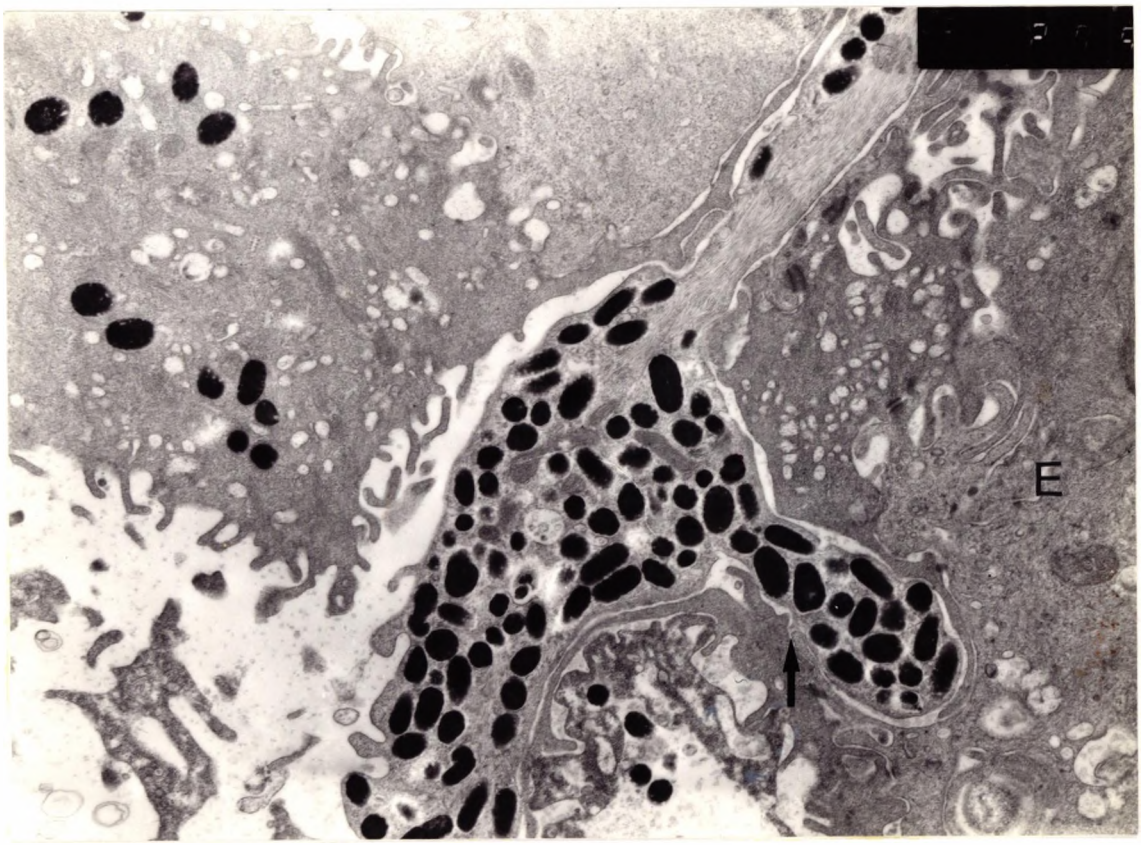


Figure 42. Direct evidence of pigment donation in the limbal conjunctival epithelium of the cynomolgus monkey. A cytoplasmic process of a melanocyte, packed with mature melanosomes (arrow), is "nipped-off" by the recipient native epithelial cell. This illustrates the process that is thought to be in operation in all the species of primate examined. (x26 062).

3.4.3. Light Microscopic Observations: Rhesus Monkey

The rhesus monkey, like the cynomolgus monkey had no structures equivalent to the palisades of Vogt in man. The organisation of blood vessels, lymphatics and nerves in the limbal stroma was similar to that previously described for the cynomolgus monkey. The most noticeable difference between the two species was the absence of "Star" cells in the suprabasal layers of the epithelium in rhesus monkey.

Transverse sections, cut parallel to the limbus suggested that star cells were absent from the limbal conjunctival epithelium of the rhesus monkey. This observation was confirmed by flat sections (Fig 43). Basal cell dichotomy present throughout the basal layer epithelium in man, was absent in the rhesus monkey, where small and large cells were freely interspersed.

Melanin was present in all layers of the limbal epithelium with the basal layer being the most densely pigmented. Like the cynomolgus monkey, pigment in basal layer was black whereas that in suprabasal and superficial layers was rustic brown. The amount of pigment contained within cells of the same layer varied ranging from dense pigmentation through to cells containing little or no pigment within their cytoplasm. Pigment heterogeneity, described in the basal layer of the limbal conjunctival epithelium of man and present in the intermediate layer of the cynomolgus monkey was absent in the epithelium of rhesus monkey.

3.4.4. Electron Microscopy (Rhesus Monkey)

Fibrocytes, blood vessels (small and large calibre), lymphatic vessels, nerves and the basal lamina all displayed feature similar to those previously described for the cynomolgus monkey.

As with the two other primate groups described, surface cells of the epithelium displayed slender microvilli and microplicae (with an associated glycocalyx coat) along their outermost surface. Like the other species investigated, adjacent cells were joined by tight junctions along the apical surface and membrane interdigitations and desmosomes elsewhere. They contained a large deeply staining central nucleus, and very few organelles and cytokeratin filaments. Some surface cells contained melanosomes which were arranged in clusters around the nucleus. These granules appeared to be finer than those within epithelial cells of the cynomolgus monkey.

