BEHAVIOURAL CHARACTERISTICS OF THE CELLS WHICH FORM EPIRETINAL MEMBRANES

PENELOPE ANN HOGG, BSc. Hons. Anat., MSc. (Lond.)
Department of Clinical Science,
Institute of Ophthalmology,
University of London,
Bath Street,
London EC1V 9EL.

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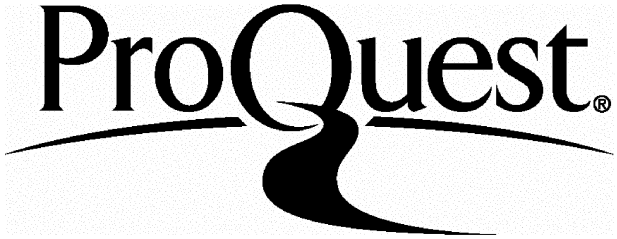

ABSTRACT

Epiretinal membranes are contractile cellular proliferations that form on the surfaces of the retina after trauma or insult to the posterior segment of the eye. Key cell types involved in membrane formation are retinal glia, retinal pigment epithelia and fibroblastic cells. A bovine tissue culture test system comprising bovine retinal glia, bovine retinal pigment epithelia and bovine scleral fibroblasts was employed in a series of behavioural studies to investigate the effect of soluble mediators and cell contact on migration, settlement and proliferation of the three key cell types in membrane formation.

Migration was assessed in vitro in a modified 48-well Boyden chamber, employing a standard chemoattraction assay. The migration of all three test cell types was stimulated by a glycoprotein found abundantly in epiretinal membranes (fibronectin), samples of subretinal fluid and a retinal crude extract. Cells grown from epiretinal membranes removed during surgery for retinal detachment also migrated to fibronectin. Retinal pigment epithelial cells showed less aptitude for migration to soluble stimuli in a Boyden chamber than the other two cell types and when the cell types were labelled and migrated together, the retinal pigment epithelium still migrated less than the other two cell types. Retinal pigment epithelium were also less responsive to platelet derived growth factor.

Static cell settlement studies demonstrated that retinal pigment epithelial cells showed more affinity for settlement onto plastic and in the presence of soluble fibronectin, than the other two cell types but the surface of a retinal glial monolayer appeared to be a less attractive substrate than serum coated plastic for the settlement of all three cell types. Video time-lapse microscopy was employed to monitor the settlement of retinal pigment epithelium onto glial and mixed cell layers. It revealed that the settlement process, in which distinct and reproducible stages could be identified, to be one of invasion and incorporation of the test cell types into the underlying cell layer. The fibroblasts entered a glial monolayer more rapidly than the pigment epithelia but the latter after entry, provoked a wave of glial mitotic activity.

The results indicated that within the confines of the test system there was interaction between the cell types, both through cell contact and remotely via soluble mediators and these

affected the behavioural responses of migration, settlement and proliferation. The findings when extrapolated to epiretinal membrane formation indicated that the glia were not a particularly good substrate for either fibroblasts or retinal pigment epithelium. The glia secreted substances attractive for the settlement of the retinal pigment epithelium and for the migration of all three cell types and media collected from cultures of retinal pigment epithelial cells was stimulatory for a whole range of activities and provoked a powerful migratory response from the fibroblasts.

So that the retinal pigment epithelium though possibly not the most important cell type in epiretinal membranes have an important role in membrane formation by releasing bioactive substances which attract and modify the behaviour of other cell types.

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PUBLICATIONS ARISING FROM THE WORK CONTAINED IN THIS THESIS

Savage, F.J., Day, J.E., **Hogg, P.** and Grierson, I., Characterisation and use of cultured retinal glial cells. Invest. Ophth. Vis. Sci., 1988, Suppl., 29, 244.

Savage, F.J., Day, J.E., **Hogg, P.** and Grierson, I., Tissue culture of retinal glial cells. Eye, 1982, Suppl., 1, 164-179.

Hogg, P. and Grierson, I., Model for the simultaneous migration of the three cell types in epiretinal membrane formation. Invest. Ophth. Vis. Sci., 1991, Suppl., 32, 767.

Grierson, I., Hiscott, P., **Hogg, P.**, Robey, H., Mazure, A., Larkin, G., Development, repair and regeneration of the retinal pigment epithelium. Eye, 1994, 8, in press.

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LIST of ABBREVIATIONS

aFGF : Acidic fibroblast growth factor
bFGF : Basic fibroblast growth factor
ANOVA : One way analysis of variance
BRG : Bovine retinal glia
BRPE : Bovine retinal pigment epithelium
BSF : Bovine scleral fibroblasts
cm^2 : Centimetres squared
CM : Conditioned media
CO_2 : Carbon dioxide
CPM : Counts per minute
°C : Degrees centigrade
DPM : Disintegrations per minute
EDTA : Ethylenediaminetetraacetic acid
EGF : Epidermal growth factor
ELISA : Enzyme-linked immunoabsorbant assay
ERM : Epiretinal membrane
F10 : Ham's nutrient media F10
FCS : Foetal calf serum
FGF : Fibroblast growth factor
Fn : Fibronectin
FSC : Forward scatter
GFAP : Glial fibrillary acidic protein
hr : Hour
ID_{50} : 50% of the initial dose
IGF I : Insulin-like growth factor one
IGF II: Insulin-like growth factor two
IL-1 : Interleukin one
IL-2 : Interleukin two
ILL : Inner limiting lamina
MEM : Minimal essential media
µg : Microgram
mg : Milligram
µl : Microlitres
M : Molar
min : Minute
ml : Millilitre
mm : Millimetre
mm^2 : Millimetres squared
Mm : Millimolar
NCS : Newborn calf serum
ng : Nanogram

NGF : Nerve growth factor
nm : Nanometre
OIF : Oil immersion fields
PBS : Phosphate buffered saline
PDGF : Platelet derived growth factor
PDR : Proliferative diabetic retinopathy
PVP : Polyvinylpyrolidone
PVR : Proliferative vitreoretinopathy
r : Correlation coefficient
RCE : Retinal crude extract
RD : Retinal detachment
RG : Retinal glia
RGD : Glycine-arginine-aspartic acid
RPE : Retinal pigment epithelium
rpm : Revolutions per minute
RRD : Rhegmatogenous retinal detachment
SEM : Standard error of the sample mean
S-N-K : Student-Newman-Keuls interval
SRF : Subretinal fluid
SSC : Side scatter
TGFβ : Transforming growth factor beta
TNFα : Tumour necrosis factor alpha
w/v : Weight per volume

Chapter 1.

GENERAL INTRODUCTION

1.1. Anatomy and Pathology

1.1.1. The Posterior Segment of the Eye

The eye is the peripheral sensory organ of vision. It develops as an outgrowth of the embryonic forebrain and as such is modified to receive light information and transmit it to the visual cortex where it is processed into an image (Warwick and Williams, 1973). The posterior segment of the eye is made up of three concentric tunics (Fig 1.1a.); the outermost is the protective sclera which consists mainly of fibrous and elastic tissue, the middle is the highly vascular and pigmented choroid and the innermost is the retina which contains the light sensitive photoreceptor cells and neuronal elements for processing the focused visual image. The vitreous body is the central gelatinous core and is adherent to the inner limiting membrane of the retina at the vitreoretinal interface. This study is concerned with epiretinal membrane (ERM) formation; a pathological condition occurring in the vitreoretinal region of the posterior segment of the eye (Fig 1.1b.).

1.1.2. The Retina

The retina is composed of neuronal, supportive and vascular elements. There are two main regions, the inner neurosensory retina and the outer retinal pigment epithelial (RPE) cell layer (Hogan et al, 1971). The neurosensory retina contains the retinal neurons, the photosensory cells and the retinal glia (Fig. 1.2.). Of particular interest in this study are the retinal glia and the RPE as they are known to be important cellular constituents of ERMs (Machemer, 1978; Grierson et al, 1987).

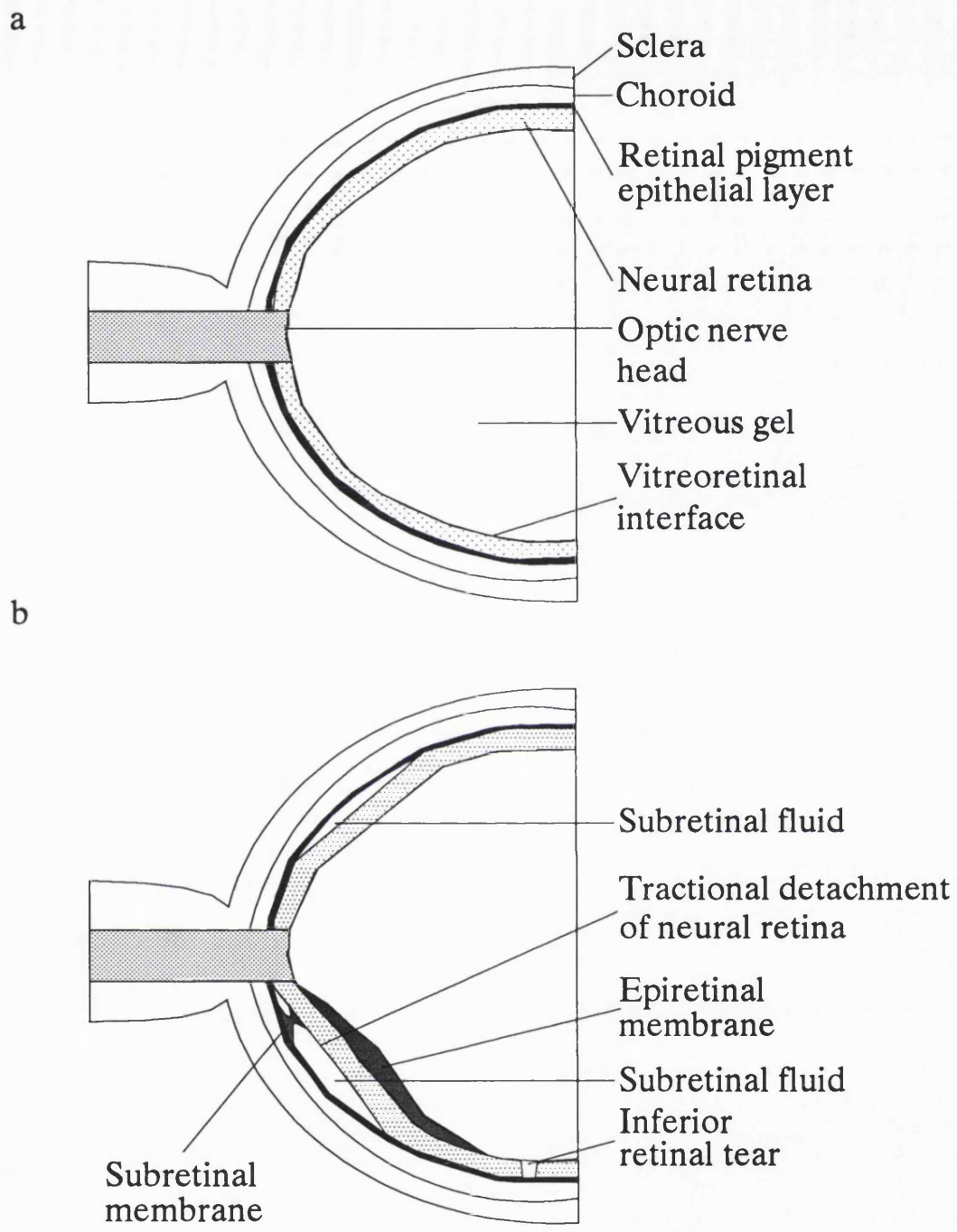


Fig 1.1. Schematic diagrams of the posterior segments of (a) the normal human eye and (b) a pathological eye with a retinal tear, epiretinal and subretinal membrane formation and tractional retinal detachment.

The retinal glia are a heterogenous group of cells derived both from neuroectoderm and from mesoderm, see below:-

1. Retinal glia derived from neuroectoderm:-
a. Müller's glia (Müller cells).
b. Astrocytes.
c. Perivascular glia.
d. Oligodendrocytes.

2. Retinal glia derived from mesoderm:-
a. Microglia.

(Savage et al, 1988; Hjelmeland and Harvey, 1988).

The Müller cells are highly specialised tall columnar glia which are radially orientated and span almost the full depth of the retina (Hogan et al, 1971). Externally they are connected to the cell bodies of the rod and cones sensory cells by gap junctions (which are historically, collectively known as the outer limiting membrane). Laterally the processes of the Müller cells branch and surround the adjacent retinal neurons and nerve fibres and serve to fill in the spaces between these structures. At the vitreal surface they expand into flattened cone shaped processes known as "end feet".

Müller cells are the most prevalent glial cell type in the retina and their radial orientation with respect to the retinal surface is accepted as a diagnostic characteristic in stained preparations of the retina (Hjelmeland and Harvey, 1988). They can be clearly outlined immunohistochemically by fluorescently labelled antibodies to the intermediate filament vimentin (Schnitzer, 1985; Grierson et al, 1987). Glial fibrillary acidic protein (GFAP) an intermediate filament which appears to be specific to glial cells is expressed strongly by retinal astrocytes (Dixon and Eng, 1981; Hiscott et al, 1984; Hjelmeland and Harvey, 1988) but is only localised to Müller cell end feet in the normal human retina (Erickson et al, 1987). Müller cells perform numerous functions in the retina (Fig. 1.3.); mainly concerned with the support and nutrition of the neurons and photosensory cells and they also play a part in the cellular response to injury of the surrounding tissues.

Cell type	Morpho-logical features	Location in retina	References
Müller cells	Columnar with central nuclei, many branching processes and vitreal end feet.	Full thickness of neural retina	Hogan et al, 1971
Astrocytes:-	Stellate:-	Plexiform, ganglion cell and nerve fibre layers.	Wolter, 1955
I Elongate	Few long branching processes.	Run parallel to nerve fibre bundles.	Ogden, 1978
II Stellate	Many short radiating processes.	Encircle nerve fibre bundles.	Ogden, 1978
Perivascular glia	Flat broad multipolar cells with few processes.	Form outer sheath of blood vessels.	Wolter, 1957 Lessel and Kuwabara, 1963
Oligo-dendrocytes	Few processes, ovoid nuclei	Not seen beyond optic papilla in vascular retinae. Form myelin sheath of nerve axons in the medullary ray of an avascular retina.	Schnitzer, 1988 Savage et al, 1988
Microglia	Phagocytic cells similar in appearance to macrophages	Inner and outer plexiform layers of the retina.	Hogan et al, 1971 Hume et al, 1983

Fig. 1.2. Classification of retinal glial cell types (Hogan, Alvarado and Weddel, 1971; Warwick and Williams, 1973).

The retinal astrocytes are most numerous in the inner retinal regions, especially the ganglion cell and nerve fibre layers and are often associated with nerve axons and blood vessels (Wolter, 1955; Ogden, 1978). Essentially they are small stellate cells with central nuclei and radiating processes and two different phenotypes have been described in the retina (Fig. 1.2.). Perivascular glia (Lessel and Kuwabara, 1963) are another class of glia and though little is known about them, they appear to be akin to the protoplasmic astrocytes of the central nervous system and are found closely associated with the retinal blood vessels. Oligodendrocytes are associated with the nerve fibres in the medullary ray of avascular retinae such as that of the rabbit (Savage et al, 1988).

The microglia have been described as forming regular arrays in the plexiform layers of the normal retina and if activated by retinal injury become amoeboid and phagocytic and share many characteristics of tissue histiocytes rather than true glia (Hjelmeland and Harvey, 1988; Gilbert et al, 1988). Evidence from studies on mouse retina suggests that they may be mononuclear phagocytes extravasated from nearby blood vessels (Hume et al, 1983).

The RPE constitute a monolayer of pigmented polygonal cells that form the outermost layer of the retina (Hogan et al, 1971; Zinn and Henkind, 1979). Their internal (apical) surfaces are covered with a dense carpet of microvilli and these surround and make close contact with the outer segments of the rods and cones. The RPE are joined at their lateral surfaces by a belt of intercellular tight junctions which prevent (all but small hydrophilic molecules) from moving passively between the cells and are thought to form part of the blood retinal barrier (Clark, 1986). The cells' functional cohesion is sustained by extensive intercellular communication in the form of gap junctions (Hudspeth and Yee, 1973). The outer surface of the RPE layer has many basal infoldings and its basement membrane together with a layer of fibrous tissue and the basement membrane of the choroidal capillaries forms Bruch's layer.

The cytoskeleton of the RPE contains a group of intermediate filaments, found commonly in epithelial cells, known as the cytokeratins (Moll et al, 1992). These have been used to differentiate the RPE from other retinal cell types and have been found to be expressed by RPE in culture and in situ (McKechnie et al, 1988). The RPE perform a vital role in the well being of the neurosensory retina by their sustenance of the photoreceptor cells, their maintenance of retinal adhesion and their part in

Functions of retinal glia
1. Structural support a. Müller cell and astrocyte processes surround the neurons and blood vessels and fill the spaces between the retinal structures. b. Müller cell processes also insulate the nerve fibres.
2. Nutrition Müller cells supply glucose to the neurons and synthesise and store glycogen.
3. Physiological support Müller cells and astrocytes play a role in the processing of information and maintenance of balance of ions, transmitters and metabolites to provide an optimal environment for neuronal activity.
4. Phagocytosis Müller cells, astrocytes and microglia are phagocytic and remove substances from the extracellular space.
5. Barrier role Astrocyte processes envelope blood vessel walls and play a modulating role in vessel development and form part of the blood retinal barrier.
6. Injury response Retinal glial cells respond to injury and inflammation by migration, cell division and secretion of soluble mediators.

Fig. 1.3. Functional characteristics of the retinal glia (adapted from Hogan et al, 1971; Apple et al, 1986).

the maintenance of the blood retinal barrier (Fig. 1.4.). The first two functions depend on close contact between the RPE and the photoreceptor outer segments and are consequently disrupted if retinal detachment occurs in that region.

1.1.3. The Vitreoretinal Interface and Vitreous

The vitreoretinal interface is the region at which the inner surface of the neurosensory retina and the cortical (peripheral) vitreous meet. It is lined by the internal limiting membrane (Salzman, 1912) which is formed partly from the basement membranes of the retinal Müller cells and astrocytes (Hogan et al, 1971) and partly from the condensation of collagenous fibrils of the cortical vitreous and vitreal ground substance (Rhodes, 1982). Bundles of vitreal collagen have been shown to insert between the retinal glia in this region (Gloor and Daiker, 1975). The basement membranes of the Müller cells are collectively known as the inner limiting lamina (ILL), which is classified as the innermost layer of the retina (Hogan et al, 1971).

The vitreous can be divided into two main regions, the peripheral vitreous (cortical vitreous) which contains the most of the formed vitreal elements and its cellular population and the more fluid central region (Hogan et al, 1971). The formed elements of the vitreous consist of a network of types II, V, IX and XI collagen (Seery and Davison, 1991; Bishop et al, 1993; Mayne et al, 1993) in a ground substance of glycosaminoglycans such as hyaluronic acid (Rhodes, 1982).

The vitreal cortex contains a sparse cellular population of various different phenotypes (Hogan et al, 1971) and they have been divided into two main groups on the basis of structure and function (Sebag, 1992). The first are the most numerous and are known as hyalocytes (Balazs, 1964). They are closest to macrophages in form and function (Teng 1969; Grabner et al, 1980) and are located in the vitreous and are spread out in a single layer 20-50 μm from the ILL posteriorly and the basal lamina of the ciliary epithelium at the pars plana and the vitreous base anteriorly (Sebag, 1992). Their functions include synthesis of hyaluronic acid, glycoproteins and collagen and also phagocytosis. The second category form 10% of the vitreous cell population and are found at the vitreous base and in the proximity of the optic disc. They are thought by some to be

Functions of the RPE
1. Maintenance of a healthy photoreceptor layer by:- a. Phagocytosis of rod and cone outer segments. b. Transference of metabolites to and from the visual cells and the choroidal circulation. c.Transferal of vitamin A to the photoreceptors.
2. Promotion of adhesion of the neurosensory retina to the to the RPE by:- a. Pumping fluids from the vitreal to the choroidal side of the retina. b. An intimate association of the apical villi of the RPE with the rod outer segments. c. Secretion of adhesion enhancing glycosaminoglycans into the subretinal space.
3. A barrier function protecting the neural retina from possibly harmful molecules in the choroidal circulation by:- The presence of tight junctions between the lateral surfaces of the RPE layer preventing the diffusion of all but the smallest molecules into the neural retinal region.
4. Absorption of scattered light by the presence of melanin in the cytoplasm which :- a. Improves the resolution of the image by minimising scatter of the transmitted light. b. Prevents degradation of the image due to leakage of light through the sclera.