Adjacent cells of the intermediate layer were attached to each other by membrane interdigitations and desmosomes. These cells had a large, central, circular nucleus, and as would appear to be the trend in all of the primate species examined, were more richly endowed with organelles and cytokeratin filaments compared to surface cells. Pigment granules in the intermediate layer appeared no different to those present in squamous cells. Basal cells were attached to their neighbours by membrane interdigitations and desmosomes and to the basal lamina by hemidesmoses. Their cytoplasm was rich in organelles and cytokeratin filaments. Basal cells were generally the heaviest pigmented and like in the other layers, perinuclear melanosomes were clustered and finer than those

in present in the epithelium of the cynomolgus monkey. Morphologically, melanosomes in basal cells appeared no different to those in the other layers.

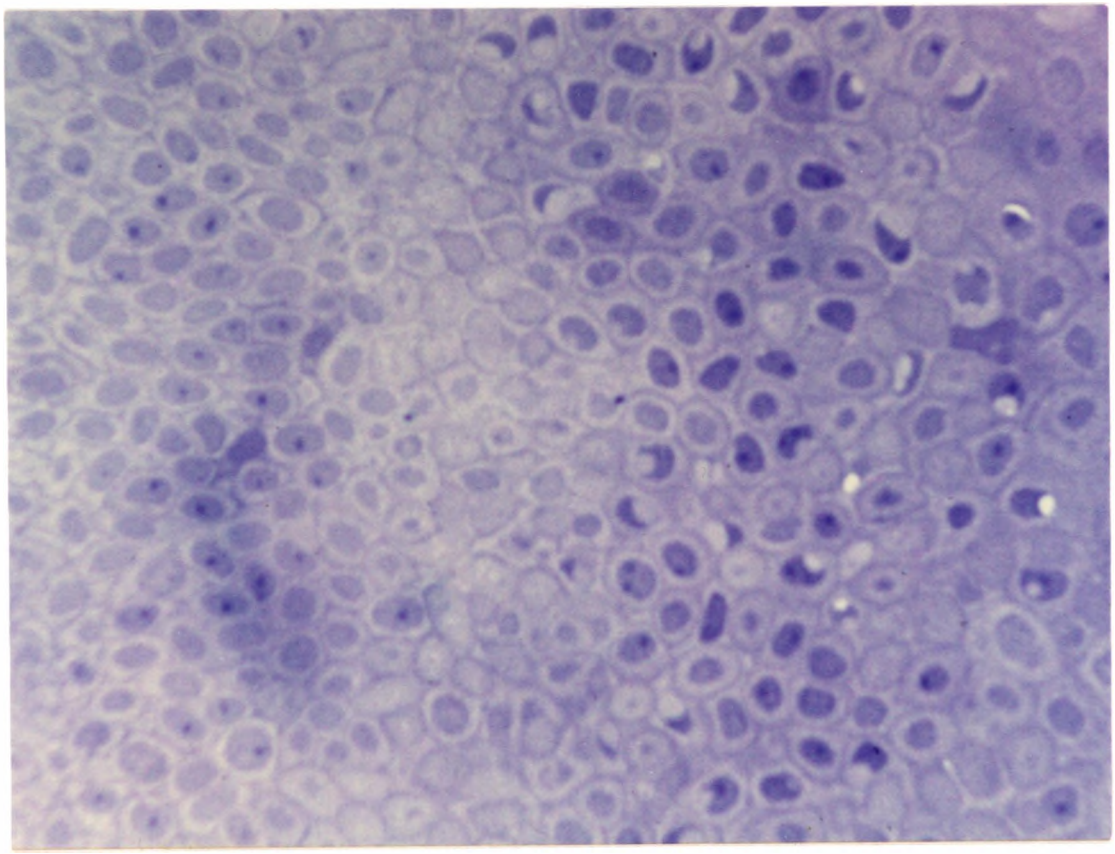


Figure 43. Light micrograph of a flat section through the suprabasal layers of the limbal conjunctival epithelium of the rhesus monkey. "Star" cells, characteristic in the suprabasal layers of the epithelium of the cynomolgus monkey are absent in the rhesus monkey. (x482).

CHAPTER 4

DISCUSSION

4.1. Anatomy and Histochemistry of the Primate Limbal Conjunctiva

Surprisingly little is known about the anatomy and functional significance of the limbal palisades. They were first adequately described by Vogt (1921) in his *"Atlas of the slit-lamp microscopy of the living eye",* although he accredited Streiff (1914) with having noted the radial stripes a few years earlier. Graves (1934) referred to palisades as "the trabeculae of the limbal conjunctiva". According to this author palisades range from 0.5 to 1.25mm in length and there are seven to ten per mm. These values relate to the more recent studies of Davanger and Evensen (1971), Goldberg and Bron (1982) and Townsend (1991). In the present study the range of palisade length was: 0.32 to 1.16mm and there were 10 to 13 palisades per mm. In addition, the magnification afforded by the photographic system used in the current study allowed accurate measurement of the width of each palisade (range 0.018 to 0.045mm). Also the average distance between adjacent palisades could be determined. The range in this parameter was 0.0058 to 0.12mm.

Palisades were recognised earlier in histological studies incidental to a quest to find Manz glands in man (Manz 1859, Ciaccio 1874, Virchow 1910, Aurrell and Kornerup

1943). These studies described a series of finger-like stromal processes, invaginating the overlying epithelium at the superior and inferior borders of the cornea. However, the observations of these studies on thick sections under relatively low magnification contributed little to understanding of the organisation of the palisades and it was not until the study of Davanger and Evensen (1971), that a possible function was suggested. Through observations of pigment slide from the limbus to the peripheral cornea, they suggested that epithelial cells migrating from the limbus to the cornea are generated by the palisade zones. This hypothesis was supported by Bron (1973) who linked the vortex pattern in various epithelial corneal epithelial disorders with the palisade pattern. With the introduction of corneal epithelial stem cell theories, Tseng (1990) hypothesised that the palisade zones may be a suitable area for housing these important progenitor cells and thus are probably involved in generating cells to replenish the corneal epithelium. This suggestion was investigated by Townsend (1991) in his comprehensive study, however, this worker failed to come to a firm conclusion as to whether or not this area is involved in epithelil cell regeneration. The apparent lack of understanding of the organisation and possible function of the palisade zones have been addressed in the present study and form the basis of the following discussion.

Those who have examined the palisades have commented on the complexity of their vasculature. Graves (1934) described a network of conjunctivally derived tight hairpin loops and arteriovenous anastomoses that run radially within and deep to the palisades. It was found in the current study that these vessels were derived from larger episcleral vessels whose walls incorporate smooth muscle that was innervated. This arrangement presumably allows these vessels to regulate blood flow to their finer tributaries. Further

evidence of vascular lumen control was suggested by the presence of open, semi-open and closed vessel lumina. Apart from reflecting the dynamic nature of the limbal vasculature, these observations strongly suggest that vascular shunting, presumably operated by episcleral perivascular smooth muscle may be in operation in the palisade zones. Iwamoto and Smelser (1965) described fenestrated capillaries in the limbus-no such vessels were found in the palisade zones. As the limbal conjunctiva is subject to shearing during eye movements and lid closure fenestrated capillaries would be at risk of trauma. Therefore, if these vessels are present, perhaps they represent a very small percentage of the total limbal capillaries and may not extend as far as the palisade zones.

The fine lymphatic capillaries which converge from the palisade zones to join larger stromal vessels are part of the large lymphatic system described by Busacca (1948). Here, vessels radiate from the limbus to drain into larger, deeper lying vessels further back in the conjunctiva where they form an incomplete upper and lower circumferential drainage ring 7 to 8mm from the limbus. The presence of lymphatic vessels and stromal mast cells together with epithelial lymphocytes, macrophages and Langerhans cells suggests that the palisade zones are an immunocompetent unit, capable of responding rapidly to foreign antigens. During investigation of the palisade epithelium (and subsequently the remaining limbal conjunctival epithelium) it was noticed that limbal Langerhans cells did not contain Birbeck granules, which are characteristic in their epidermal counterparts. Mommas et al (1994) presented a case report of a healthy 29 year old male whose epidermal Langerhans cells lacked Birbeck granules. When challenged, these cells functioned normally. Therefore these workers concluded that the presence of these granules is not a prerequisite for normal Langerhans cell function.

There is no reason to question the immunocompetent nature of the limbus as responses in the form of conjunctival injection and oedema are readily visible even to the naked eye. Hence it can be concluded that Langerhans cells, which lack Birbeck granules, are functional and play an important role in the presentation and processing of antigens.

The organisation of the palisade epithelium was of greatest interest. Many of the features seen in the palisade epithelium were similar to the remainder of the limbal conjunctiva. Tight junctions girdling surface cells apical perimeters of the palisade epithelium presumably prevent the intercellular passage of solutes from the tear film. Microvilli and microplicae, present on the outermost face of these surface cells increase the area of attachment for a fine, branching glycocalyx network, which is important in tear film adherence (Nichols et al 1983, Dilly 1985). In sharp contrast to the results of the present study, Bron et al (1985), reported wide intercellular spaces in the superficial layers of the limbal conjunctival epithelium. In the present study adjacent surface cells were found to be strongly adherent to their neighbours. The apparent disparity in results may represent variety. Alternatively as the limbal conjunctiva is subject to shearing during blinking, lid closure and eye movements, weakly linked epithelium would be at risk of constant trauma. Therefore it would appear unreasonable that the limbal conjunctival epithelium should be attached in such a manner.

A dichotomy of native basal cells was present throughout the limbal conjunctival epithelium, most noticeably in the vertical meridian where the palisades are most prominent. In his study Townsend (1991) found that larger basal cells outnumbered their smaller counterparts. These results are in sharp contrast to those of the current

study in which the ratio of small cells to large was approxiamtely 10:1. The difference in results seem unlikely to reflect variation but as this was a consistent finding in the current study it can only be speculated that Townsend was mistakenly describing a non-native cell in the epithelium. The argument for error by this worker is strengthened because of the apparent confusion in that paper in describing the palisade zone. In the early parts of his thesis, based on clinical findings, Townsend suggest that palisades in the inferior position are linear. However, later in his study of the anatomy and cytology of this area this worker suggests that palisades in the superior position are linear. This apparent confusion is not cleared in the discussion section of that paper.