Fig.1.4. Functions of the RPE (adapted from Zinn and Henkind, 1979 and Clark 1986).

fibroblastic cells (Teng, 1969; Balazs et al, 1980) and have been linked with the production of collagen (Birk and Zycband, 1989) and glycosaminoglycans (Bleckmann, 1984; see also Section 1.3.1.).

1.1.4. ERM Formation and Proliferative Vitreoretinopathy

The vitreous, vitreoretinal interface, neurosensory retina and RPE are to some extent privileged areas. They are protected from external influences present in the systemic circulation partly by the avascularity of the RPE, the outer retinal layers and the vitreous and also by blood retinal barriers. The barriers are situated externally between the choroidal circulation and the retina, internally between the retinal circulation and the retinal cells and anteriorly between the ciliary circulation and the ciliary epithelium. Damaging stimuli such as breaks in the wall of the eye caused by penetrating trauma or retinal reattachment surgery or tears in the retina, which occur in situations such as posterior vitreal detachment and retinal degeneration, may cause irreversible disruption to the normal functioning of the tissues in these regions, by evoking an injury response.

The injury response may take the form of acute or chronic inflammation which will in turn result in the production and release of substances that stimulate the migration of cells such as inflammatory cells and retinal glia (Hiscott et al, 1988). The glia are stimulated to proliferate and either remain as asymptomatic simple glial sheets which are known as simple ERMs (Fig. 1.5a.) or they are joined by more cells types and eventually organise into a contractile fibrous tissue present on both surfaces of the detached retina, on the posterior surface of the detached vitreous and within the vitreous itself. In the latter instance the fibrous tissue is referred to as a complex ERM (Fig. 1.5b.) and the mechanism of its formation is not as yet fully understood. The presence of an ERM is threatening to vision, since it becomes contractile and causes tractional retinal detachment with consequential blindness and surgery is required to remove it and reattach the retina.

The contractile fibrous outgrowths of cells may be divided into two main types. Firstly there are the vascularised membranes which can result from ischaemic eye diseases such as retinal

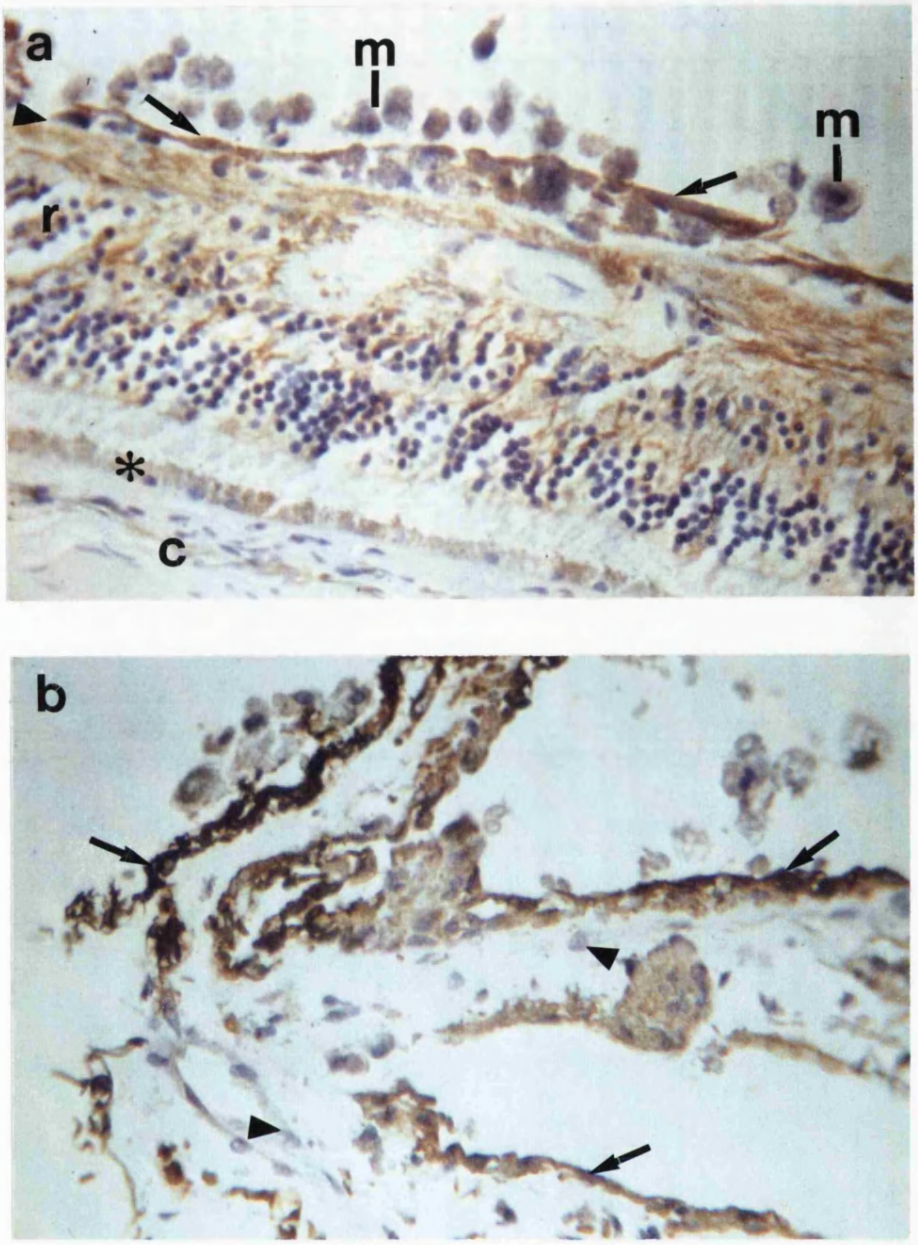


Fig. 1.5. Light micrographs (a) and (b) are stained by the immunoperoxidase procedure using antibodies against GFAP. The reaction product stains glial cells brown. The sections are counterstained with haematoxylin and eosin. The micrograph (a) shows a section of human retina with a simple ERM, consisting of large elongated glial cells (arrows) on the vitreal surface of the ILL (arrow heads). In addition numerous macrophages (m) are present in and around the membrane. The underlying retina (r), RPE layer (asterisk) and choroid (c) are undisturbed. The fibrocellular (complex) ERM shown in (b) has a substantial glial component (arrows) together with fibrous scar-like tissue (arrow heads) and other cellular constituents. Only glial cells are positive for GFAP. (Magnifications:- (a) x 300, (b) x 400).

venous occlusion and diabetic retinopathy. The damage to cellular elements sets up an inflammatory response followed by angiogenesis and scar tissue formation. Secondly there are the non-vascularised retinopathic membranes which are associated with a wide range of conditions (Foos, 1978; McLeod et al, 1987) and can be simple asymptomatic glial membranes or multicellular contractile scars. This study did not investigate vascular endothelial cell behaviour therefore its findings are directed towards understanding the formation of complex multi-cellular non-vascularised proliferations.

The conditions in which complex ERMs will appear and tractional retinal detachment occur (Fig. 1.6.), have been defined as proliferative vitreoretinopathy (PVR) by the Retina Society Terminology Committee in 1983 (Hilton et al, 1983). The definition was based on the what was felt to be the main disease process, namely proliferation and its location, the vitreoretinal surfaces. Until that time, the condition had been variously described as massive vitreous retraction (MVR; Cibis, 1965), massive preretinal retraction (MPR; Tolentino et al 1967; Schepens, 1967) and massive periretinal proliferation (MPP; Machemer and Laqua, 1975), each term reflecting the changing ideas as to the structural basis of the disease process.

In addition the committee identified four main stages in the development of ERMs from the initial inflammation to the final scarring and complete retinal detachment (Fig. 1.7.). This classification has subsequently been modified to accommodate progress in the understanding of the disease (Lean et al, 1989; Machemer et al, 1991). If the condition is untreated or if surgical intervention is unsuccessful, the disease progresses until the neurosensory retina is completely detached by the contraction of the scar tissue, except in the regions of the ora serrata and optic nerve head and it appears funnel or cone shaped. The end result is a blind painful eye full of contracted scar tissue.

Complex ERMs are essentially fibrocellular in composition and the cellular components of the proliferations are thought to consist of non-neural elements from the retina together with fibroblasts from an, as yet, unknown source (Newsome et al, 1981; Grierson et al, 1987). The vitreous in all probability, as well as providing a scaffold for the further development of complex membranes, acts as a major source and store for many of the stimulating factors that attract cells to the site of membrane formation.

Complex non-vascularised ERM formation	
Condition	**References**
Rhegmatogenous retinal detachment (RRD).	Kampik et al, 1981 Michels, 1982
As a complication of surgery for RRD.	Rachal and Burton, 1979
Idiopathically, often from simple glial membranes.	Foos, 1978

Fig. 1.6. The main conditions giving rise to complex non-vascularised ERMs.

Grade	Name	Clinical signs
A	Minimal	Vitreous haze, vitreous pigment clumps.
B	Moderate	Wrinkling of the inner retinal surface, rolled edge of retinal break, retinal stiffness and vessel tortuosity.
C	Marked	Full thickness fixed retinal folds, first in one then in all four quadrants of the posterior eye cup.
D	Massive	Fixed retinal folds in four quadrants and a funnel shaped retinal detachment which becomes progressively more closed until the optic nerve is no longer visible.

Fig. 1.7. The classification of retinal detachment with PVR as defined by the Retina Society Terminology Committee in 1983.

The behavioural characteristics of the cell types found to be present in complex ERMs have been investigated in this study. With an ultimate aim to provide knowledge which will help in future attempts to halt the progression of the membranes and to prevent their development, by therapeutic or pharmacological means. Initially pharmacological treatments are likely to be an adjunct to surgery but ideally we should aim to provide an alternative to surgical intervention.

1.1.5. A Comparison of Complex ERM Formation with Wound Healing

Formation of an ERM between the peripheral vitreous and the inner surface of the retina can be regarded as a maladaptive wound healing response, due to the disruptive effect of inflammation and scar formation on the surrounding tissue. In many sites of the body these two processes are essential to restoring homeostasis and preserving life but within the eye they have a gross deleterious effect on the fragile architecture of the vitreal cortex and the neurosensory retina. (Gloor et al, 1975; Cleary et al, 1981; Weller et al, 1990).

Wound healing has been described as "a frustrated attempt by the organism to recapitulate selected developmental events: first, to re-establish tissue integrity and secondly, to re-establish tissue function" (Davidson and Broadley, 1991). Wound healing is one of the homeostatic control mechanisms by which multicellular organisms maintain their viability. The process of wound healing can be divided into three main overlapping phases (Peacock, 1984; Tahery and Lee, 1989; Borel and Maquart, 1991; Hunt et al, 1991); these are:-

1. Inflammation.
2. Cellular proliferation.
3. Scar formation and remodelling.

The phases take place in all injury responses to a greater or lesser extent, dependent on the type and extent of the tissue disruption and the site in the body in which wound healing occurs. Useful parallels can be drawn from the large body of literature on the cellular and pharmacological events taking place in the healing of epidermal wounds and also in

developmental processes such as morphogenesis. To some extent these comparisons help to clarify the mechanisms which contribute to ERM formation (Weller et al, 1990; Kovacs, 1991) where the inflammatory response coupled with the development and contraction of fibrous tissue impairs rather than restores the normal functioning of the particular tissue or organ.

The events occurring in ERM formation resemble wound healing, especially that which occurs in the skin and other tissues. The main differences are a lack of clot formation, no granulation tissue and the absence of vascularisation in ERM formation. In skin wounds there is a short initial wave of proliferation but in ERMs cell division continues well into the postcontractile phase of membrane formation (Hiscott et al, 1985). Finally in skin wound repair there are six main cellular elements (Peacock, 1984; Weller et al, 1990) as follows:-

1. Platelets.
2. Neutrophils.
3. Mononuclear phagocytes.
4. Fibroblasts.
5. Endothelial cells.
6. Keratinocytes.

In ERMs although all the participating cells are not exactly known, the elements that appear important are inflammatory cells, fibroblastic cells from an unknown derivation and cells which have migrated from the retina (see Sections 1.2.1 - 1.2.4.).

1.2. Cell Types Found in Complex ERMs

The importance of cellular involvement in ERMs was recognised in the latter half of the 19th century and since then, many different cell types (in fact all the possible ones from the neighbouring tissues) have been implicated in their formation (reviewed by Hiscott, 1986). The use of experimental animal models by Machemer and coworkers (Laqua and Machemer, 1975), light and electron microscopical analysis of surgical specimens (Kampik et al, 1981) and the more recent development of immunohistochemical techniques (Hiscott et al, 1985), has led to the general acceptance that the types of cells observed can vary

according to the different types of PVR and generally include inflammatory cells as well as retinal glia, RPE and fibroblastic cells (Newsome et al, 1981; Grierson et al, 1987; Fig. 1.8.).

1.2.1. Inflammatory Cells

Inflammation appears to play an essential role in the initiation and early stages of ERM formation (Hiscott et al, 1988; Gilbert et al, 1988; Weller et al, 1989). Damage to the retina can precipitate extravasation of inflammatory

cells (Hiscott et al, 1985) and auto-immune processes involving retinal elements can also promote the inflammatory process (Grisanti et al, 1991). Macrophages (Kampik et al, 1981; Hiscott et al, 1985; Miller et al, 1986) and T lymphocytes (Charteris et al, 1992) have been reported in some ERMs. Platelets, when infused into the vitreous of the rabbit and owl monkey, can trigger inflammation leading to formation of ERMs (Constable et al, 1975).

Macrophages are essential cells in reparative processes. They perform the functions of phagocytosis and secretion of myriad factors amongst which are platelet derived growth factor (PDGF), fibronectin, transforming growth factor beta (TGFβ), (reviewed by Nathan, 1987). Macrophages have been found to be present in both early and late ERMs in the rabbit as well as the human (Gilbert et al, 1988). Such macrophages might be derived from the following sources:-

1. The blood.
2. Vitreal hyalocytes.
3. The microglia of the retina.

Blood borne macrophages could enter the vitreal cavity as monocytes infiltrating from damaged retinal blood vessels (Cleary and Ryan, 1979; Weller et al, 1990), the ciliary vessels (Grierson and Forrester, 1980), or the choroidal vasculature (Johnson and Foulds, 1977). Some hyalocytes are macrophage-like in appearance and behaviour and possibly of either blood or microglial origin (Grabner et al, 1980; see section 1.3.5.). Retinal microglia are thought to belong to the fixed tissue macrophage group of cells and are phagocytic in function (Gilbert et al, 1988).

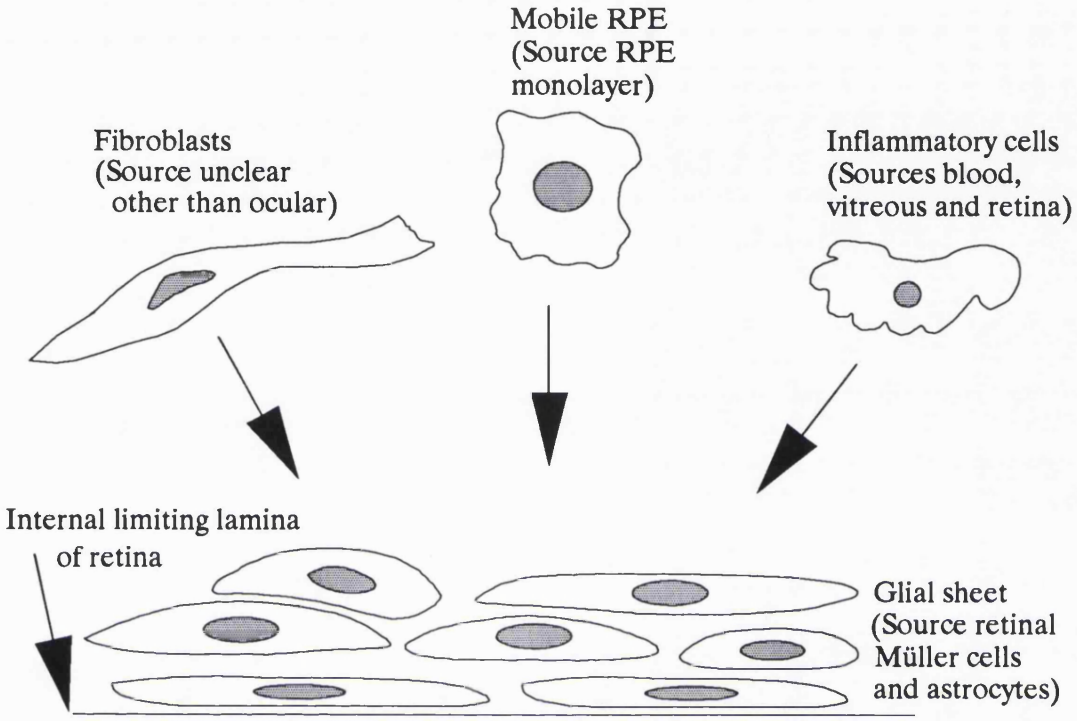


Fig. 1.8. Schematic diagram demonstrating the main cell types implicated in the formation of a complex ERM.

1.2.2. Glial Cells

The retinal glia, as already stated in section 1.1.2., are a heterogenous population of cells, comprising three main types in the human retina; the microglia, Müller cells and astrocytic cells. The possible role of the microglia in ERM formation as tissue histiocytes, has already been mentioned in section 1.2.1. Both the Müller cells and astrocytes are believed to play an important structural role in membrane formation, but due to lack of positive distinguishing characteristics there is still controversy as to their relative prominence in complex ERMs (Grierson et al, 1987; Hjelmeland and Harvey, 1988). On the other hand they are along with a few inflammatory cells the sole constituents of simple ERMs.

It was originally held that astrocytes were the major glial cells in complex ERMs (Foos, 1977) and this identification was based on morphological criteria. The cells had oval to spindle shaped nuclei, many processes and abundant intermediate filaments in their cytoplasm. However there were also less differentiated cells of glial types as well. Now there is increasing evidence that Müller cells may be just as important, if not more so, than astrocytes. They are not only known to be involved in the earliest stages of membrane formation by extending their processes through deficits already present in the ILL but studies in rabbit animal models have also shown that Müller cell processes can force their way through intact regions of ILL (Hitchins et al, 1985) and Müller cell nuclei have been shown to incorporate ^{3}H thymidine in the detached retina and under epiretinal proliferations. Cultures of retinal Müller cells adopt a similar phenotype to the cells in glial membranes. The rabbit retina, though it is largely without astrocytes, forms similar ERMs to humans (Schnitzer, 1985; Hitchins et al, 1985 and 1988). Finally the glial component of subretinal membranes is unlikely to be anything other than of Müller cell origin, as the astrocytes are confined to the inner layers of the retina and the subretinal cellular components have a flattened plate-like appearance, comparable to glial sheets on the ILL.

The intermediate filament vimentin is expressed by Müller cells and retinal astrocytes however it is present in many other cell types including fibroblasts (Ben Ze'ev, 1986; Issacs et al, 1989) and RPE (McKechnie et al, 1988) and is therefore of no value as a marker of cell type. GFAP appears to be specific to glial cells and is expressed by astrocytes (Hiscott et al, 1984)

and by Müller cells in retina which has suffered trauma (Bignami and Dhal, 1979; Hiscott et al 1984; Erickson et al, 1987). Thus GFAP is a useful marker for epiretinal glia which have been identified in ERMs (see Fig .1.5.). Although using antibodies to GFAP has identified glial cells as a major component of complex ERMs (Grierson et al, 1987) it does not distinguish between astrocytes and Müller cells.

Simple glial proliferations (simple ERMs) are asymptomatic and almost transparent but they can become complex ERMs, which are fibrous and contractile, by acquiring a fibroblastic element (Hjelmeland and Harvey, 1988). However the mechanism by which the "complexing" takes place is obscure and the origins of the fibroblastic elements is in doubt. It is possible that the glial component (Hiscott et al, 1985) may act as a cellular scaffold for the fibroblast-like cells. It has been considered by some that the glia may adopt a fibroblast-like phenotype, however the substantial fibroblastic component in ERMs stains negatively for GFAP so this would rule out fibroblast-like glia unless true metaplasia had taken place.

The glial cells may also be involved in cell-cell signalling which induces migration and proliferation of fibroblast and/or RPE cells. This intercellular communication may either take the form of direct cell to cell contact with fibroblasts and RPE (Puro et al, 1989), or by autocrine and paracrine modulation through the influence of soluble mediators secreted by the glia (Burke and Foster, 1985; see also Section 1.5.).

1.2.3. The Retinal Pigment Epithelium

If a simple rhegmatogenous retinal detachment is not successfully treated then changes take place in the RPE under the detachment. The RPE enter the cell cycle (Anderson et al, 1981), detach from their basement membrane and adopt a "macrophage" or "fibroblastic" phenotype (Machemer and Laqua, 1975; Johnson and Foulds, 1977). The proliferating RPE can migrate into a developing ERM as PVR develops Machemer et al, 1978). Machemer and co-workers in the 1970s induced retinal detachment in the owl monkey eye and observed "pigment-containing macrophages" migrating from the RPE monolayer and rapidly multiplying to eventually form clusters of cells on the exposed surfaces of the retina and vitreous in some of their experimental detachments.