In addition to the obvious morphological difference between the two native basal cell types described in the current study, in pigmented conjunctivae, each accumulated different amounts of melanin within their cytoplasm. These observations led to the investigation of the whole process of pigmentation of epithelial cells in the limbal conjunctiva which appears not to have not described before.

Animal models frequently serve as a useful tool in understanding anatomical and physiological processes in man. The structural similarities between the three primate groups studied suggested monkeys are a suitable model to investigate pigment donation in the primate limbal conjunctival epithelium. The main structural difference between the species studied was the absence of palisades in the monkeys, hence structures associated with them, such as corpuscular nerve endings were also absent. Lawrenson (1991) suggested these receptors may serve a sensory function and that a specific concentration of them around the limbus may promote awareness of low grade stimuli

that would endanger the cornea. The absence of these receptors in monkey reflects species variation, however, as monkeys are more prone to foreign bodies entering their eyes from their environment and so may be detection of low grade stimuli would be a hindrance, and so is absent.

Incidental to the quest to find palisades in monkeys it was noticed that the relationship between melanocytes, their processes and native limbal epithelial cells in these primates was similar to that present in man. Melanocytes were only present adjacent to the basal lamina and their processes did not extend beyond this level. In man there was evidence suggesting the presence of pigment donation; whilst in monkey the evidence was more direct. This process seemed to be similar to that described in skin by Cruikshank and Harcourt (1964). Melanocyte processes were regularly seen to be actively accepted into the cytoplasm of recipient epithelial cells. Although direct evidence of pigment donation was not found in man, the similar arrangement of the melanocyte/epithelial "unit" in the primates examined suggests that pigment donation is probably also in operation in the human limbal conjunctival epithelium. Evidence from the current study would suggest that in the limbal conjunctival epithelium this process occurs at basal level only.

One of the most noticeable differences between the epithelium of man and monkey is the density of pigment accumulated within cells. Monkeys have developed densely pigmented conjunctivae presumably to protect the ocular surface from the intense assaults of UV from sunlight in their environment. In both species of monkey examined, pigment was present in all layers of the epithelium whereas in man, even in

the most densely pigmented conjunctivae, it is confined chiefly to the basal layer. This difference may be because the process of pigment donation is more active in monkeys compared to man and/or more rapid vertical migration of cells that have received pigment in the basal layer in this species.

"Star" cells represent an interesting morphological variant that are described for the first time in the current study. These cells were demonstrated to be exclusive to the limbal conjunctival epithelium of the cynomolgus monkey. Their processes suggest a capacity for movement and coupled with the scarcity of melanin granules within their cytoplasm, these cells would make ideal candidates for migration to the cornea where the lack of pigment would be mandatory. However, further work is needed to elucidate their characteristics fully and substantiate this hypothesis.

Although pigment heterogeneity has been described in other epithelia (Lavker and Sun 1983), the current study is the first to document this feature in primate limbal conjunctival epithelia. These authors correlated the density of pigment basal cells of the monkey palm epidermis to cell cycling time and suggested that less densely pigmented cells were faster cycling than their more heavily pigmented neighbours. This hypothesis can only be true if pigment donation is in operation at basal level in the tissue under investigation, As the focus of the present study was on human tissue, this relationship was investigated in the limbal conjunctival epithelium of man, where pigment donation was assumed to be in operation and where a distinct heterogeneity in the pigment accumulation characteristics of the two native basal cell types was observed.

Using immunohistochemiccal techniques, positive nuclear staining was demonstrated in two areas: basally and perhaps somewhat surprisingly to an equal proportion, suprabasally. In the former, large cells appeared to preferentially stain indicating that they were more actively entering cell division in comparison to neighbouring small cells. As large cells characteristically accumulate less melanin in pigmented conjunctiva, it is suggested that the hypothesis put forward by Lavker and Sun is also applicable to the limbal conjunctival epithelium.

There now exists a strong body of evidence that suggests that a proportion of basal cells of the limbal conjunctival epithelium represent corneal epithelial stem cells. Cell cycle time has been a major factor used to characterise these cells. Cotsarelis et al (1989) demonstrated that presumptive stem cells in the limbal conjunctival epithelium of SENCAR mice were slow cycling. Additionally, these workers described these cells as anatomically "primitive" as they found that their perikaryon was chiefly occupied by a large, deeply staining central nucleus that left little space for other organelles and cytokeratin filaments. By contrast, a few other basal cells were found to be fast cycling and displayed a cytoplasm that was rich in organelles and cytokeratin filaments. These cells were assumed to be the immediate offspring of stem cells - transient amplifying cells. The perikaryon of large basal cells of the limbal conjunctival epithelium of man was found to be rich in organelles and cytokeratin filaments. Additionally, the evidence of immunohistochemical labelling demonstrated these cells to be fast cycling. Small basal cells, by contrast could be defined as anatomically "primitive" and were assumed to be slow cycling as they rarely labelled positively. Hence, applying stem cell concepts to these observations, it would not seem unreasonable to suggest that in man, large basal

cells conform to the anatomy and proliferation characteristics of transient amplifying cells and by inference, small cells conform to the properties of corneal epithelial stem cells.

Schofield (1983) suggested that stem cells reside in an optimal "niche" or microenvironment that maintains them in an undifferentiated condition. The **exact** location of the corneal epithelial stem cells remains to be defined. Several workers have suggested the limbus may represent the domain of these cells (Schermer et al 1985; Tseng 1990; Zeiske 1994). Lauweryns and co-workers (1993) suggested that the peripheral cornea may house these cells. It is now accepted however, that the corneal epithelial stem cells are housed in the basal region of the limbal conjunctival epithelium, hence it would seem that the cell type described by these workers is likely to represent a transient amplifying cell and not a stem cell as initially thought. It resembles the cell described by Zieske and Wasson (1992) as expressing alpha enolnase.

The current study has demonstrated that the palisade zones are highly organised and possess many features that suggest they would be capable of supporting such important progenitor cells and their offspring. Therefore, it may be fair to assume that the corneal epithelial stem cell "niche" extends out as far as the palisades zones and that these areas serve to enhance the local concentration of these cells.

Advocates of corneal epithelial stem cell theories have suggested that the majority of cell division and proliferation occurs in the basal layer of the limbal conjunctival epithelium where stem and transient amplifying cells are thought to reside (Tseng 1989;

Lavker et al 1991).The presence of positive immunohistochemical labelling in the suprabasal layers of the limbal conjunctival epithelium in man appears to conflict with these theories. The significant amount of positive suprabasal labelling probably reflects cells in S-phase of cell division which are synthesising DNA for repair rather than true cell division. Using antibodies specific only to the early stages of cell division such as cyclin dependent kinases (CDK's) would help to differentiate the two possibilities. However, division of basal cells of the limbal conjunctival epithelium alone may not be sufficient to satisfy the demands of the corneal epithelium therefore, perhaps suprabasal cell division is present to meet this need. Moreover, as the basal layer of the limbal conjunctival epithelium has a finite volume, perhaps some dividing cells leave the basal lamina and migrate vertically where they continue to proliferate (and hence label) where their offspring may be more readily accommodated.

Using immunohistochemistry to label cytokeratins, Schermer et al (1986) demonstrated that the entire basal layer of the corneal epithelium consisted of cells conforming to the biochemical properties of transient amplifying cells. In man, the basal layer of the central corneal epithelium consists primarily of columnar cells which are similar in morphology to the "large" cells described in the current study. Their number decreases gradually across the profile of the cornea to the limbus until only a few are present in the basal layer of the palisade epithelium. It has been demonstrated in the current study that these cells conform to the properties of transient amplifying cells. These cells originate from the limbus, therefore in order to reach the cornea they must migrate centripetally. The presence of a gradient of large cells across the ocular surface suggests that centripetal movement may be selective and specific to these cells only. This may occur

in two ways (Fig 44); large limbal epithelial cells may remain basal and migrate centripetally to become the basal layer of the corneal epithelium. However, this may be difficult as their passage may be obstructed by neighbouring cells, so in addition, perhaps those suprabasal cells which label positively are also migrating centripetally to the corneal epithelium, where some may re-attach to the basement membrane to join the basal layer.

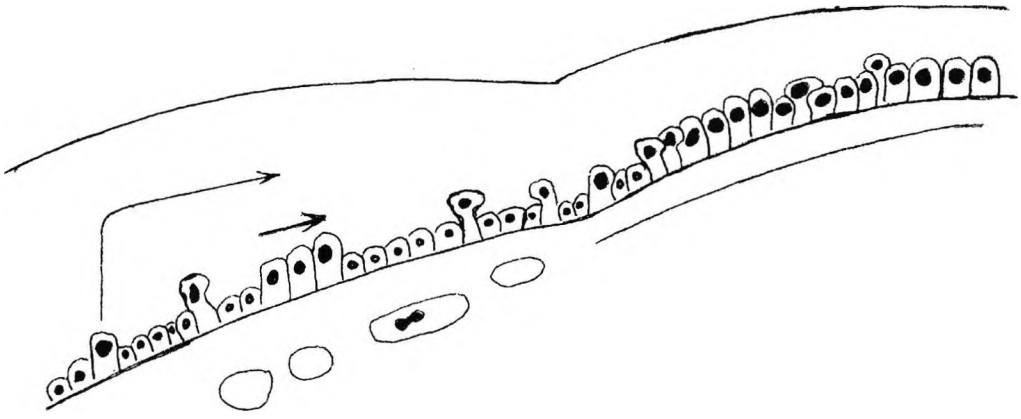


Figure 44. A schematic representation of the new model of centripetal migration from the limbal conjunctiva to the cornea. Conventional theories suggest that cells migrate along the basal lamina (short arrow). Although feasible cells further away from the cornea would have to bypass much 'traffic'. In order to compensate some cells may leave basal level and migrate in supra basal levels to the cornea where they may reattach to the basal lamina. It is postulated in the new theory that both systems are in operation in primates.

4.2. The Stability of the Limbal Palisades and Their Response to Hydrogel Contact Lenses

The former discussion suggested that the palisade zones are highly organised areas that house cells which are involved in generating cells for the corneal epithelium. Perhaps somewhat surprisingly there is no literature regarding the stability of the palisade pattern that is visible by slit-lamp biomicroscopy. Palisades are stromal structures and the potential for the initiation of change in the their pattern must lie primarily in the collagen. Alteration in the palisade pattern may however, also arise as a consequence of epithelial activity constraining normal collagen function. Therefore, any hindrance to the normal production of cells and their subsequent placement may result in clinically visible alterations in the palisade pattern.