Groups of fibrocyte-like cells, also assumed to be RPE in origin, were seen. The authors assumed that the RPE underwent "metaplasia" to change to a macrophage or fibroblast-like cell. However subsequent studies by Vidaurri-Leal (1984) and others (Müller-Jensen et al, 1975; Kirchof et al, 1988; Vinores et al, 1990) have since shown that cultures of hexagonal RPE change to an elongated fibroblast-like cell when overlaid with vitreous or collagen gel. A similar situation occurs in the embryo, known as epithelial mesenchymal transformation, where epithelial cells undergo a reversible transformation to a mesenchymal phenotype and migrate to a different location (Vallés et al, 1991). Cultured RPE in a polarised mosaic culture, when placed in the middle of a collagen gel, will take up the fibroblastic phenotype and once they have migrated to the surface of the gel will revert to a discoid shape and if the cell population is dense enough will form a mosaic again (Mazure et al, 1992).

The macrophage-like form of the RPE is also active and migratory and exhibits non specific phagocytosis. Exhibition of the three different phenotypes appears to depend on the surrounding environment. The RPE adopt a macrophagic form in fluid aqueous environments such as the subretinal fluid (SRF) and the vitreous, a fibroblastic form in three dimensional matrices such as formed by collagen and in sparse cell numbers on substrates such as fibronectin and an epithelioid form in densely populated regions (Grierson et al, 1994). Immunocytochemical techniques employing antibodies to cytokeratins (Mc Kechnie et al, 1988) have confirmed the presence of different phenotypes of RPE in ERMs, ranging from cuboidal to spindle shaped (Hiscott et al, 1984).

Involvement of RPE in PVR appears to depend on the type of membrane and on its location. Animal models of PVR indicate that RPE play a dominant role in membrane formation (Machemer et al, 1978) and this is true for subretinal membranes (Hiscott et al, 1991). However investigations on human surgical specimens show that on average, only 25% of the cellular content of the ERMs stained for cytokeratin (Grierson et al, 1987), occasionally the membranes consisted of 90% RPE but much more frequently the RPE are only present as scattered cells with large groups of fibroblast-like cells that are negative to cytokeratins and GFAP (Hiscott et al, 1983). The origin of these negatively staining cells is still obscure and is discussed in Section 1.2.4.

1.2.4. Fibroblasts and Fibroblastic Cells

The fibroblast is the activated form of the fibrocyte (Kang, 1978) and is mesenchymal in origin. The resident fibrocyte is a stationary quiescent cell found in small numbers in connective tissue, especially around blood vessels (reviewed by Grierson et al, 1988). Ocular fibrocytes are known to be present in Tenon's capsule (Joseph et al, 1988) and the corneoscleral and uveal coats (Hogan et al, 1971). They are also possibly present in the vitreous (Balaz, 1964; Sebag, 1991), the adventitia of the ocular blood vessels (Grierson et al, 1987) and in the optic nerve region (Newsome et al, 1981).

Fibroblast morphology in vivo depends to some extent on their surrounding environment. When surrounded by bundles of collagen fibres they are elongated fusiform bipolar cells, when the substrate is less uniform they may appear as flattened stellate cells with slender cytoplasmic processes (Bloom and Fawcett, 1975). Their electron microscopic appearance is that of an actively synthesising and secreting cell, in that the cytoplasm contains prominent rough endoplasmic reticulum, numerous Golgi and mitochondria and the nucleus is relatively large, lightly staining with multiple nucleoli (reviewed by Dunphy, 1963; Ross, 1968). In culture actively moving fibroblasts in sparse population on a flat substrate take up a flattened kite shaped appearance (reviewed by Abercrombie, 1978).

Fibroblasts become activated early in the response to tissue injury and stimulation can result from the presence of denatured extracellular materials and various factors secreted by macrophages and inflammatory cells. Activated fibroblasts migrate towards the focus of injury and undergo mitosis and synthesise extracellular matrix proteins (Grierson et al, 1988).

Cells with a fibroblastic phenotype and mode of action have long been known to be present in ERMs (Parsons, 1905), together with sub-groups of cells known as myofibroblasts (Grierson et al, 1987; Smiddy et al, 1989; see also section 1.3.6.) which display smooth muscle like intracellular organelles (Gabbiani, 1981). In PVR that arises as a complication of perforating injury the fibroblasts are recruited from the choroid and sclera into the periretinal region and form part of the contractile scar tissue (Constable et al, 1974; Winthrop et al, 1980). However in idiopathic membranes where Bruch's layer remains intact the origin of the fibroblast-like cells remains a subject for debate. The problem is that there are no easily identifiable adventitial

fibrocytes associated with the retinal vasculature. There are a number of possible candidates whose eligibility is based on their anatomical proximity to the vitreoretinal region and to morphological and behavioural characteristics which they share with true fibroblasts (reviewed by Grierson et al, 1987). The likely candidates are retinal glia, RPE, macrophages and the cells of the vitreous, also the endothelium, pericytes and smooth muscle cells of the retinal vascular system.

The retinal glia and/or RPE have been considered to be likely contenders for adopting a fibroblastic role in complex ERMs (Machemer and Laqua, 1975). There is as yet no direct immunohistochemical evidence for either cell type acting as a fibroblast or precursor cell in ERMs, unless it is possible that their phenotype is radically altered (see also sections 1.2.2. and 1.2.3.). Unfortunately fibroblast-like cells to date have no specific cell markers and have only been distinguished in ERMs by their lack of staining with monoclonal antibodies to the intermediate filaments GFAP and cytokeratins (Grierson et al, 1987).

Hyalocytes have been described by Harada and coworkers (1981) as being components of ERMs. They are known to adopt several different phenotypes in their normal location in the cortical vitreous (Sebag, 1992). They have also been demonstrated to secrete glycosaminoglycans but not collagen (Grierson et al, 1987) and are very sparse with no obvious replicative capacity. Due to their secretory and phagocytic properties in culture they are thought to form part of the tissue monocyte/macrophage system and are thus more likely to be activated to form terminally differentiated macrophages rather than fibroblast-like cells (Grabner et al, 1980).

In the classic literature on wound healing, one of the possible sources of fibroblasts has long been thought to be mononuclear phagocytes from the blood (Petrakis et al, 1961) and macrophages are known from animal studies (Hitchins et al, 1985) and human pathologic material (Kampik et al, 1981) to be associated with ERMs (see section 1.2.1.). However there has been no evidence to date that macrophages can become fibroblasts (Grillo and Potsaid, 1961) and their role in ERM formation is most likely to be that of modulating the proliferative behaviour of the constituent cells (Grierson et al, 1987).

The retinal vasculature is also a potential source for the fibroblastic cells in ERMs. Not only are endothelial and smooth muscle cells capable of migration, proliferation and differentiation in response to chemical stimuli but also the

multipotential pericytes divide in response to injury and are known to be contractile (Kelley et al, 1987). Endothelial cells can be identified by locating Factor VIII in their cytoplasm. When used on sections of ERMs, antibodies against Factor VIII picked out the few organised vessels which were present but nothing else (McLeod et al, 1987). Some workers have attempted to identify α actinin and desmin in the cells of ERMs (Shirakawa et al, 1989; Weller et al, 1992) but these markers for smooth muscle cells have not provided clear cut evidence that smooth muscle cells are a substantial component of ERMs. Pericytes have been distinguished from endothelial cells in culture with antibodies to isoforms of muscle and non-muscle actins, α actinin and 3G5 (Herman and D'Amore, 1985; Capentandes et al, 1990). Pericytes in common with smooth muscle cells express enzymes such as cyclic GMP-dependent protein kinase (Hogan et al, 1971). However pericyte identification and localisation in ERMs has not yet been published so it is not known whether they are present in any great numbers.

Finally it is possible that the fibroblast-like cells in idiopathic ERMs are indeed true fibroblasts from nearby sources such as the cortical vitreous, the adventitia of retinal blood vessels or the optic nerve region. It is believed by some that the vitreous contains a small population of fibrocytes as well the hyalocytes (Sebag, 1992) (see section 1.1.3.). However there is little evidence for their ability to respond rapidly to injury stimuli by proliferation, as is normal for fibroblasts. For example whole blood was introduced into the vitreous of rabbits (Lean, 1987) and resulted in the proliferation of glial cells but not fibroblasts.

Connective tissue fibrocytes are known to be closely associated with the outer regions of blood vessels and though none as yet have been identified in the retinal area, their presence may have been obscured due to the dense cellularity of that region. The retinal blood vessels have been found to be closely associated with ERMs so that cells such as fibrocytes, if situated in the vessels' outer walls would be in an ideal position to infiltrate the membrane (Grierson et al, 1987). Other authors have suggested a potential mesenchymal source from the optic nerve region (Newsome et al, 1981) and fibroblasts certainly can come from this region in the rabbit to form complex ERMs alongside RPE and glia (Hiscott et al, 1988).

1.3. Cell Behaviour during Complex ERM Formation

The sequence of cellular events in the pathogenesis of ERM formation can be summarised as follows:-

1. Activation of cell types such as macrophages, retinal glia and RPE following inflammation and/or retinal damage.
2. Migration of retinal glia, RPE and fibroblast-like cells to the vitreoretinal region.
3. Cellular adhesion, settlement and invasion.
4. Proliferation.
5. Contraction of the cellular membrane.
6. Synthesis of an extracellular matrix.
7. Remodelling and contracture of scar tissue.

(Hilton et al, 1983; Verdoorn et al, 1986; Gilbert et al, 1988; Grierson et al, 1988).

In the following paragraphs all the aforementioned activities are outlined briefly. Formation of ERMs is course, far from being the simple sequence of biological processes listed above. Migration of the activated cell types to the vitreoretinal interface is believed to be one of the initial processes in membrane development. However it must be borne in mind that retinal glia, RPE and fibroblast-like cells are all anchorage dependent and have to be already adherent to a surface for migration to occur. Also the retinal glia display what can be termed as initial invasive behaviour as they migrate out from amongst the neuronal cells (through the intact ILL in some cases) and into the vitreal region. There is also evidence from animal models that the RPE migrate through the intact neurosensory retina, as well as through full thickness retinal tears, to the vitreal surface (Hitchins, et al 1988).

In the present study the emphasis of the research is on cell migration, settlement, invasion and proliferation. Relatively little is known about these cellular activities in the context of ERM formation and they present promising target areas for therapeutic agents. A halt to these would hopefully prevent the later more damaging processes of membrane contraction, extracellular matrix synthesis and remodelling and contracture of scar tissue.

1.3.1. Activation

In PVR, activation of the normally quiescent stationary retinal cells to an active motile state is thought to take place in response to an insult such as a retinal tear. This evokes a local inflammatory response and allows for the collection of fluid between the neurosensory retina and the RPE layer (see Fig. 1.9.), cytokines secreted into the fluid further stimulate the cells.

Müller cells and astrocytes are thought to be the first cells to be activated. Studies on animal models (Laqua and Machemer, 1975; Foos and Gloor, 1975) have shown glia to hypertrophy and accumulate intermediate filaments in response to pathogenic stimuli. When, for example bacterial endotoxin was injected into the rabbit vitreous body, a vigorous inflammatory response took place during which glial-like processes, staining positively for GFAP, were seen to extend through a hitherto intact ILL (Hiscott et al, 1988). Machemer and coworkers (1975) induced experimental retinal detachments in the owl monkey and the RPE showed a change in morphology from that of a polarised cell in the monolayer to that of a more macrophagic phenotype in response to the insult. Fibroblasts in the region of an injury show an increase in the number and types of cell surface receptors, microspikes and membrane ruffles together with a reorganisation of the cytoskeleton and an increase cytoplasmic organelles. Activated fibroblasts also exhibit a greater amount of euchromatin in the nucleus, which probably indicates a transition from the non-dividing G_0 state into G_1 stage of the cell cycle (Grierson et al, 1988).

1.3.2. Migration

With the progression of PVR, as in any wound healing response, cells are attracted to the area of inflammation from the surrounding tissues. In order to the reach the site their receptors are up-regulated and the cells become motile and migrate in response to a variety of chemoattractive stimuli (Verdoorn et al, 1986). As already mentioned, activated retinal glia migrate onto the inner surface of the retina and there is evidence that erstwhile stationary RPE migrate through the intact

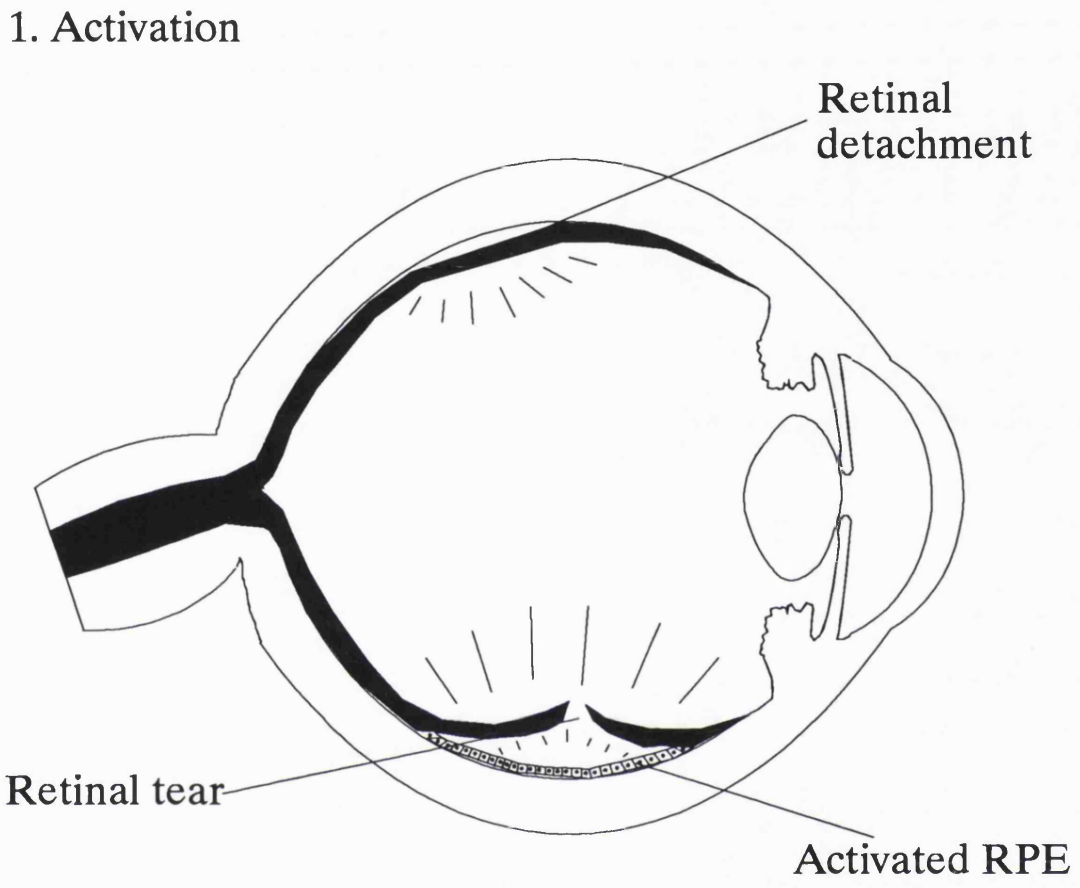


Fig. 1.9. Schematic representation of a mid-sagittal section of the human eye to illustrate activation in ERM formation.

retina (Hitchins et al, 1988; Fig. 1.10.).

The migration of the cell types present in ERM formation is of course one of the key factors in the accumulation of cells at the ectopic site and as such it would be an ideal area to target with therapeutic agents including those compounds which inhibit cellular locomotion. However first it is necessary thoroughly to investigate the migratory capabilities and susceptibilities of the cell types important in ERM formation. The migration of cells in response to substances produced during tissue injury is known as chemoattraction. The presence of chemoattractive substances in ERM formation is believed to be one of the mechanisms by which cells are attracted to the vitreoretinal region (Verdoorn et al, 1986). The chemoattractive migratory response has several likely components which have been identified by in vitro investigations.

1. Chemotaxis.
2. Chemokinesis.
3. Haptotaxis.

Chemotaxis is receptor mediated and involves directional movement of cells down a concentration gradient to a soluble stimulus. Chemokinesis is the random movement of cells to a soluble stimulus. Haptotaxis is the directional and random movement of cells to the excitatory molecules which are sequestered to a substrate so that the cells crawl along this (Lackie, 1986; Grierson et al, 1988; McCarthy et al, 1988). In the process of attraction of cells from the surrounding retinal tissues to the vitreoretinal interface there are likely to be potential chemoattractants attached to substrates such as the vitreous collagen as well as those present in soluble form in the extracellular fluids. Given this, all three migratory components are likely, to an extent yet to be determined, to influence the movement of cells in vivo. The present study has concentrated on the possible role of soluble stimuli on cell behaviour ie chemotaxis and chemokinesis.

The amount of chemoattractant required to produce a migratory response from a particular cell type can be measured accurately by means of a simple in vitro chemoattraction assay developed in the early 1960s by Stephen Boyden (1962). Boyden investigated the influence of antibody/antigen mixtures on the locomotory behaviour of polymorphonuclear leucocytes and to do this developed a precise method for quantifying the migration of cells to soluble chemoattractants. The technique involved the use of a polycarbonate chamber divided into two compartments by a

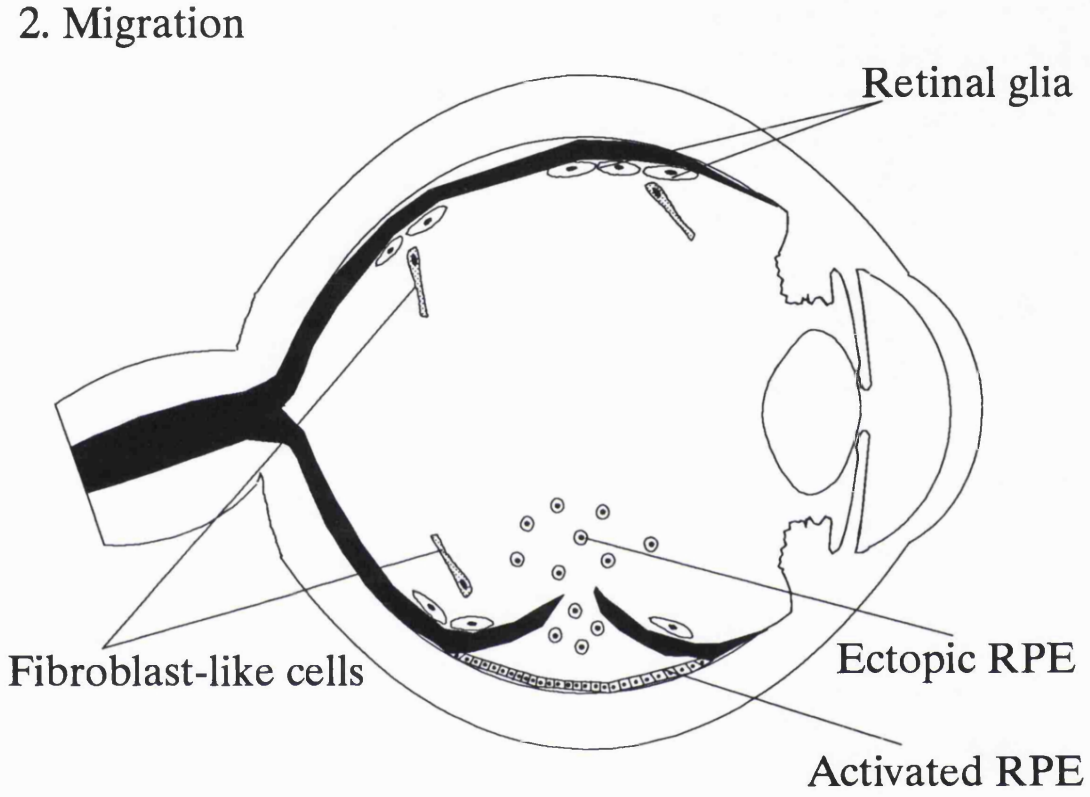


Fig. 1.10. Migration of the cells types in epiretinal membrane formation.

membrane with pores only large enough to allow cells to pass through by active migration. The cells were placed in one section of the chamber, the chemoattractant in the other and the stimulating effect was assessed by counting the number of cells that had migrated to the side of the membrane nearest the chemoattractant.

The single, open ended chamber system devised by Boyden, was later modified in many different ways. The microchemoattraction chamber used in these studies was developed by Falk and coworkers in 1980 and provided a set of 48 "mini" Boyden chambers with the lower half of each chamber closed to form a rounded well. The wells were set in apposing polycarbonate blocks and separated by a polycarbonate membrane. The array of 48 wells allowed different concentrations of one chemoattractant, different chemoattractants and/or different cell types to be assessed simultaneously under nearly identical conditions.

To some extent the initial migratory behaviour of glial cells for example which in vivo push their way through breaks (sometimes of their own making) in the ILL could be modeled in the chemotaxis chamber. For any cell type the migration process has not been studied in "real time" or time-lapse yet, due to the depth of the wells and the poor optics of the polycarbonate. Much of what we know about migration in chemotaxis chambers is either surmised from other migration assays or based on static analysis of scanning electron micrographs of cells caught in the act of migrating through polycarbonate membranes (Fig 1.11.).

The settled cell will ruffle its leading membrane and move slowly about the surface. Any pores contacted will be examined with exploratory pseudopodia. Microspikes are extended into the pore and sometimes retracted but if retraction does not take place then larger processes may push into the opening. Eventually the rest of the cell body will follow and the nucleus is often last because it is the bulkiest part of the cell. Microspikes and larger processes are extended onto the other surface of the membrane and the rest of the cell squeezes out of the pore (Calthorpe et al, 1990). The cells on the migrated side of the membrane adopt a much more spread out configuration than the cells on the upper surface (Robey et al, 1992).

The Boyden chamber system can be employed to assess migration on its simplest level. The cell is stimulated or not by the chemoattractant and migrates from point A to point B with the implication that there is little or no interaction between the cells which may influence their behaviour. However, in the formation of ERMs, there is more than one cell type involved and

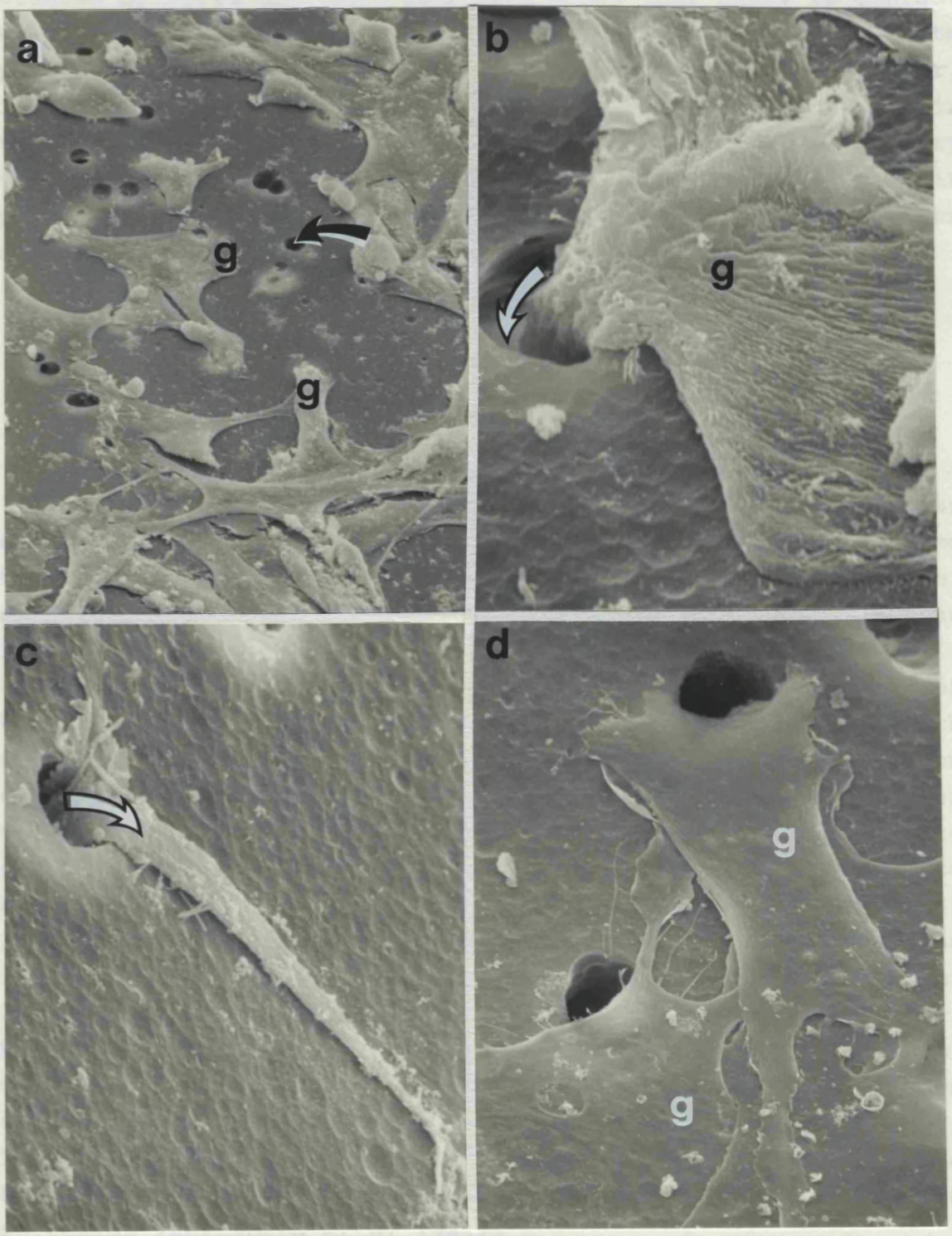


Fig. 1.11. Scanning electron micrographs of the settlement and migration of bovine retinal glia from the upper to the lower surface of a polycarbonate membrane through pores (curved arrows) 10 μm in diameter. Glial cells (g) are shown settled and spread on the upper (a) and lower (d) surfaces of the membrane and cytoplasmic processes are extended through the pores on the upper (b) and underside (c) of the membrane. (Magnifications:- (a) x 350, (b) x 2,000, (c) x 1,250 and (d) x 1,200).

studies by Burke and coworkers have shown that there is contact mediated interaction between the key cell types which affects proliferation and settlement (Burke, 1983; Burke and Foster, 1985). As a consequence, it is a more realistic situation to mix the cell types together and look at their combined migratory response and developments in the present study have made it possible to investigate migratory activity in the Boyden chamber assay.

1.3.3. Adhesion, Settlement and Invasion

Cell adhesion is a fundamental property of multicellular organisms (Grinnell, 1978). Strong adhesion is essential during the wound healing response for the migration, proliferation, contraction and synthesis of anchorage dependent cells (such as fibroblasts) to take place.

The process of adhesion is largely mediated by a family of transmembrane receptors known as integrins (Hynes, 1987) which are heterodimeric glycoproteins that link the cell cytoskeleton of one cell with another or with components of the extracellular matrix (Hogg, 1991). Cells can either adhere to each other (cell-cell adhesion) or to an underlying substratum (cell-substrate adhesion) (Ruoslathi, 1991).

Initial cell to cell adhesion involves the binding of the integrin receptors of one cell to cellular adhesion molecules (known as CAMs) on the membrane of another cell (Ruoslathi, 1991; Vallés et al, 1991). More permanent connections take the form of tight cell-cell interactions via cell junctional molecules (known as CJMs; Gherardi, 1991) and/or gap junction formation (Warner, 1988). Gap junctions allow exchange of ions between cells and therefore have within their domains inter-cellular linking molecules (Hertzberg et al 1981).

Cells adhere to an underlying substratum via the anchorage of integrin and other cell surface receptors to recognition sites on glycoproteins such as fibronectin, vitronectin, laminin and also collagen (Hook et al, 1988). Each extracellular matrix protein has a tripeptide arginine-glycine-aspartic acid (RGD) binding site (Ruoslathi and Pierschbacher, 1987). Certain integrin receptors will only bind to the RGD sequences of a specific adhesive molecule but others are less specific and will recognise groups of molecules. The integrins provide a flexible

signal recognition system which allows for anchorage, traction in migration and signals for polarity, position, differentiation and mitogenesis (Lévesque et al,1991).

Integrins have been found to mediate cell to cell adhesion in RPE (Chu and Grunwald, 1990) and cell-substrate adhesion in RPE and dermal fibroblasts (Chu and Grunwald, 1991). Integrin receptor distribution and regulation is important for a full understanding of settlement and adhesion mechanisms and $\alpha 5\beta 1$ the classical fibronectin receptor integrin has been located on the component cells of ERMs (Weller et al, 1991). In addition monoclonal antibodies against the $\beta 1$ integrin subunit have been shown to inhibit migration but not spreading of RPE in organ culture (Hergott et al, 1993). However integrin identification was considered to be outside the scope of this project which concentrated on the behavioural rather than molecular and structural aspects of cellular interaction.

Under culture conditions cells adherent to the surface of the substrate settle, flatten and spread. The cells undergo a transition from being contracted, free floating and non-motile to a spread configuration by which they are able to move along a rigid surface (Hook, 1988). When suspended in culture their cell surface, although seemingly smooth, displays small round bleb-like protrusions and relatively long thin projections known as filopodia or microspikes. Shown by immunostaining to be rich in actin and actin is essential for cell adhesion and locomotion (Middleton and Sharp, 1984).

Cell settlement is to some extent dependent on the underlying substrate and settlement onto a rigid substrate such as serum coated issue culture plastic follows a number of distinct stages (Fig. 1.12.). In Stage 1 the cell is still free floating and is contracted and rounded. However the outer surface of the cell is not smooth but covered in blebs and protrusions. In Stage 2, the cell makes contact with the underlying surface and attaches via non-specific receptors and the cells changes shape from rounded to a flattened and spread configuration (Hook, 1988). Flattened and spread cells in sparse culture have the look of a "fried egg" when seen by DIC (differentiation interference contrast) optics. On the other hand if seeding is at high density, then cell-cell interactions come into play so that the "fried egg" shape is not seen. At Stage 4, filopodia extend, touch and adhere to the substratum and form focal contacts, visible by reflective interference microscopy (Izzard and Lochner, 1976), which are in fact anchorage points for actin containing stress fibres and are linked to the extracellular

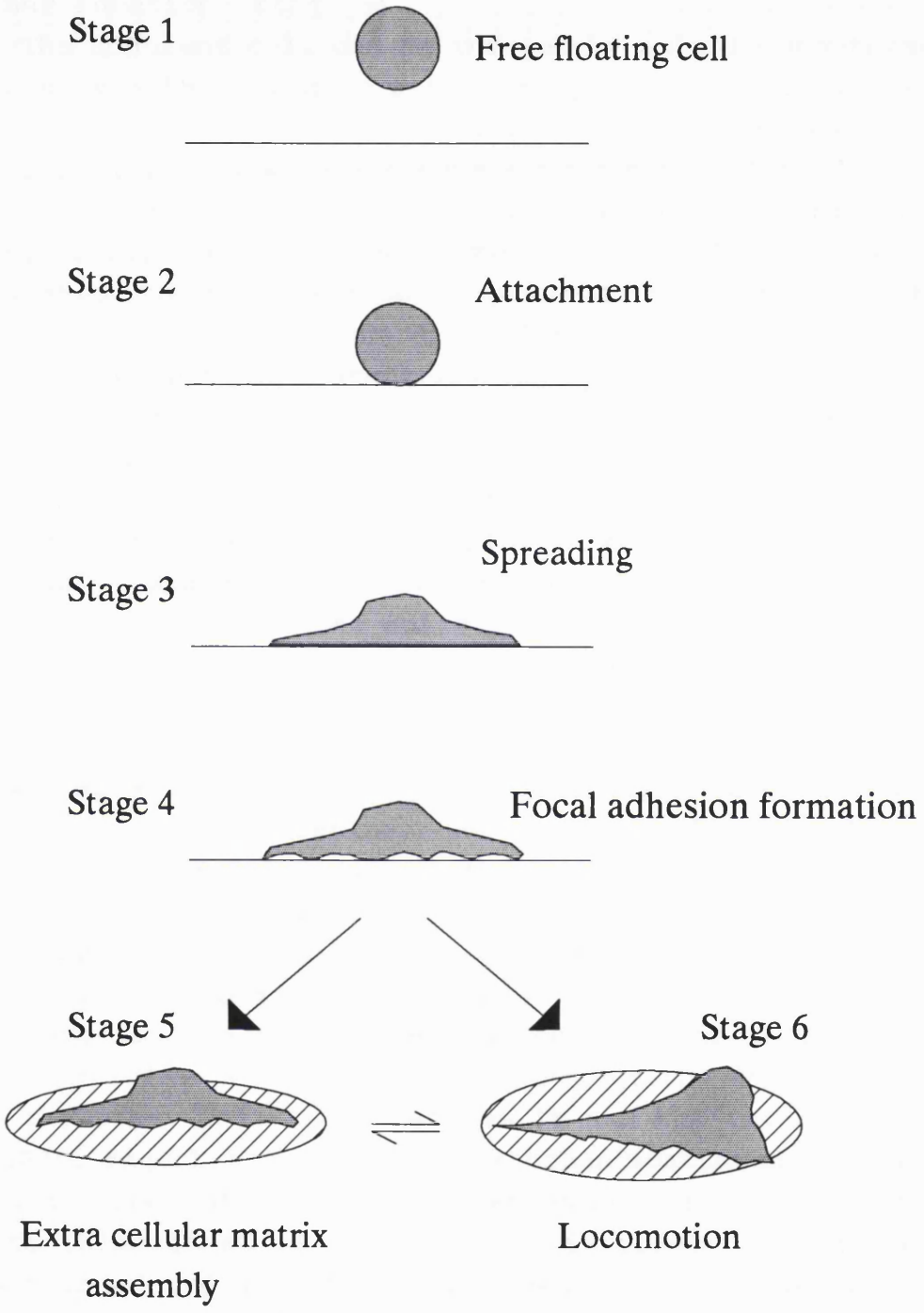


Fig. 1.12. Schematic representation of the different stages in cell-substrate adhesion and settlement on a rigid substrate (adapted from Hook, 1988).

matrix via intermediary proteins (Singer et al, 1989). The cell secretes extracellular matrix proteins and remain stationary (Stage 5) or under the influence of various stimuli migrate to another location (Stage 6).

The adherent cell can be induced by soluble growth/motility factors, via the integrin receptor system and the cytoskeleton, to change from a stationary cell to a motile one (Stage 6; Gherardi, 1991), displaying extensive membrane lamellae, ruffles and pseudopodial protrusions (Abercrombie, 1961; Middleton and Sharp, 1984; Oster, 1988). The motile cell can be further stimulated to move and when present on a flat surface to continuously spread in one direction (Vasiliev, 1982).

In the in vivo state the underlying substrate is unlikely to be flat and rigid but is more likely to be a mixture of basement membrane collagen combined with laminin and fibronectin (such as the ILL) or a three dimensional latticework of collagen fibrils (such as the cortical vitreous) or a scaffold of previously migrated cells. One of the potential cellular substrates in PVR are the epiretinal glia on the vitreal surface of the retina (Grierson et al, 1987), form layers of flat plate-like cells and are thought to act as a scaffold on which the other cell types form a complex ERM (Fig. 1.8. and Fig. 1.13.). The RPE as mentioned already are possibly carried to substrates such as the damaged retina, the glial scaffold or the collagen of the vitreous (Fig. 1.13.) as a part of the debris known as "tobacco dust" (Hamilton and Taylor, 1972; Machemer, 1978). This is possibly one of the vectors of spread of the RPE. Adhesion to and settlement on a substrate is necessary before migration and proliferation can take place. As the membrane increases in size the glial (GFAP positive cells) remain as the inner lining layer with the other cell types between it and the retina (Hiscott et al, 1885; Fig. 1.13.). The RPE are often scattered throughout the cellular mass of fibroblast-like cells, singly, as foci and sheets (Hiscott et al, 1984) so it is plausible that they have both invaded and infiltrated between the glial cells.

The term invasion in this context, is used to describe the incorporation of one cell type into the already established sheet of another cell type. Although disruptive to the delicate retinal tissue it is a natural part of the wound healing process and of course it is benign. It is not the same as the malignant invasion of underlying tissue by tumour cells. Both processes involve active migration of the invading cell and inhibition of contact inhibition of locomotion (as shown in normal monolayering behaviour) (Armstrong and Lackie, 1975). However the former

entails the migration of cells through a cellular substrate and integration of one group of cells with another and the latter involves penetration by neoplastic cells of their basement membrane and consequent infiltration and disruption of the underlying tissues (Liotta and Barsky, 1983).

Cell settlement in vitro is often assessed by estimating the number of cells settled on a substrate over a fixed time period (Herbst et al, 1988). Direct or indirect methods of estimation can be employed. Visual counting under a light microscopy is the most obvious direct assay and an indirect assay is to label the cells with a radioisotope and measure the resultant activity in a scintillation counter. Static settlement assays have limited value in assessing the interaction of one cell type with an underlying monolayer as they will only record the events in one time frame. Time-lapse filming of the interactive process is of more value because although the number of samples are small of necessity, it allows the observer to follow the entire progress of a single cell or group of cells over a prolonged time period. Procedures used to investigate the effects of cell density and age on the adhesion of lymphocytes, macrophages and smooth muscle cells to bovine aortic endothelial monolayers (de Bono and Green, 1983), have been adapted in this investigation to study the settlement and invasive behaviour of the cell types involved in the formation of complex ERMs.

1.3.4. Proliferation

Cells will often only exhibit a pronounced migratory response when they are actively dividing (Abercrombie, 1978) so that proliferation can be considered to be a combination of cell division and migration. Growth factors such as PDGF will stimulate both cell division and migration and this implies that the same receptors are involved for both processes. In PVR, proliferation of the cell types present is an important step in membrane formation (Machemer et al, 1978) as it leads to increase in bulk of the membrane and, combined with contraction and extracellular matrix synthesis, leads to disruption and dislocation of the neural elements of the retina (Fig. 1.14.). Cell division can be provoked by a variety of stimuli including inflammation and mechanical injury (Peacock, 1984) and both of these are present during PVR development (Gilbert et al, 1988).

3. Settlement and Invasion

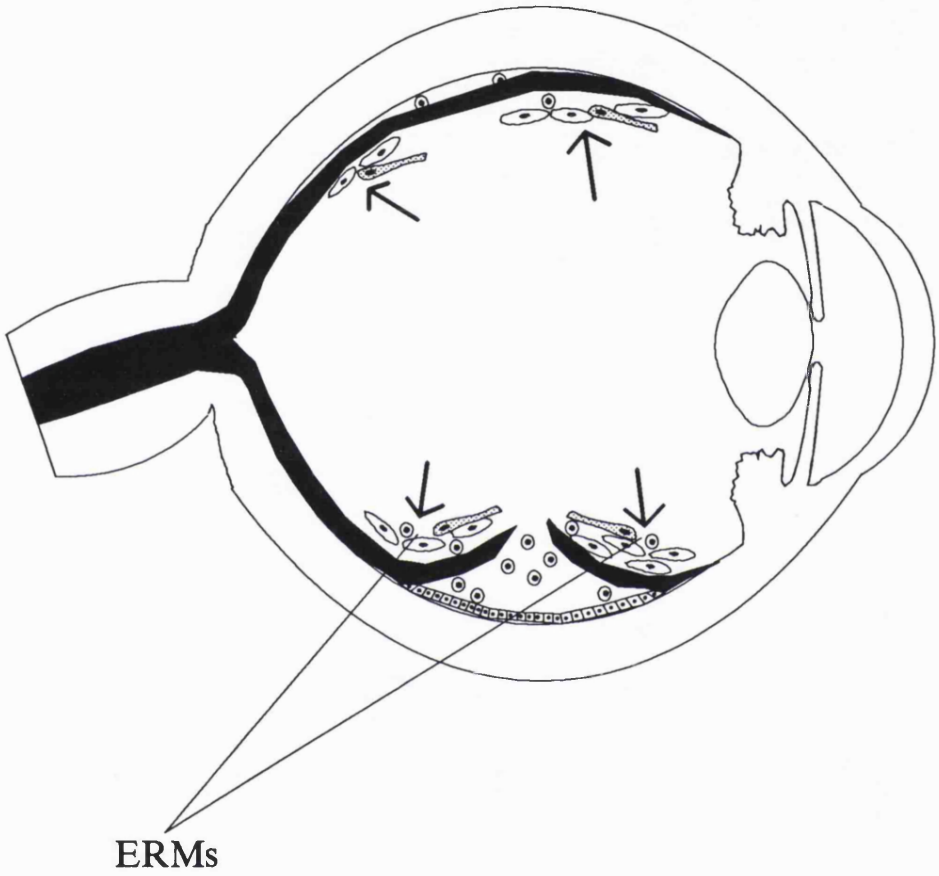


Fig. 1.13. Settlement and invasion (arrows) of the cell types in ERM formation.

Cell division in PVR is one of the key factors contributing to the advancement of the condition and in recent years much attention has been paid to developing antiproliferative drugs with a view to therapeutic intervention. Substances tested for controlling proliferation in PVR have included corticosteroids (e.g. triamcinolone), antimetabolites (e.g. 5-fluorouracil), compounds which disrupt the cytoplasmic microtubular network (e.g. colchicine) and naturally occurring fatty acids (e.g. sodium butyrate) which are differentiating agents (Grierson et al, 1988; Weller et al, 1990). The main problems associated with these agents have been systemic side effects, toxicity to the retinal tissue and difficulty in maintaining a therapeutic concentration at the vitreoretinal interface.