The stability of the palisade pattern was investigated in the current study over a period of one year under normal and experimental conditions. In the latter hydrogel contact lenses were fitted in an attempt to challenge the stability of the palisade pattern. The results of each study will be discussed separately.

4.2.1. Normal (Control) Group

No significant changes were detected photographically in the gross palisade pattern under normal conditions ($p>0.05$). An explanation of this observation may also lie at cellular level. Under normal conditions, forced blinking and shearing forces do not alter the connective tissue forming the palisade. Also, these forces are unable to alter cell

division in the palisade epithelium. Subsequently, the arrangement of epithelial cells in the palisade epithelium is exact. If none of these factors are altered under normal conditions, then there is no reason for alteration in structure, hence the palisade pattern remains stable.

It has been argued that the palisade epithelium harbours cells conforming to the properties of corneal epithelial stem cells. Zieske (1994) suggested that many regulatory factors are present in an area containing stem cells in order to protect these cells and to ensure that their division is precise. One such factor is protection by the eyelids. The palisade zones are present only in the vertical meridian where protection from the eyelids is available. Lid coverage ensures access to a good vascular supply as well as maintaining a constant temperature. Therefore, stem cells are habitant in the palisade zones, then any regulatory mechanisms, present to protect these cells can only serve to enhance the stability of the palisade pattern.

Other minor factors that would result in no changes being detected in the palisade pattern include: the sensitivity of the apparatus used for monitoring and the duration of the study being too short to notice any change.

There is currently no evidence on the affect of ageing on the palisade pattern. However, Goldberg and Bron (1982) speculated that the palisade pattern may alter with age. They suggested that any changes may be linked to a greater incidence of corneal pathology in the elderly. If the palisade pattern alters with time then any modification may be as a result of senescent changes in the palisade collagen or its epithelium. Age-related

changes in the palisade collagen is unlikely to be detrimental to the cornea but if stem cells located in the palisade epithelium undergo senescent change, then this may not only in alteration of the palisade pattern but also in pathological changes in the cornea.

4.2.2. Experimental (Contact Lens Wearing) Group

No significant changes were found in palisade gross structure over the course of this study ($p>0.05$). These findings indicate that in the short-term the palisade zones are able resist the influence of hydrogel contact lenses. The current study reflects that the palisade pattern can be disrupted by constraining collagen activity or by effecting epithelial cell turnover. The ability of the palisade pattern to resist alteration suggests that well fitting hydrogel contact lenses, in the short term, do not have any detrimental effect on the palisade stroma, or perhaps more importantly, as far as the cornea is concerned, its epithelium. Once again, the sensitivity of the monitoring apparatus and the duration of the study must also be a minor point of consideration when discussing the stability of the palisade pattern.

The complete lack of symmetry in the palisade pattern of subject KP, who had previously worn a poorly fitting soft contact lens, reflects an inconsistent result that was not part of the controlled study. Further investigation, under controlled conditions would be required to fully evaluate the effects of poorly fitting contact lenses on the palisade pattern.

Kruse et al (1990) suggested that corneal epithelial stem cells in the limbus form a barrier, preventing invasion of the cornea by the conjunctiva. These workers showed that any breakdown of this barrier resulted in fibrovascular ingrowth of the cornea. As stem cells may be housed in the palisade epithelium, disruption of this barrier may be reflected by alteration in the palisade pattern which may serve as an "early warning" of imminent corneal pathology.

CHAPTER 5

SUMMARY AND CONCLUSIONS

The objectives of this study were to investigate the morphology and possible functional significance of the palisades of Vogt in the human limbal conjunctiva. Their existence has been known since the middle of the last century, when Manz described a series of finger-like projections at the upper and lower border of the cornea. Although the clinical appearance of these structures has been well documented, the present study is the first to investigate the cellular organisation of these structures at both light and electron microscopic level. In addition, evidence of limbal palisades in other primate species was sought for the first time

Palisades were found to be exclusive to the limbal conjunctiva of man. They exist in zones which consist of a closely packed radial array of connective tissue ridges with an epithelial covering that lie adjacent to the corneal margin. Palisades are present predominantly in the vertical meridian where the conjunctival/scleral overlap of the cornea is most marked. A variety of palisade forms exist; those located inferiorly generally are linear, whereas superiorly, they often appear less regular and may be formed from interrupted papillae. Palisades terminate largely in register 0.3-0.5mm with Bowman's layer of the cornea.

It can be argued that the most interesting aspect of the palisade zones is the arrangement of their epithelium. Previous workers have proposed that this may serve as a repository

for generating epithelial cells for the corneal epithelium. Light and electron microscopy were used to examine this area and investigate this hypothesis. The palisade epithelium was found to be highly organised. The basal layer of this area displayed a distinct dichotomy of native cells. These cells differed morphologically; the more numerous small cells appeared anatomically primitive in that their cytoplasm displayed few organelles and cytoskeletal fibres, whereas their less numerous counterparts, the large cells displayed a richer cytoplasm. Further investigation showed that basal cell dichotomy was present throughout the limbal conjunctiva.

In pigmented conjunctivae, these cells were found to accumulate unequal amounts of melanin. Pigment donation by non-native melanocytes is documented in skin. The present study is the first to report evidence of this process in cells of the limbal conjunctival epithelium. This process was found to be in operation at basal level only. Previous work in the monkey palm epidermis has correlated the density of pigment to cell-cycle time, finding that sparsely pigmented cells are faster cycling than those that were heavily pigmented. Immunohistochemical techniques and pigmentation were used to confirm this relationship in the limbal conjunctival epithelium of man. Star cells in the limbal conjunctival epithelium of the cynomolgus monkey are described in the present study for the first time. These cells appear to be adapted for movement and their lack of pigment would make them ideal candidates for migration to the cornea. However, further work is required to characterise these cells more accurately.

It is now largely accepted that the limbal conjunctival epithelium houses putative corneal epithelial stem cells. The present study reports that the palisade zones in man

may represent a part of this locus. It has been hypothesised that the small and large basal cells of limbal conjunctival epithelium in man conform respectively, in terms of their anatomy and proliferation characteristics to stem cells and their immediate off-spring- transient amplifying cells.

The study of the stability of the palisade pattern under normal and experimental conditions indicates that these structures were unchanging over a duration of one year. In the latter the palisade pattern remained unaltered by hydrogel contact lens wear. Future work possibly over a longer duration, would seek to examine further the effects of other types of contact lenses on the palisade pattern and how any change may serve as a precursor to observing any change in the cornea.

The results of the present study support the hypothesis of Davanger and Evensen (1971) which suggested that the palisade zones may represent areas which are involved in generating epithelial cells for the cornea. Palisade epithelial cells with the expected properties of stem cells and transient amplifying cells were found. In addition, it was shown that the palisade zones displayed characteristics which make them a suitable niche for housing such important progenitor cells. With the increasing frequency of therapeutic corneal epithelial stem cell grafting, the results of the present study may provide a useful tool to clinicians involved in this procedure.

APPENDIX I

Details of Histological Specimens

Human:

Specimen Reference	Age	SEX	RACE	Fixative
HW23	69	F	C	GLUT./PARA.
HW27	63	M	C	GLUT./PARA.
HW28	70	F	C	GLUT./PARA.
HB 1and 2	39	M	A	GLUT.
HC1	4	F	C	GLUT.

Table 1. Anterior eye segments used to study the cellular organisation of the limbal palisades.

Monkey:

Specimen Reference	Species	Fixative
MR91	Cynomolgus	GLUT/PARA
MR89	Cynomolgus	GLUT/PARA
MR86	Cynomolgus	GLUT/PARA
MR56	Rhesus	GLUT/PARA
MR56 C and E	Rhesus	GLUT/PARA

Table 2. Anterior eye segments used for the primate comparative anatomy study.

APPENDIX II

Subject details for palisade stability: control and experimental groups.

			Control Group - Average Width				Control Group - Average DBP				Control Group- Average Length			
	AGE	E.ORIG.	B.L.	3MTH	6MTH	12MTH	B.L.	3MTH	6MTH	12MTH	B.L	3MTH	6MTH	12MTH
1 AJ	20	N	0.0244	0.0244	0.0244	0.024	0.0755	0.076	0.076	0.076	0.762	0.756	0.774	0.764
2 DP	19	A	0.0282	0.0282	0.0282	0.0278	0.07775	0.08075	0.079	0.078	0.506	0.514	0.508	0.512
3 BD	20	A	0.0372	0.0366	0.0366	0.0366	0.10525	0.106	0.10175	0.101	0.356	0.36	0.352	0.352
4 SD	23	A	0.0296	0.0298	0.0294	0.0296	0.0815	0.0815	0.0815	0.0815	0.476	0.472	0.476	0.472
5 SS	22	A	0.024	0.023	0.0226	0.0236	0.0745	0.07475	0.0775	0.07625	0.444	0.452	0.444	0.436
6 VP	21	N	0.0186	0.019	0.019	0.02	0.0875	0.08675	0.08725	0.08625	1.156	1.156	1.15	1.154
7 MP	21	SJ	0.0332	0.0324	0.0322	0.0336	0.07575	0.0765	0.0765	0.07525	0.6	0.588	0.602	0.598
8 RK	19	N	0.0298	0.0298	0.0288	0.0298	0.057325	0.0573	0.05725	0.05725	0.324	0.318	0.322	0.322
9 KP	19	A	0.0312	0.032	0.0324	0.0324	0.095	0.09305	0.09475	0.09475	0.434	0.426	0.426	0.434
10 BE	21	C	0.0226	0.023	0.0244	0.0222	0.0815	0.082	0.0815	0.08125	0.42	0.428	0.428	0.424
11 KM	20	A	0.0308	0.0306	0.0306	0.0308	0.26175	0.26675	0.261	0.26	0.724	0.716	0.722	0.722
12 JD	22	N	0.0364	0.0358	0.0356	0.0364	0.08075	0.08125	0.08275	0.083	0.396	0.4	0.384	0.416
13 SB	19	N	0.024	0.0224	0.0234	0.0232	0.106	0.10875	0.10425	0.1055	0.53	0.558	0.556	0.55
14 SG	23	C	0.0184	0.0186	0.0188	0.0188	0.06975	0.072	0.072	0.07175	1.074	1.078	1.07	1.074
15 BJ	21	A	0.0242	0.0236	0.024	0.0236	0.075	0.07525	0.072	0.07225	0.384	0.384	0.376	0.388
16 DV	24	A	0.0244	0.0224	0.0216	0.0222	0.08175	0.082	0.08125	0.08325	0.534	0.536	0.542	0.532
17 CR	23	A	0.0364	0.0368	0.0364	0.0362	0.08425	0.08325	0.0825	0.08225	0.564	0.564	0.568	0.566
18 MS	22	A	0.0284	0.0288	0.029	0.0294	0.094	0.09975	0.09975	0.09475	0.548	0.558	0.566	0.564
19 ST	22	A	0.0288	0.0288	0.0288	0.0288	0.07375	0.07775	0.078	0.07475	0.774	0.788	0.788	0.784
20 HR	20	A	0.0302	0.0292	0.0302	0.0298	0.07025	0.07025	0.07275	0.07025	0.564	0.564	0.568	0.564