1.3.5. Contraction

Contraction of the granulation tissue in a healing wound was originally thought to be achieved by shortening of the collagen fibres but subsequent studies have shown it to be a cellular event, with the fibroblast as the chief protagonist (reviewed by Majno, 1979). Experimental studies on tendon healing led to the hypothesis that sub-populations of fibroblasts adopted the characteristics of smooth muscle cells and these groups of cells with prominent contractile microfilaments organelles were termed "myofibroblasts" (Gabbiani, 1981). It was also hypothesised that their active coordinated contraction resulted in wound closure. However, a large amount of the actin in myofibroblasts is aggregated into tight bundles called stress fibres (cables). Since the formation of stress fibres is a feature of cells in their non-motile phase (Middleton, 1984), it is now in doubt that their contraction produces the shortening. Myofibroblasts probably act more in the function of staples holding the wound together and in doing this they eventually become entombed in collagen and die (Hiscott et al, 1985; Grierson et al, 1988).

It seems more likely that the tractional forces are produced by the migration of the cells through the underlying substratum and this causes wound contraction (Lewis, 1984, Andujar et al, 1992). This view is supported by the experimental observation that migrating fibroblasts produce traction lines on thin sheets of silicone (Harris, 1980). Moreover time-lapse studies show migrating RPE pulling on collagen threads (Glaser, 1987) and

4. Proliferation

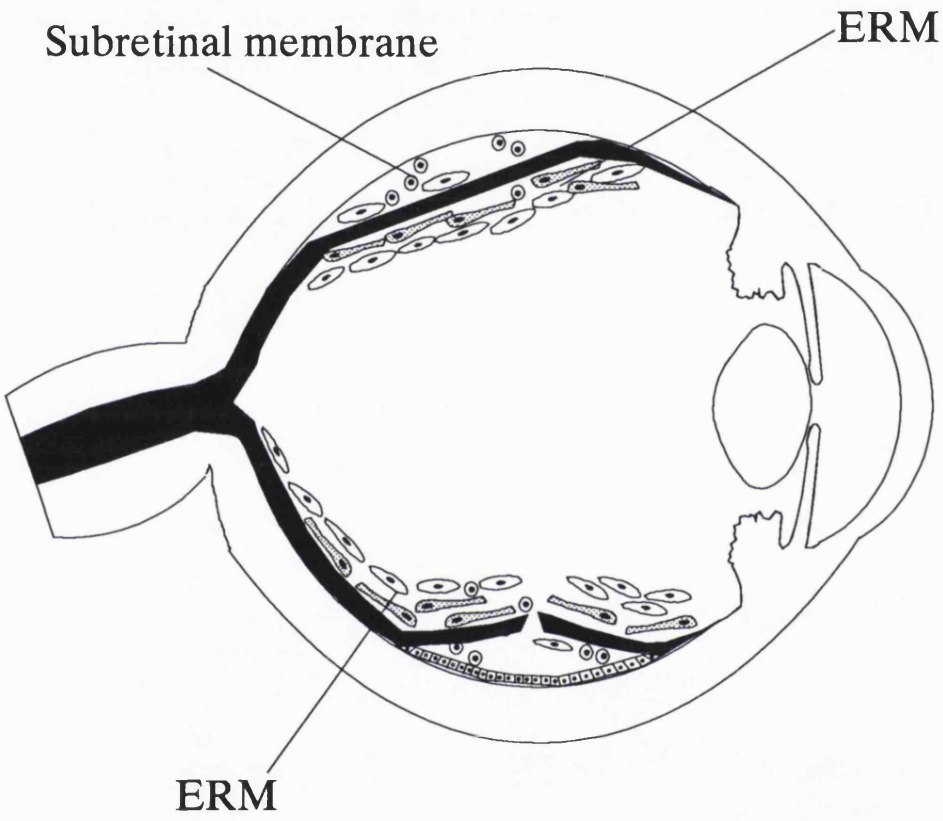


Fig. 1.14. Proliferation of the cell types in ERM formation.

fibroblasts doing the same to fibronectin threads (Grierson, 1988). In addition, seeding of fibroblasts and RPE into three dimensional collagen gels results in contraction of the gel as the cells migrate through it (Mazure et al, 1992).

Unlike the beneficial effect of contraction in a skin wound which closes an exposed cleft and promotes healing, an ERM in PVR causes tractional retinal detachment and puckering with subsequent death of the photoreceptors and ensuing loss of sight (Fig. 1.15.).

1.3.6. Synthesis

Synthesis of extracellular matrix molecules is an integral part of wound healing. The extracellular matrix provides a scaffold for adhesion, migration and of division of the constitutive cells (Fig. 1.16.). Synthesis commences towards the end of the inflammatory phase and is carried out in the main by fibroblasts under the influence of macrophages (Pollack, 1984). The actively synthesising fibroblast can be characterised morphologically by a cytoplasm rich in rough endoplasmic reticulum, Golgi vesicles, coated pits and vesicles, mitochondria and lysosomes.

Cells with features in common with synthesising fibroblasts are seen in ERMs (Grierson et al, 1987). extracellular matrix molecules found in ERMs include glycoproteins such as fibronectin, laminin, vitronectin and thrombospondin (Hiscott et al, 1985 and 1992, Weller, 1990), collagen types I, III, IV and small amounts of vitreal collagen type II (Morino et al, 1090; Weller, 1990) and elastic tissue precursors (Alexander et al, 1992) have been identified.

The fibronectins comprise a closely related family of dimeric glycoproteins with molecular weights in the range 400-500 kilo daltons (Akiyama and Yamada, 1987) and they are thought to play an important role in the pathogenesis of PVR (Wiedemann et al, 1988). Fibronectin is usually associated with the early stages of wound healing and unlike dermal wounds (where it disappears after a few weeks), it persists in ERMs for more than four months duration (Hiscott et al, 1985). Cellular fibronectin which consists of large insoluble molecules is known to be produced by retinal glia (Hiscott et al, 1992), RPE (Campochiaro, et al, 1986; Yamakawa et al, 1987) and fibroblasts in vitro

5. Contraction

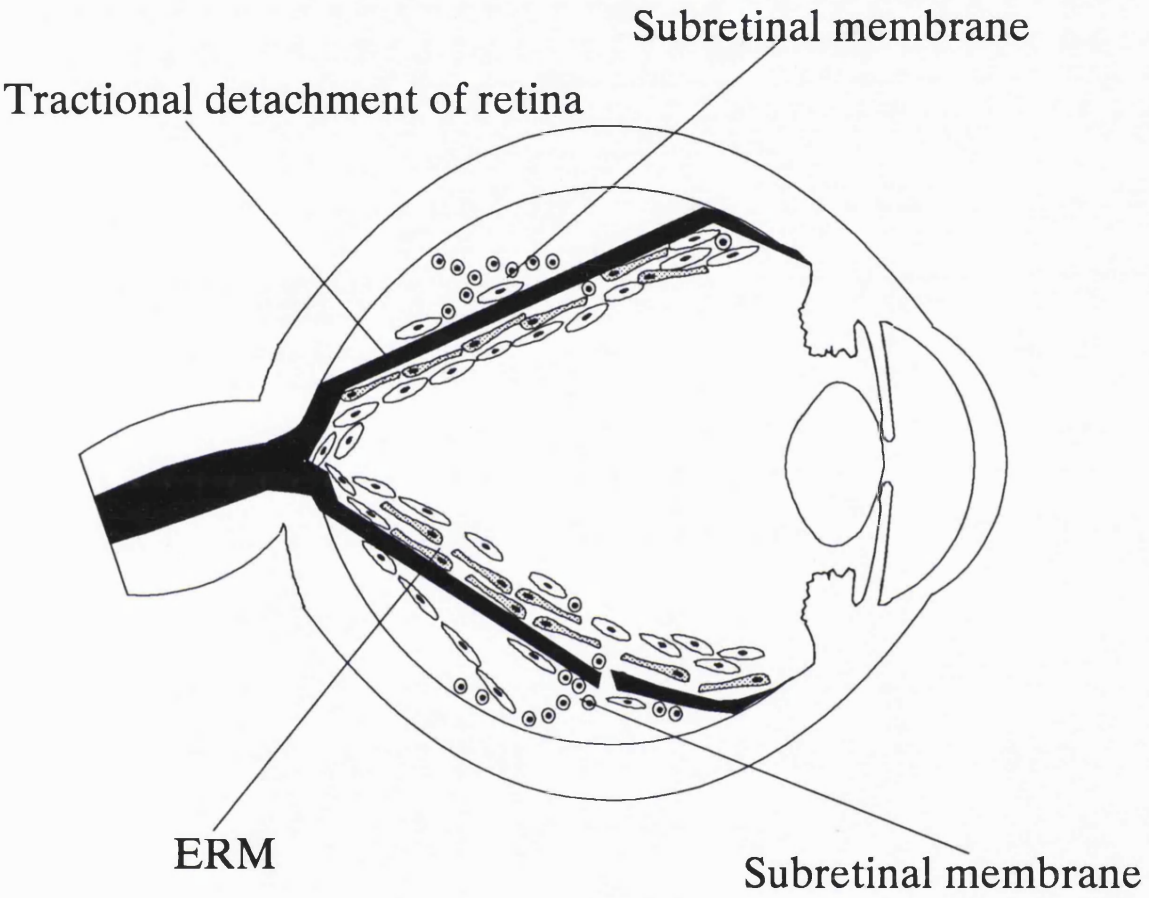
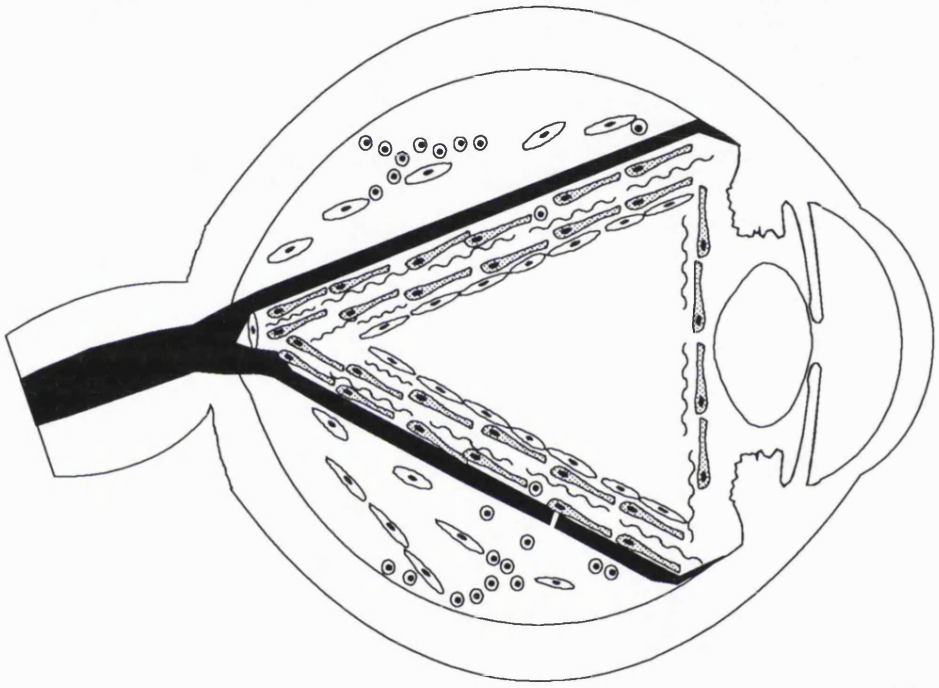


Fig. 1.15. Contraction of an ERM.

6. Synthesis



Extracellular matrix material

Fig. 1.16. Synthesis of extracellular matrix in ERM formation.

(Vaheri et al, 1976).

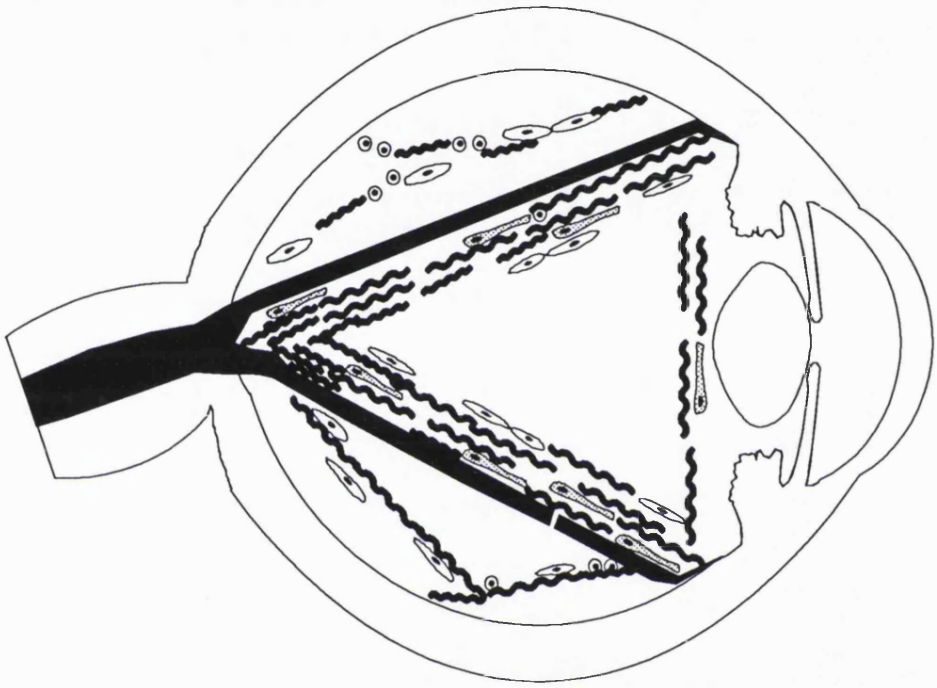
Retinal glia, RPE and fibroblastic cells have also been found to be associated with fibronectin in ERMs (Hiscott et al, 1985). Use of monoclonal antibodies that differentiate plasma fibronectin (which is a relatively small soluble molecule) from cellular fibronectin (a large insoluble molecule found on cell surfaces) have shown that both types are present in ERMs (Grisanti et al, 1993). In situ hybridisation studies have further demonstrated cellular fibronectin mRNA in all forms of ERM cell (Hiscott et al, 1992). The results suggest that ERM fibronectin has a dual origin;

1. from the retinal circulation by extravasation and
2. local production by all the cell types present in ERMs.

1.3.7. Remodelling

Ultimately in a healing wound, the bulk synthesis of extracellular connective tissue is complete and the remaining cells ie the fibroblasts decline in number. Even in ERMs where the proliferative phase is relatively prolonged, the waves of proliferation die down and histology and immunohistochemistry of late stage ERMs (four months or more duration) has shown a predominance of collagen with less cellular content than the earlier membranes (Morino et al, 1990). The cell types that persist into the later stages seem to be the glia as well as the fibroblastic cells rather than the RPE (Fig. 1.17.). The scar tissue undergoes contracture which is a late stage process rather than contraction which occurs relatively early in the wound healing process. Contracture is a collagen remodelling process which consists of dissolution and reformation of collagen with concurrent production of GAGs. The fibroblasts as well as producing collagen, can dissolve it with proteolytic enzymes such as collagenases and metalloproteinases (reviewed by Grierson et al, 1988).

7. Remodelling



Extracellular matrix material

Fig. 17. Remodelling of the ERM.

1.4. Factors Influencing Cell Behaviour in Complex ERM Formation

The different cells types involved in the development of ERMs, as in other wound healing responses, are thought to act in concert (Burke, 1983). It can be inferred therefore that they influence each others' behaviour by means of intercellular communication, the signalling either taking the form of direct transmission by cell-cell contact (Burke, 1989) or, indirectly, by the secretion of soluble mediators which evoke autocrine and paracrine stimulation.

1.4.1. Direct Signalling: Cell-cell Contact

Cellular proliferation and movement are known to be influenced by cell contact (Pontén et al, 1969; Middleton 1973; Bunge et al, 1979). Cell-cell contact stimulates DNA synthesis in subconfluent cultures of retinal glia and co-cultures of retinal glia and RPE but not in co-cultures with dermal fibroblasts (Burke, 1983). Coordination of processes such as capillary endothelial migration during new blood vessel formation (Pepper et al, 1989) are carried out by the passage of signalling substances via intercellular gap junctions. In addition gap junctions have been shown to be present between the RPE and the cells of the primordial neural retina during mammalian (Dr. Glen Jeffry, personal communication) and human (Fisher and Linberg, 1975) retinal development. However it is not known to what extent cell contact mediated interactions affect migration in ERM formation and although gap junction formation helps cell-cell communication it is only a part of the process of exchange of information. An aim of the present thesis is to study the effect of short term co-culture of glia, RPE and fibroblasts on their proliferation and migration in vitro.

1.4.2. Indirect Signalling: Factors in the External Environment

Factors that are considered to influence the migration and proliferation of the cell types involved in ERM formation are the peptide growth factors and extracellular matrix protein molecules. There are also other substances known as motility factors (not within the scope of this investigation) that promote locomotion of epithelial and mesenchymal cells (Warn and Dowrick, 1989). Cellular motogens and mitogens exert their influences on the affected cells through the activation of cell surface receptors and their targets can either be autocrine, juxtacrine or paracrine (Wiedemann, 1991).

One of the likely sources of these factors is the tissue fluid known as SRF that accumulates between the neurosensory retina and the RPE layer due to the break down in the RPE pumping system that draws fluid from the retina and vitreal region to the choroidal circulation. Another important source of stimulatory substances is likely to be products released by injured and dying neurons in the degenerating retina in the region of the retinal break. SRF and retinal extracts have been shown to stimulate migration and proliferation of retinal cells (see Fig. 1.18.) but the investigation remains incomplete and comparison of the relative rates of migration of the three main cell types has not been done.

One major factor distinguishing PVR from most wound healing responses, as stated earlier in this text, is that cellular proliferation in ERMs persists well into the postcontractile phase, which might indicate the presence of sub-populations of actively dividing cells or continuing cellular recruitment (Hiscott et al, 1985). That most ERMs do not become vascularised would tend to preclude the former explanation on the grounds that cellular activity in most wounds is dependent on an adequate blood supply. Continued recruitment seems more likely since the pathological vitreous as well as fluid from the subretinal space and retinal extracts are known to contain chemoattractants and mitogens that induce retinal glia and RPE to migrate and proliferate (Fig. 1.18.). In addition, media collected from cultures of retinal glia, RPE, fibroblasts, endothelial cells and pericytes has been shown to stimulate migration and proliferation of glia, RPE, ocular fibroblasts, retinal capillary endothelial cells and pericytes (see Fig. 1.19.).

Fluid/ tissue source	Target cell	Activity	References
Wash of bovine subretinal space	Bovine RPE and retinal glia	pro	Lemor et al, 1987
Human SRF	Human RPE	mig/pro	Hackett et al, 1988
Bovine retinal extract	Human RPE Rat brain astrocytes Rat retinal glia	pro pro mig/pro	Campochiaro et al, 1986 Bryan et al, 1987 Harvey et al, 1987
Bovine vitreous	Pig RPE Rat RG Bovine foetal dermal fibroblasts	pro mig pro	Wiedemann et al, 1985 Harvey et al, 1987 Raymond et al, 1982
Rabbit vitreous	Human RPE Rabbit RPE	mig/pro mig/pro	Campochiaro et al, 1986 Burke et al, 1987
Human:- vitreal aspirates	Rat brain astrocytes Human RPE	mig mig	Rowen et al, 1986 Campochiaro et al, 1985
vitrectomy specimens	Human RPE Rabbit RPE Human scleral fibroblasts	mig/pro mig/pro mig	Burke et al, 1985 Burke et al, 1985 Wilson-Holt et al, 1992

Fig 1.18. Reported observations when cultured cells are exposed to ocular tissues and fluids. * Activities indicated are migration (mig) and proliferation (pro). Retinal glia (RG). (Adapted from Burke, 1989)

Source of cell conditioned media	Target cell	Activity*	References
Rabbit retinal glia	Human RPE	pro	Burke et al, 1985
	Rabbit RPE	pro	Burke et al, 1985
	Bovine RPE	pro/mig	Burke et al, 1989
Human retinal pigment epithelium	Rat retinal glia	mig	Harvey et al, 1987
	Rat brain astrocytes	mig	Bryan et al, 1987 Rowen et al, 1985
	Rat brain astrocytes	pro	Bryan et al, 1986
	Human RPE	mig	Bryan et al, 1986
	Bovine corneal fibroblasts	mig	Rowen et al, 1985
	Bovine retinal capillary endothelial cells and pericytes	pro	Wong et al, 1988
	Bovine choroidal endothelium	pro	Morse et al, 1989
Bovine corneal fibroblasts	Rat brain astrocytes	mig	Rowen et al, 1985
Rabbit Tenon's fibroblasts	Rabbit Tenon's fibroblasts	mig	Joseph et al, 1987
Bovine retinal capillary pericytes	Bovine retinal endothelial cells	pro	Wong et al, 1987

Fig. 1.19. Reported migratory and proliferative responses of cultured cells when exposed to media collected from cultures of ocular cells. * Activities indicated are migration (mig) and proliferation (pro). (Adapted and extended from Burke 1989)

Peptide growth factors such as PDGF, the fibroblasts growth factors (FGFs), TGFβ and insulin-like growth factor one (IGF I) have long been implicated in ERM formation (Connor et al, 1988; Gaudric et al, 1988; Burke 1989; Leschey et al, 1990) and a number of these have been tested for their ability to stimulate the in vitro migration and proliferation of the cell types found in ERM tissue (as summarised in Figs. 1.20 and 21). RPE respond well to a variety of growth factors, PDGF, basic FGF and IGF I,(see Fig. 1.21.) which have been identified in extracts of RPE cells, retinae and vitreal samples and stimulate migration as well as proliferation (Burke et al, 1989). Retinal glia are responsive to a more limited range of factors, mainly PDGF and basic FGF. Ocular fibroblasts from the posterior segment have not been studied to date but motogenic and mitogenic effects of growth factors have been examined using fibroblasts from other parts of the eye and body (see Fig. 1.22.). They show migrational and proliferative responses to a similar range of growth factors as the RPE.

The extracellular matrix has always been recognised as having essentially a passive role as a structural scaffold for cells in tissues and organs in health and following injury. However it is now known to play an active role in essential processes such as cell activation, adhesion, migration and proliferation (Lévesque et al, 1991). extracellular matrix molecules affect migration and proliferation of the cell types found in ERM formation. While fibronectin has not been found to have mitogenic properties for the cell types in ERM formation (de Juan et al, 1988) it does stimulate the in vitro migration of RPE and fibroblasts (see Fig. 1.23.). Although cultures of Schwann cells from the central nervous system (Baron-Van Evercooren et al, 1982) and immature rat retinal glia (de Juan et al, 1988) have been stimulated to migrate by fibronectin, cultures of adult retinal glia tested so far appear to be insensitive (Hjelmeland et al 1988)(Fig 1.23.).

Retinal Glia			
Factor	**Species**	**Activity***	**References**
PDGF	Human	mig, +	Hjelmeland et al, 1987 Uchihori et al, 1991
	Human	pro, +	Uchihori et al, 1991
	Rat	mig, +	Harvey et al, 1987
	Rat	pro, +	de Juan et al, 1988
	Rabbit	pro, -	Burke et al, 1982
EGF	Rat	mig, -	Harvey et al, 1987
TGFβ	Rat	mig, -	Harvey et al, 1987
aFGF	Rat	mig, -	Harvey et al, 1987
bFGF	Rat	mig, -	Harvey et al, 1987
	Cat	pro, +	Lewis et al, 1991
	Rabbit	pro, +	Lewis et al, 1991
IL-1	Rat	mig, -	Harvey et al, 1987
IL-2	Rat	mig, -	Harvey et al, 1987

Fig. 1.20. The effect of peptide growth factors on the migration and proliferation of cultured retinal glia.

* Absence (-) or presence (+) of migration (mig) and/or proliferation (pro) activities. (Adapted from Burke 1984)

Retinal Pigment Epithelium			
Factor	**Species**	**Activity***	**References**
PDGF	Human	mig, +	Campochiaro et al, 1985
PDGF	Human	pro, +	Bryan et al, 1986
(+EGF, insulin)			Leschey et al, 1990
EGF	Human	mig, -	Campochiaro et al, 1985
	Human	pro, +	Bryan et al, 1986
			Leschey et al, 1990
	Bovine	pro, +	Hicks et al, 1991
AFGF	Human	pro, +	Leschey et al, 1990
	Bovine	pro, +	Hicks et al, 1991
BFGF	Human	mig, +	Campochiaro et al, 1985
	Human	pro, +	Leschey et al, 1990
	Bovine	pro, +	Hicks et al, 1991
IL-1	Human	mig, +	Kirchof et al, 1987
	Bovine	mig, +	Kirchof et al, 1987
IGF I	Human	mig, +	Grant et al, 1990
	Human	pro, +	Grant et al, 1990
TNFα	Human	pro, +	Burke 1989
			Bresgen et al, 1991
NGF	Human	mig, -	Campochiaro et al, 1985

Fig. 1.21. The effect of peptide growth factors on the migration and proliferation of cultured RPE. * Absence (-) or presence (+) of migration (mig) and/or proliferation (pro) activities. (Adapted from Burke 1984)

Factor	Species	Activity*	References
PDGF	Human dermal fibroblasts	mig	Seppa et al, 1982
	Rat lung fibroblasts	pro	Brody et al, 1992
EGF	Human foreskin fibroblasts	pro	Carpenter et al, 1976
	Rat granulation tissue fibroblasts	mig	Buckley-Sturrock et al, 1989
TGFβ	Human dermal fibroblasts	mig	Postlethwaite et al, 1987
aFGF	Human dermal and bovine ligament fibroblasts	mig	Senior et al, 1986
	Human and murine fibroblasts	pro	Gospodarowicz et al, 1975
bFGF	Mouse 3T3 fibroblasts	mig	Diegelman et al, 1986
	Rat granulation tissue fibroblasts	mig	Buckley-Sturrock et al, 1989
	Human and murine fibroblasts	pro	Gospodarowicz et al, 1975

Fig.1.22. The effect of peptide growth factors on the migration and proliferation of cultured fibroblasts. These are some of the many that have been used. *Activities indicated are migration (mig) and proliferation (pro).

Cell Type	Migration	References
Adult rat retinal glia	-	Harvey et al, 1987
Immature rat retinal glia	+	de Juan et al, 1988
Human Schwann cells	+	Baron-Van Evercooren et al, 1982
Human retinal pigment epithelium	+	Campochiaro et al, 1984
Adult human skin fibroblasts	+	Postlethwaite et al, 1981
Foetal human skin fibroblasts	+	Mensing et al, 1983
Rabbit Tenon's capsule fibroblasts	+	Josephs et al, 1987

Fig 1.23. The effect of fibronectin on the migratory activity of glia, pigment epithelia and fibroblasts.

1.5. Aims and Purpose of Study

It is apparent from the literature, that there is a patchy understanding of the factors influencing the behaviour of the cell types which form ERMs and there are fundamental questions that so far have remained unanswered. For example why simple glial proliferations can either remain as asymptomatic retinal outgrowths or become transformed into multicellular contractile membranes or why surgery to remove an ERM is often complicated by a recurrence of the condition. As a consequence, the current methods employed for the treatment and prevention of PVR are only partially successful. The early activities of the cells include migration to the ectopic site, settlement on the retinal surface and proliferation in the membranes. However little is known about which cells are the most efficient at these activities. Once it is known which of the cell types are most active then they form a potential target for selective inhibitory treatments.

The signalling systems that regulate cell behaviour in ERMs are also poorly understood. Signals are thought to be transmitted via diffusion of substances in the surrounding tissue fluids and by cell-cell contact. The fluid that collects under the damaged neurosensory retina (SRF), the damaged retina itself and proliferating cells in the membrane are likely sources of such substances. It is poorly understood to what extent they influence the cellular activities of migration, settlement and proliferation and whether or not some cell types are more responsive than others. When there is an improved understanding of these signalling systems it is possible that cellular receptors for chemoattractants and/or mitogens could be blocked using neutralising antibodies or receptor antagonists.

It is probable that the retinal glia act as a scaffold for the later more tractionaly active cell types. The evidence from cell-cell contact studies of the cell types in ERMs suggests that the initial monolayer of glial cells may affect the subsequent activities of the migrating and settling cells (Burke, 1983).

Taking the previous factors into account the aims of this investigation are:-

1. To assess the migratory abilities of the cells in single and mixed populations and the implications of these evaluations to ERM formation.

2. To assess the potential of SRF and a retinal crude extract as chemoattractants for the test cells.

3. To examine the hypothesis that ERMs are self perpetuating, because the proliferating cells in ERMs will produce substances that attract cells to the site of membrane formation and enhance their settlement and proliferation and so continue the cycle.

4. To investigate the relative attractiveness of a glial cell substrate for the test cell types and to assess the theory that retinal glia are an effective scaffold for the other two cell types.

5. To compare RPE and fibroblast invasion into glial monolayers.

Chapter 2.

MATERIALS AND METHODS

2.1. Tissue Culture I: Isolation of Primary Cultures

2.1.1. Culture Media

The bovine ocular cell cultures; bovine retinal glia (BRG), bovine retinal pigment epithelium (BRPE) and bovine scleral fibroblasts (BSF) were grown and maintained in Minimum Essential Medium (MEM) with Earle's salts (Eagle, 1959) (Gibco BRL, Life Technologies, European Division, Uxbridge, UK). MEM was employed as this was found by Burke and Foster (1984) to be best medium for the culture of rabbit retinal glial cells and all three bovine cell types grew well in it. They were grown in either 25 cm^2 or 75 cm^2 tissue culture flasks (Bibby Sterilin, UK Ltd.). The human cells showed less vigorous growth in culture than the bovine cells and required a richer medium, therefore Ham's F10 Nutrient Media (Gibco) was adopted (Ham, 1963). Both media were supplemented with L-glutamine (2 mM), Penicillin (100 units/ml) with Streptomycin (100 μg/ml) solution, Fungizone (Amphotericin-B 2.5 μg/ml) and either 10% foetal calf serum (FCS) for the bovine cultures or 20% for the human cells (Gibco, UK). The MEM solution was buffered with 2.2 mg/ml sodium bicarbonate and F10 with 1.2 mg/ml sodium bicarbonate and 20 mM HEPES (Gibco BRL).

2.1.2. Bovine Retinal Glia

BRG cells were isolated in a laminar flow hood (Bassaire Ltd., Southampton, UK) according to the method of Savage and co-workers (1988). Bovine eyes were obtained from a local abattoir and transported to the laboratory chilled over ice and kept at a temperature of 4°C for 24 hours post-mortem to allow the neural retina to separate more easily from the RPE. The intra-orbital

fat and extra-ocular muscle were removed and the eyeballs washed briefly in 70% methylated spirits and soaked in sterile phosphate buffered saline containing 1% Penicillin (100 units/ml) with Streptomycin (100 μg/ml) solution and 1% Fungizone (Amphotericin-B 2.5 μg/ml) for 30 minutes, to minimise possible bacterial and/or fungal contamination.

The anterior portion of the eye was removed, the retinae were then dissected out and washed several times in sterile phosphate buffered saline (PBS) to remove any adherent retinal pigment epithelial cells. The retinae were cut into pieces of approximately 1 cm^2 then floated in 75 cm^2 tissue culture flasks (Cell Cult, Sterilin, UK) containing an excess of MEM media plus 15% newborn calf serum (NCS) prewarmed to 37°C in a water bath (Grant, Jencons Scientific Ltd., Leighton Buzzard, UK; see Fig. 2.1a). NCS was employed initially in order to discourage the growth of neuronal and vascular elements in the retina, as glia are known to store glycogen (Magalhaes, 1976) and therefore would be less likely to suffer in a relatively poor culture medium. The cultures were incubated for a minimum of two weeks at a temperature of 37°C in an atmosphere of 5% CO_2 /95% air in a humidified incubator (GA2N; LEEC Ltd., Nottingham, UK). When cells settled on the bottom of the flask (Fig. 2.1b.) a proportion of the media was removed leaving about 5 ml behind and 30 ml of pre-warmed fresh media was added.

When colonies of cells were visible, the serum component of the media was changed from 15% NCS to 10% FCS to encourage growth. The populations of glial cells obtained in this way were thought to consist mainly of Müller cells for the following reasons (Savage et al, 1988). The phenotype was of elongated plate-like cells with distinct cell boundaries (Fig. 2.1c.) identical in appearance to rat and rabbit Müller cell cultures (Burke and Foster, 1984), with no obvious neural retinal contaminants. Autoradiography, employing ^3H thymidine of the floating retinal tissue showed large round cells which incorporated thymidine after seven days and these were the only cells in the retinal explant to do so, there was no labelling around the blood vessels. The large cells also stained positively with an antiserum to GFAP (a gift from Dr. U. Chakravarthy, Belfast; Chakravarthy et al, 1983) and for vimentin (Monoclonal antibody, Eurodiagnostics DV, Holland) in all cultures tested.

Astrocytes also stain positively with antibodies against GFAP but they retain positivity through passaging whereas bovine Müller glia become GFAP negative after several passages in culture. Also astrocytes do not have such a strong reaction to

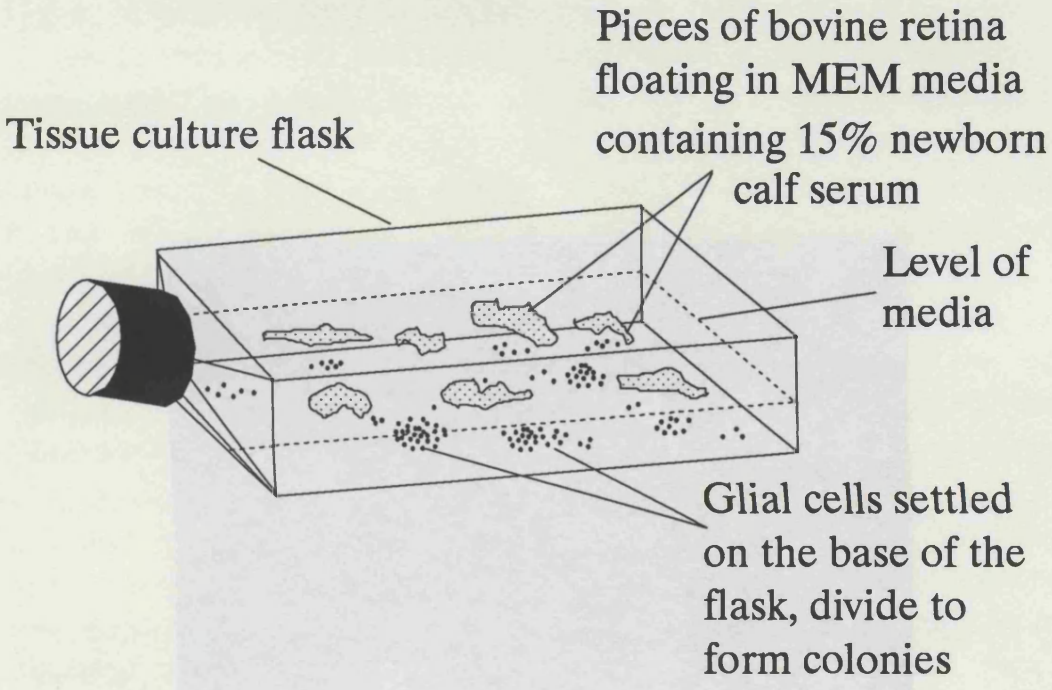
vimentin as the Müller glia and they have a stellate rather than a plate-like phenotype. In addition immunohistochemical staining to demonstrate the cytokeratins using broad spectrum antisera (ICN Biomedicals, High Wycombe, UK), positive against a range of basic and acidic cytokeratin subtypes characteristic of RPE, were negative in these cultures, as were antibodies against desmin (Monoclonal antibody, Eurodiagnostics DV, Holland) and factor-VIII (Antiserum, Dako, High Wycombe, UK) used to detect endothelial cells from retinal blood vessels (Savage et al, 1988). In addition there was no DNA synthesis associated with blood vessels in the explants which argued against the involvement of either pericytes or endothelium in the subsequent cultures. Lastly neuronal cells are known not to survive well in culture so neuronal contamination seems highly unlikely. Adler and coworkers (1982) demonstrated that the neuronal cells in cultures grown from embryonic chick retinae ceased proliferation after the sixth day in culture and surviving cells were few in number the cultures being predominated by large flat cells which were negative for tetanus toxin a neuronal marker.

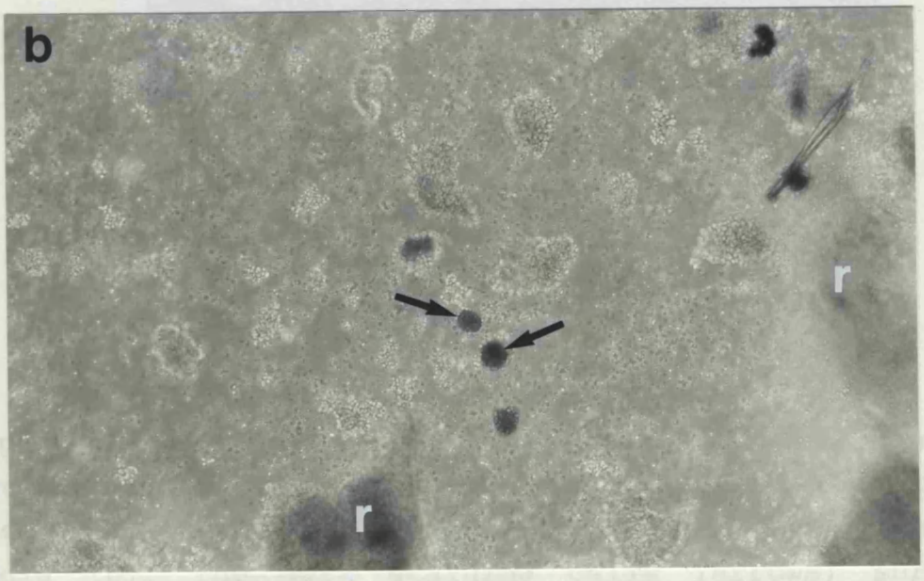
The cells were passaged (sub-cultured) at confluence (see section 2.2.1.) and used between second and fifth passage. as growth curves had been established on cells from first to fifth passage by seeding 7.25×10^3 cells per cm^2 (Savage et al, 1988). Higher passage cells were found to reach confluence (2×10^6 cells per 25 cm^2 flask) faster than lower passage cells, first passage cells taking 16 days to reach confluence as opposed to eight days for third passage cells. The cells not required for immediate use or further passaging, were stored in liquid nitrogen and thawed as required (see section 2.2.2.).

2.1.3. Bovine Retinal Pigment Epithelium

The BRPE were isolated according to the method of Basu and coworkers (1983), which was modified in our own laboratory. Bovine eyes were acquired from a local abattoir on ice and prepared in the same way as the retinal glia (see section 2.1.2.). The eyes were used as fresh as possible and always within six hours of death because it was found that unlike the retinal glia, BRPE were particularly sensitive to post mortem delay. The anterior portions of the eyes together with the vitreous and the retina were removed and the posterior eye cups

a


Pieces of bovine retina floating in MEM media containing 15% newborn calf serum
Tissue culture flask
Level of media
Glial cells settled on the base of the flask, divide to form colonies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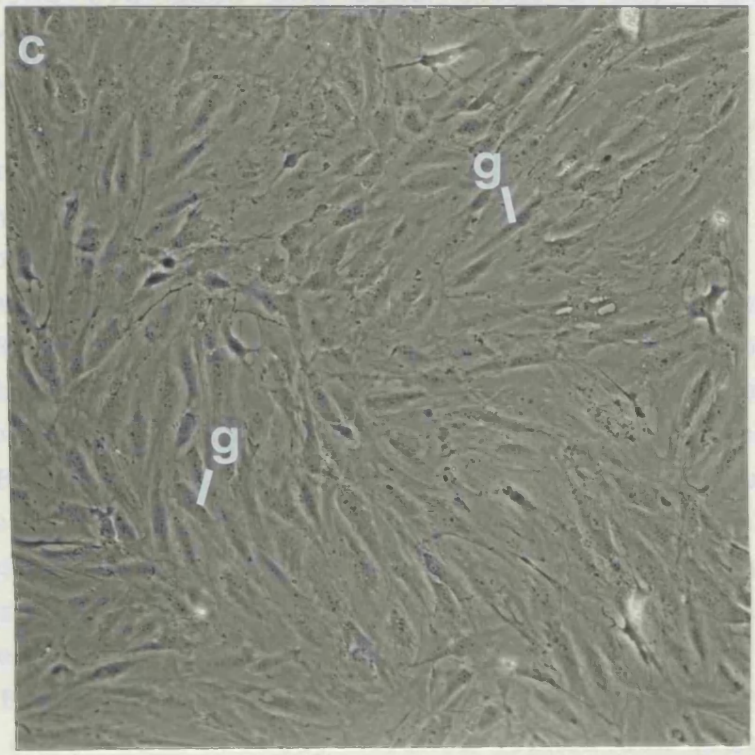


Fig. 2.1. Diagram to show the technique for the isolation of bovine retinal glia (a). Phase contrast light micrograph of pieces of bovine retinae (r) floating in excess tissue culture media (b). The dark staining cells (arrows) surrounded by refractile photoreceptor debris were observed to have the morphology of Müller cells as they settled and spread. A confluent culture of fourth passage bovine retinal glia (c), which are large well spread elongated cells (g). (Magnifications:- (b) x 100, (c) x 150).

were placed in groups of three in a sterile 88 mm diameter glass Petri dish lined with filter paper to prevent them from slipping. The eye cups were washed with sterile PBS to remove any remaining fragments of retina.