			Experimental Group- Average Width					Experimental Group- Average DBP					Experimental Group- Average Length				
			B.L.	1MTH	3MTH	6MTH	12MTH	B.L.	1MTH	3MTH	6MTH	12MTH	B.L.	1MTH	3MTH	6MTH	12MTH
1 NP	22	N	0.022	0.0206	0.0186	0.0218	0.0198	0.0695	0.066	0.069	0.0655	0.07175	0.418	0.434	0.424	0.418	0.434
2 KO	24	N	0.0194	0.0208	0.0206	0.0192	0.0196	0.0735	0.07325	0.07475	0.0765	0.0765	0.324	0.318	0.344	0.322	0.328
3 NA	22	A	0.0408	0.0408	0.0414	0.0408	0.04	0.0715	0.07175	0.074	0.07525	0.07325	0.424	0.436	0.43	0.432	0.43
4 SN	19	A	0.0204	0.0194	0.0196	0.02	0.0188	0.07175	0.07125	0.072	0.0725	0.06975	0.424	0.43	0.42	0.414	0.428
5 SS	18	A	0.0224	0.0224	0.022	0.022	0.022	0.05825	0.06475	0.06475	0.065	0.065	0.906	0.906	0.848	0.912	0.868
6 PR	21	A	0.0252	0.0236	0.0248	0.0238	0.0238	0.077	0.07875	0.07675	0.07875	0.07675	0.374	0.38	0.388	0.394	0.386
7 JM	33	SJ	0.0424	0.0432	0.0438	0.0452	0.0434	0.04625	0.04625	0.04625	0.04625	0.04625	0.382	0.388	0.384	0.386	0.384
8 SG	20	A	0.0352	0.0352	0.0338	0.034	0.0332	0.077	0.077	0.0775	0.07675	0.07675	0.578	0.578	0.578	0.572	0.586
9 BN	22	A	0.023	0.023	0.023	0.0236	0.022	0.10025	0.10025	0.09925	0.10175	0.1025	0.364	0.37	0.36	0.356	0.358
10 HS	20	A	0.022	0.022	0.022	0.022	0.022	0.0885	0.0885	0.08525	0.08475	0.0885	0.49	0.498	0.492	0.488	0.478
11 WB	22	C	0.0252	0.023	0.0256	0.0252	0.0252	0.09975	0.09975	0.097	0.1025	0.10225	0.724	0.716	0.722	0.722	0.716
12 DB	22	C	0.021	0.022	0.022	0.022	0.022	0.0945	0.10075	0.0945	0.097	0.1025	1.136	1.14	1.128	1.13	1.16
13 RP	24	A	0.0322	0.033	0.0298	0.0326	0.0332	0.07875	0.076	0.08025	0.07725	0.07725	0.55	0.558	0.548	0.56	0.562
14 AD	25	A	0.0356	0.0346	0.0364	0.0354	0.0356	0.1215	0.1215	0.1215	0.1215	0.12325	0.4	0.38	0.42	0.4	0.4
15 YB	19	C	0.0298	0.0296	0.0308	0.0288	0.029	0.0785	0.0785	0.0785	0.0785	0.08675	0.476	0.488	0.5	0.456	0.468
16 BL	26	C	0.0314	0.0302	0.0312	0.0314	0.033	0.0745	0.07475	0.07575	0.07675	0.0805	0.374	0.382	0.37	0.374	0.366
17 RN	22	A	0.0292	0.0278	0.0278	0.0292	0.0292	0.064	0.065	0.069	0.060333	0.064	0.384	0.384	0.384	0.378	0.388
18 ST	24	A	0.022	0.022	0.022	0.0248	0.022	0.0945	0.092	0.0925	0.095	0.09675	0.402	0.402	0.402	0.4	0.4
19 HE	22	A	0.0252	0.027	0.0254	0.0264	0.0264	0.0875	0.09	0.091	0.0875	0.08675	0.48	0.476	0.48	0.488	0.488
20 DQ	26	C	0.0232	0.0256	0.022	0.0244	0.0232	0.053	0.0545	0.053	0.0555	0.053	0.6	0.588	0.602	0.598	0.58
21 ZS	21	A	0.0244	0.0232	0.0244	0.0244	0.0256	0.07925	0.07925	0.07925	0.07925	0.07925	0.538	0.542	0.548	0.534	0.532

E.ORIG=Ethnic Origin: N=Negro, C=Caucasian, A=Asian, SJ=Sino-Japanese

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