A plastic tripod was constructed from a plastic Petri dish (80 mm in diameter) with three upturned blood collecting tubes glued with an epoxy resin (Araldite) to its underside (Fig. 2.2a.). The tripod structure was placed so that each one of its hollow legs covered the optic nerve head region of one of each of the three eye cups and also covered any remnants of the attached retina (Fig. 2.2a.). A small volume (2-3 ml) of 0.25% trypsin and 0.02% EDTA (tetra sodium salt) solution was pipetted into each eye cup. The legs of the tripod prevented the trypsin from acting on the cells of the optic nerve head region, so eliminating contamination of the pigment epithelial cultures with cells such as optic nerve astrocytes.

The eye cups were then placed in a 37°C incubator in a humidified atmosphere of 5% CO_2 /95% air and left for 45 minutes. When pipetted samples showed a dark colouration indicating the presence of BRPE, the cells were pipetted into flasks containing 5 ml of MEM media and 20% foetal calf serum and left to settle for 1 to 2 weeks. When colonies of close packed cells forming a pavement of round and polygonal profiles were seen (Fig.2.2b.), the cultures were fed with MEM containing 10% FCS and passaged at confluence.

The BRPE phenotype at confluence was a mosaic of largely polygonal cells with clearly defined boundaries (Fig.2.2c.). They were used from passage two to six, as preliminary studies had shown the RPE to lose their pavement-like appearance in further passages (Del Monte and Maumenee, 1981).

2.1.4. Bovine Scleral Fibroblasts

BSF were isolated according to the method of Calthorpe and coworkers (1991) from eyes within twelve hours of death. The extraneous tissue including Tenon's capsule was carefully removed. The eyes were then lightly sprayed with 70% methylated spirits and soaked for ten minutes in sterile PBS supplemented with 1% Penicillin (100 units/ml) with Streptomycin (100 µg/ml) solution and 1% Fungizone (Amphotericin-B 2.5 µg/ml). The anterior segment, the vitreous, retina and choroid were all

removed. The remaining sclera was cut into pieces 3 mm^2 or less in area and the inner and outer layers removed with a razor blade. The pieces of tissue served as explants for culture.

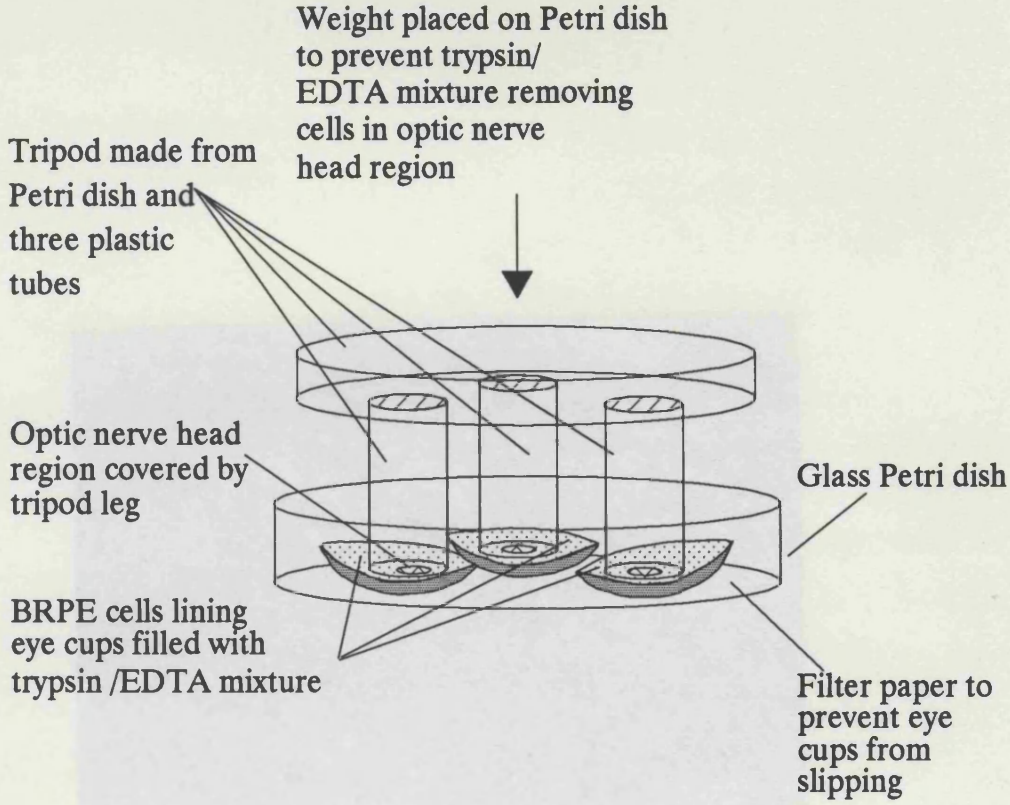
The scleral explants were placed in 25 cm^2 tissue culture flasks and left to dry onto the plastic for 20 minutes after which they were fed with 10 ml MEM and 10% FCS added. They were left to incubate at 37° C in 5%/95% CO_2/air for a week to 10 days, until cells were seen growing out of the scleral tissue (Fig.2.3a.). When the primary cultures were established (Fig.2.3b.) they were fed with MEM and 10% FCS, and passaged at confluence at a split ratio of 1:4. The explant was usually washed away in the process, if it remained firmly attached to the flask the primary culture was refed and continued to produce cells which were passaged on in turn. The fibroblasts retained their classic spindle shaped phenotype (Fig.2.3c.) with no evidence of senescent changes such as cytoplasmic vacuolation and gross cell enlargement within twelve passages or more. However for consistency with the other cell types, fibroblasts up to sixth passage but not beyond, were used in this study.

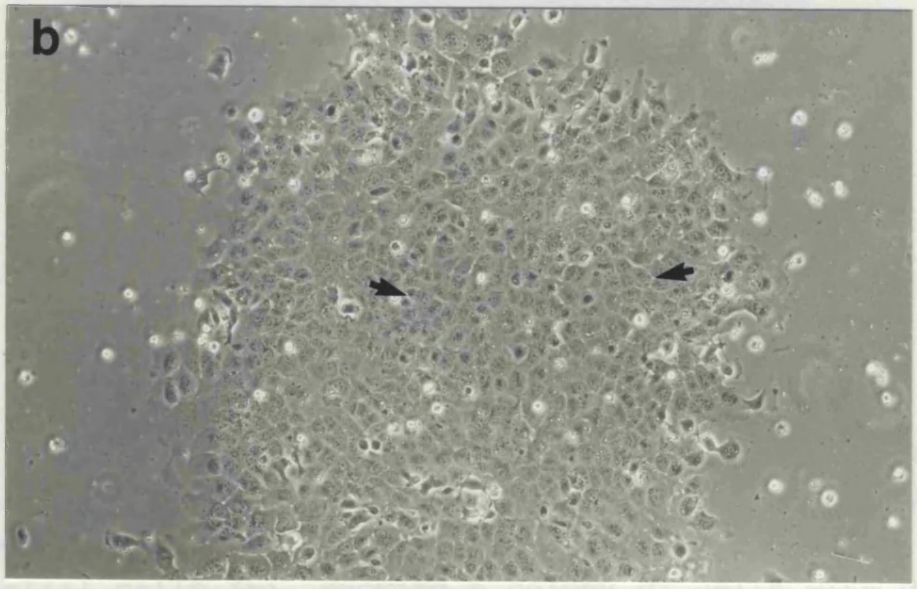
2.1.5. Epiretinal Membranes

ERMs were removed at surgery for complex retinal detachment by membrane peeling and were cultured according to the method of Hiscott and coworkers (1983). They were placed in 25 cm^2 tissue culture flasks in a small drop of neat newborn calf serum (NCS) and covered with a small glass coverslip (9 mm by 35 mm), which was fixed to the flask with sterile preheated beeswax (Fig 2.4a.). The membrane explants were fed with F10 media and 20% FCS. After one to four weeks, cells were seen to grow out of the explants (Fig.2.4b.).

A mixture of cell types were noted and these included small round cells possibly macrophages, stellate shaped cells, epitheliod cells, spindle-shaped fibroblastic cells and large spread plate-like cells (Fig.2.4b.). Although on the basis of phenotype alone we could no more than suspect which cells were present, immunohistochemistry conducted on selected primary cultures showed that a proportion of cells were GFAP positive (glia) and others were cytokeratin positive (RPE) (Hiscott et al, 1983).

a


Weight placed on Petri dish to prevent trypsin/ EDTA mixture removing cells in optic nerve head region
Tripod made from Petri dish and three plastic tubes
Optic nerve head region covered by tripod leg
Glass Petri dish
BRPE cells lining eye cups filled with trypsin /EDTA mixture
Filter paper to prevent eye cups from slipping


b

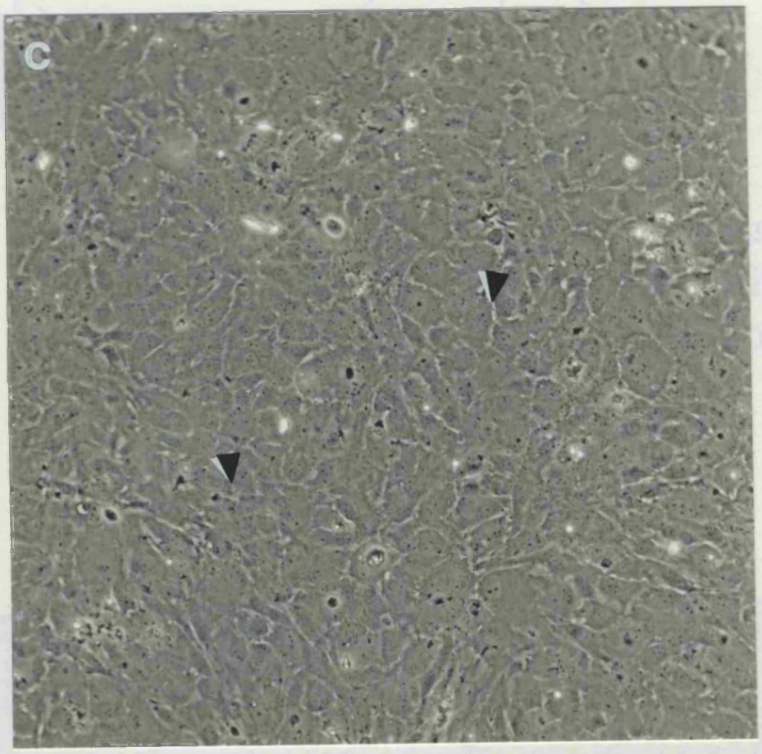
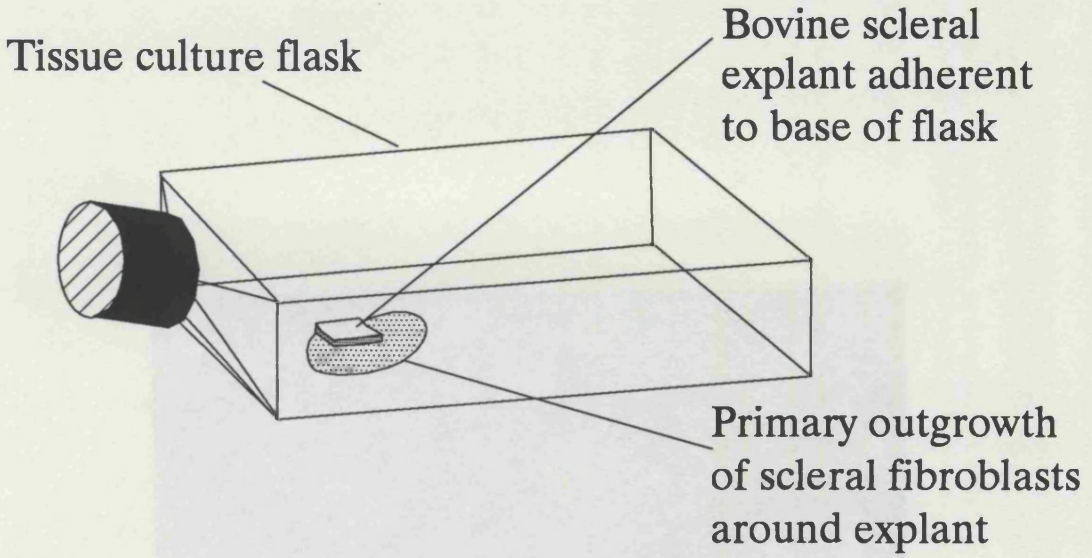
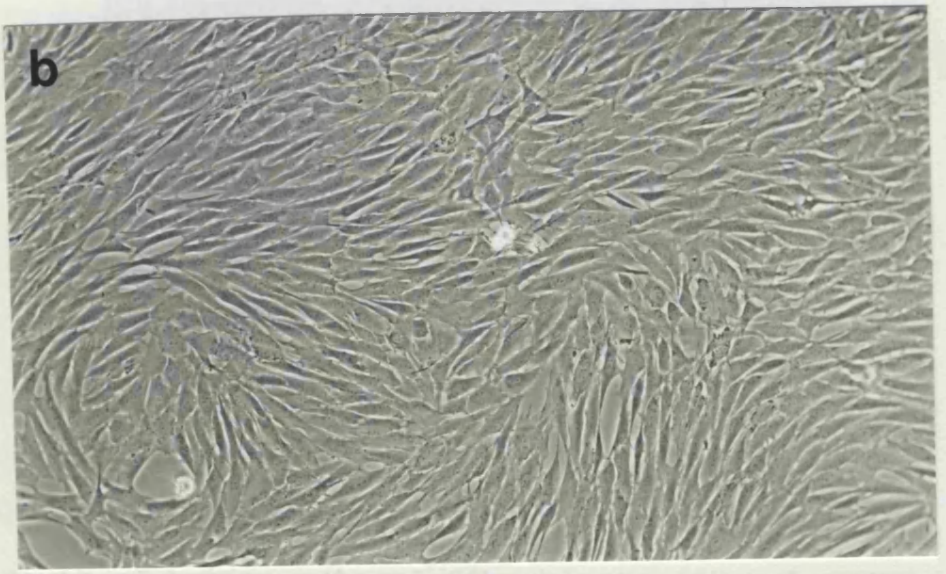


Fig. 2.2. Diagram to show the technique for the isolation of bovine retinal pigment epithelium (a). A phase contrast light micrograph of a primary culture of bovine retinal pigment epithelial cells (b), showing a colony of cells with polygonal profiles and clearly defined intercellular boundaries (arrows). A confluent culture of fourth passage bovine retinal pigment epithelium (c), the cells are more irregular in shape than the primary culture but just as well delineated (arrow heads). (Magnifications:- (b) x 100, (c) x 200).

a


Tissue culture flask
Bovine scleral explant adherent to base of flask
Primary outgrowth of scleral fibroblasts around explant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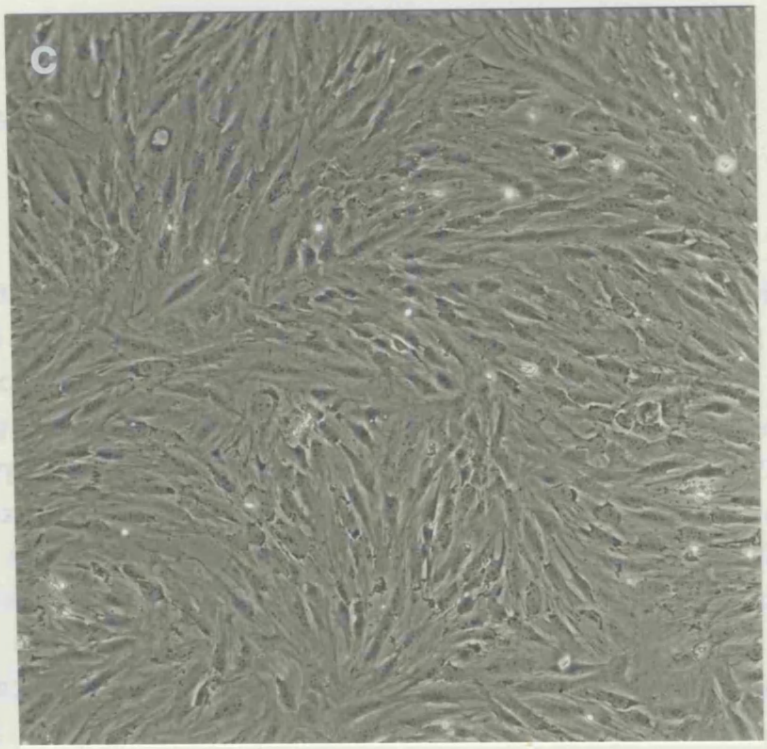


Fig. 2.3. Diagram to show a bovine scleral explant with an outgrowth of bovine scleral fibroblasts (a) and a phase contrast light micrograph of a near confluent culture bovine scleral fibroblasts (b). A confluent culture of fourth passage bovine scleral fibroblasts (c). They are clearly defined elongated bipolar cells, which grow in lines which gives a streaming effect. (Magnifications:- (b) x 100, (c) x 150).

When the ERM cultures reached confluence they were passaged. They were used at second passaged as they rarely grew beyond that. The phenotypes at second passage were still heterogenous but spindle-shaped cells, epithelioid cells and the large plate-like cells predominated while the small macrophagic cells failed to become established after replicating (Fig.2.4c.). Three ERM specimens were employed in this study, all were from male patients with PVR (Fig. 2.5.).

2.2 Tissue Culture II: General Techniques

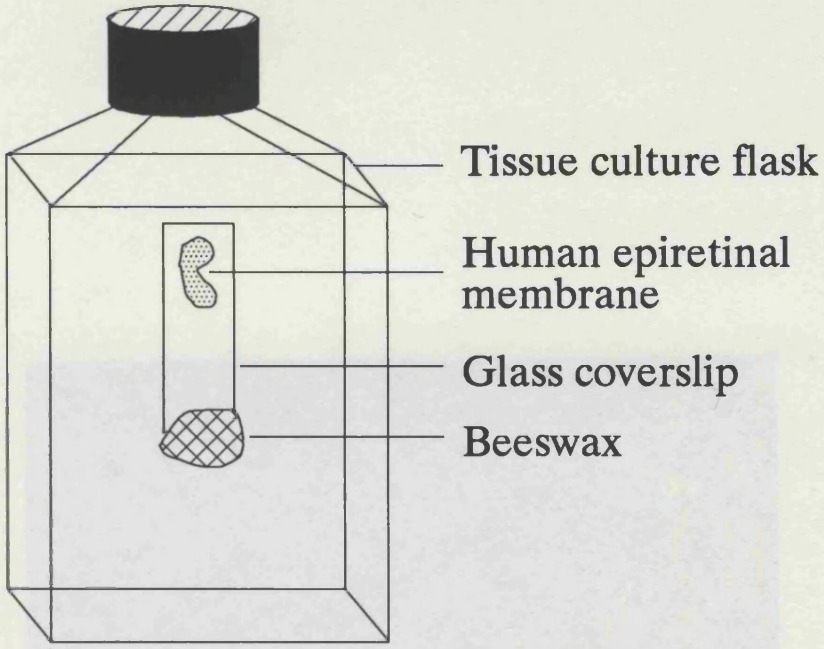
2.2.1. Subculture

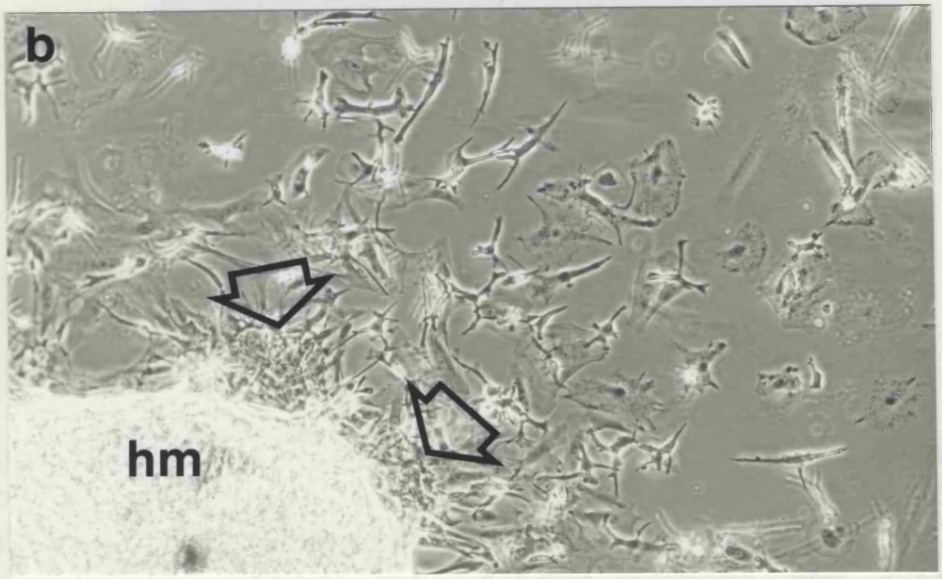
The cultures were washed three times with magnesium and calcium deficient PBS (Unipath, Basingstoke, UK),warmed to 37°C in a water bath, to remove any traces of serum and to aid in the removal of cells. The cells were detached from the flask by gentle agitation after exposure to equal amounts of warm (37°C) 0.25% trypsin and 0.02% of the disodium salt of EDTA (Sigma). The cells were viewed with the low power objective of a phase contrast inverted phase microscope (Olympus IMT, Olympus Optical Co., Japan). When the majority were seen to be rounded up (usually after a period of two to three minutes) the action of the trypsin was stopped by adding media containing serum.
The cell suspension was then divided amongst the required number of fresh flasks and the cultures fed as normal. The split ratios of the cell types depended on their growth rates, generally the slower growing BRG and ERM cultures were split one flask to two or three new flasks and the BRPE and BSF one to four or five.

2.2.2. Freeze Storage in Liquid Nitrogen and Thawing for Regrowth

The cells were removed from the flasks using the same method as for subculture (see section 2.2.1.). The cell suspension was placed in a sterile centrifuge tube and centrifuged (Centaur 1,

a


Tissue culture flask
Human epiretinal membrane
Glass coverslip
Beeswax


b
hm

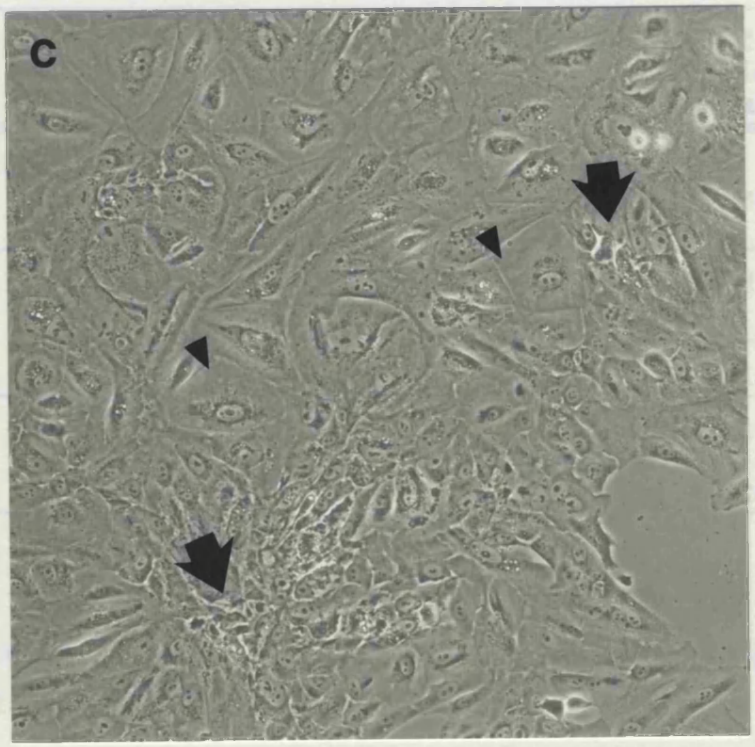


Fig. 2.4. Diagram to show the technique for the culture of cells from an ERM (a). Phase contrast light micrographs illustrating cells emerging (arrows) from an explanted human ERM (hm), after 4 weeks in culture (b). Second passage ERM cells (c), the cellular phenotypes have a heterogenous appearance consisting of large well spread irregularly shaped cells (arrow heads) surrounding nests of small epithelioid cells (arrows). (Magnifications:- (b) x 100, (c) x 200).

Patient Number	Sex	Age	Diagnosis	Type of Migration Experiment
1	M	36	PVR (Grade B)	Dose response curve
2	M	21	PVR (Grade C)	Zigmond Hirsch chequerboard analysis
3	M	55	PVR (Grade C)	Conditioned media harvested

Fig. 2.5. Table of ERM patient details and subsequent migration experiments in which the ERMs were used.

MSE Centrifuges Ltd, Leicester,UK) at a speed of 1000 rpm for 10 minutes. The pellet was suspended in the appropriate media for the cell type (see sections 2.1.2-5.) containing 20% serum and 10% dimethylsulphoxide (DMSO, Sigma) and pipetted into cryo-vials (Nalgene, Nalge Company, Sybron Corporation, USA). The cryo-vials were placed in a freezing tray in a liquid nitrogen storage vessel (35HC, Taylor Wharton, Jencons Scientific Ltd., Leighton Buzzard, UK) and the tray was wound slowly into the liquid nitrogen over a period of four hours for bovine and six hours for human cells. The slow decrease in temperature and the presence of DMSO was to prevent ice crystal formation and to ensure the viability of the cells on return to normal culture conditions. The frozen cells were then stored in liquid nitrogen until required.

When the cell cultures were required, the cryo-vials were removed from the liquid nitrogen and rapidly thawed in a 37°C water bath, suspended in media and centrifuged as above, to remove the DMSO. The supernatant was discarded and the suspended cells placed in flasks and cultured in the appropriate manner.

2.3. Migration

2.3.1. Micro Chemoattraction Assay

The microchemoattraction assay as originally developed by Boyden (1962) is a valuable guide to the potential responsiveness of cell types to various different active substances present at tissue injury sites. The migratory behaviour of the cells was assessed in a 48-well microchemoattraction chamber (Neuroprobe Ltd., Cabin John, Maryland, USA.) developed by Falk and coworkers (1980), based on the original single well system devised by Boyden in 1962. The modified 48-well system provided a rapid and accurate method for the simultaneous assessment of the response of the test cells to a number of different potential chemoattractants at a range of concentrations.

The apparatus (see Fig. 2.6.) consists of a clear plastic chamber, the lower part containing 48 wells of 25 μl capacity and corresponding upper wells of 50 μl capacity. The advantage of using small wells was that the substances to be tested were often only available in very small volumes (such as subretinal fluid)

and/or were extremely expensive (such as growth factors like PDGF). The upper and lower wells were separated by a silicone gasket and a semi-permeable polycarbonate membrane, 25 x 80 mm, 10 μm thick and pores 10 μm in diameter (Nucleopore Inc., USA), coated with the wetting agent polyvinylpyrolidone (PVP) and porcine gelatin (Type 1 300 bloom, Sigma).

Membranes without the wetting agent or coated with PVP alone had not been found to make a satisfactory settlement surface for cells (Joseph et al, 1987), therefore additional coating of the membranes was necessary. Postlethwaite and coworkers in 1976 found that gelatin was found to increase the settlement of human foreskin fibroblasts on a polycarbonate membrane. Subsequent studies by Joseph and coworkers in 1987 investigating the migration of rabbit Tenon's capsule fibroblasts demonstrated the same was true for ocular cells. The membranes used were coated with gelatin after the method of Postlethwaite and coworkers (1976). They were heated to 60°C in a solution of 0.5% glacial acetic acid for 20 minutes. Then they were washed twice with distilled water and exposed to a solution of 5 mg/ml of gelatin in distilled water at 100°C for one hour, air dried and finally dried for a further hour at 100°C.

To prepare the chamber for a migration assay, cell culture media containing dilutions of the test substance were pipetted into the lower wells. The membrane, the gasket and the upper part of the chamber were fitted and secured with thumb nuts. The upper wells were covered with a glass slide to prevent evaporation of the solutions and formation of air bubbles, then the chamber was equilibrated at 37°C in an atmosphere of 5%/95% CO_2/air for 30 minutes.

To prepare the cells for a migration assay, their media was changed 24 hours before the assay and the cultures were examined and photographed on an inverted phase contrast microscope (Nikon Diaphot TMD, Nikon, UK) before use. The cells were detached from the flask by trypsinisation (section 2.2.1.) and care was taken to keep trypsinisation time below three minutes since longer exposure is considered to reduce chemotactic activity (Postlethwaite et al, 1976; Melchiori et al, 1986). The action of the trypsin was stopped with media containing serum and the cell suspension was centrifuged (1,000 rpm for 10 minutes). The supernatant was discarded and the cells suspended in serum free media and centrifuged for a second time. The final pellet was suspended in one ml of serum free media to exclude chemoattractants present in the foetal calf serum which might influence the experiment. An aliquot of the cell suspension was

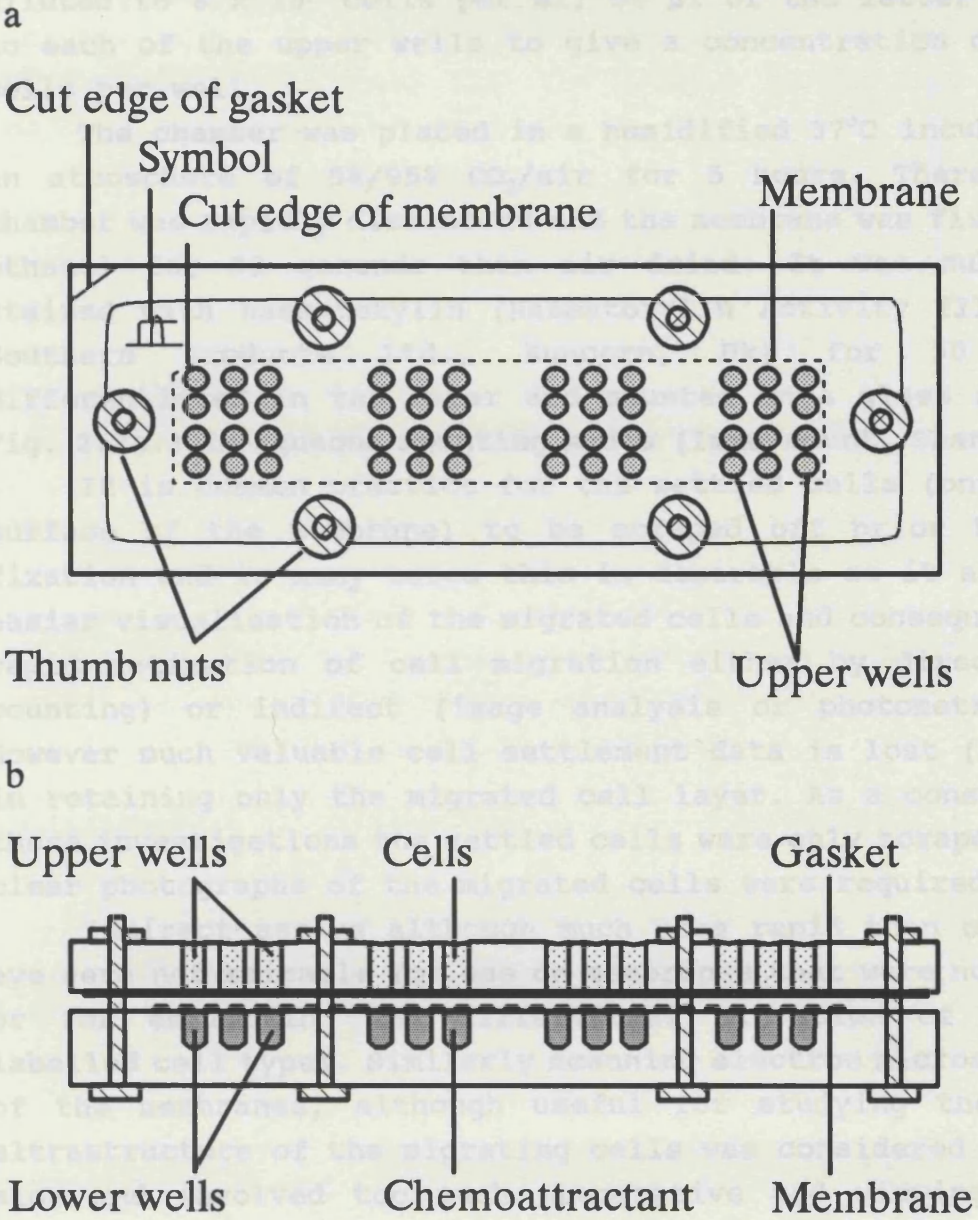


Fig. 2.6. Diagrams of a top (a) and side (b) view of a 48-well modified Boyden chamber used for the migration studies.

diluted (40 μl of cell suspension in 20 ml of Isoton diluting fluid) on a Coulter diluter and counted with a Coulter counter (Coulter Electronics Ltd., Luton, UK). The suspension was then diluted to 8 x 10^5 cells per ml; 50 μl of the latter was added to each of the upper wells to give a concentration of 40, 000 cells per well.

The chamber was placed in a humidified 37°C incubator with an atmosphere of 5%/95% CO_2/air for 5 hours. Thereafter the chamber was rapidly dismantled and the membrane was fixed in 100% ethanol for 30 seconds then air dried. It was subsequently stained with haematoxylin (Haematoxylin Activity III, Shandon Southern Products Ltd., Runcorn, Uk) for 30 minutes, differentiated in tap water and mounted on a glass slide (see Fig. 2.7a.) in aqueous mounting media (Immu-mount, Shandon, UK).

It is common practice for the settled cells (on the upper surface of the membrane) to be scraped off prior to alcohol fixation and in many cases this is desirable as it allows much easier visualisation of the migrated cells and consequently more rapid estimation of cell migration either by direct (visual counting) or indirect (image analysis or photometry) means. However much valuable cell settlement data is lost (see below) in retaining only the migrated cell layer. As a consequence in these investigations the settled cells were only scraped off when clear photographs of the migrated cells were required.

Indirect assays although much more rapid than counting by eye were not suitable for use on membranes that were not scraped or for estimating the differential migration of the mixed labelled cell types. Similarly scanning electron microscopy (SEM) of the membranes, although useful for studying the detailed ultrastructure of the migrating cells was considered to be too slow and involved too much preparative and viewing time for effective and efficient quantitation and again was not suitable for showing the differences between latex, carmine and unlabelled cells in the mixed migration studies.

The migration of the cells was assessed by the direct method of counting the number of nuclei of cells migrated to the underside of the membrane by light microscopy (Vickers Instruments, UK). In order to differentiate clearly between the migrated and settled cells the membranes were mounted with the settled cells facing downwards so that the migrated cells were in the upper planes of focus. Another distinguishing feature was that the settled layer of the membrane contained many dark staining rounded cells and the migrated cells were more sparsely distributed and spread in profile. The cell nuclei (stained dark

blue by the haematoxylin) were counted using a high power (x 100) oil immersion objective and x 10 wide field eyepieces (Fig. 2.7b). The slide was moved in a snake rasta pattern to avoid counting the same field twice. The nuclei were counted rather than the cell bodies as SEM and transmission electron microscopy (TEM) studies have shown that the nucleus is the last portion of the cell to be pulled through the pore (see section 1.3.2.) (Calthorpe et al, 1990).

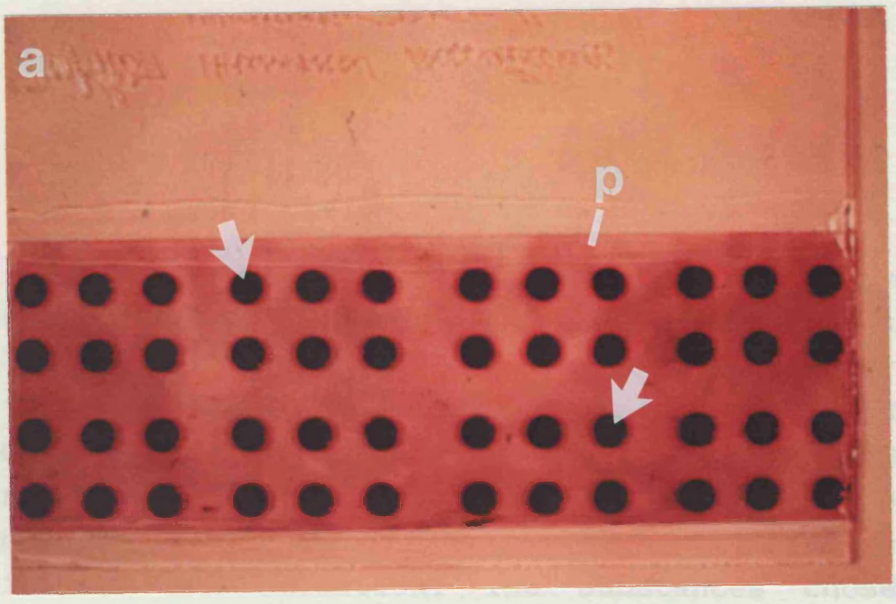
In view of the high power of the objective used it was not practical to count all the migrated cells in each well. Twenty fields were examined for each of the 48 wells as this was consistent with much of the literature on this subject (Postlethwaite et al, 1976). Twenty high power fields represented 0.54 mm^2 or 1/15th of the area of each well. Each concentration of chemoattractant was run at least in quadruplicate and each experiment was repeated at least three times. Inter-observer error was checked by an additional experienced observer counting the same membrane again and intra-observer error was calculated by the same observer counting the same wells a second time. The percentage error for intra- and inter- observer counts was calculated from the following equation:-

$$\text{Percentage error} = \frac{\frac{X_1+X_2}{2} - X_1}{\frac{X_1+X_2}{2}} \times \frac{100}{1} \%$$

Where X_1 is the first count and X_2 the second count.

Settlement counts were also carried out in order to account for any variation in migration due to differences in settlement. The total number of cells settled was counted in one random field per well and the number was divided by the number of cells migrated. Percentage migration of the cell numbers settled was then calculated. The one way ANOVA F test was employed to test the significance of the migratory responses (see section 2.5.4.).

To ensure that the migratory behaviour of the cells was not affected by any limitations of the assay imposed by variations in size and bulk between the three cell types, the following experiments were performed. Hitherto the standard membrane pore size of 8 μm had been used to assess the migration of fibroblasts and similar cell types (Postlethwaite et al, 1976). We initially performed comparative migration assays with membranes of pore sizes of 8 and 10 μm and found the latter to be more suitable for our cell types. In addition flow cytometry was employed to



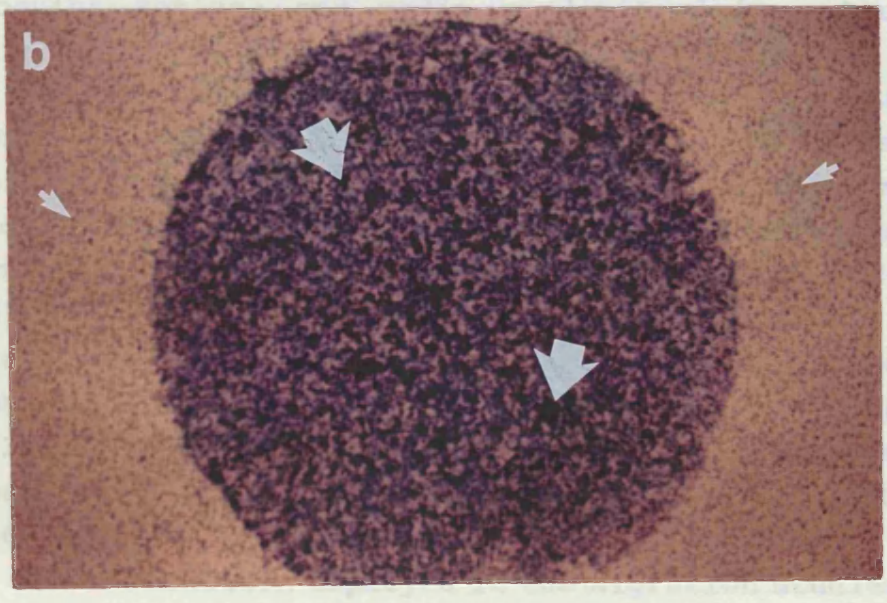


Fig. 2.7. A polycarbonate membrane (p) with wells of migrated and settled cells (arrows) stained with haematoxylin (a). Enlargement of one well of cells (b), in which groups of dark staining cells (large arrows) are visible in the area of the well and the pores of the membrane appear as lightly staining dots in the surrounding regions (small arrows). (Magnifications:- (a) x 1.5, (b) x 26).

estimate the relative sizes of the three cell types in suspension by using the parameters of forward scatter and side scatter of the cells (see Appendix I).

2.3.2. Chemoattractants

The substances employed as chemoattractants were selected on the basis of the following criteria. Their possible role in the migration of cell types to the site of ERM formation, that is to say their known chemoattractive potential for the cell types involved. Their presence at the injury site either in extracellular fluids or damaged tissues and their production in response to injury by the cell types known to be present in the region of ERM formation. The substances chosen were the attachment factor fibronectin, the growth factor PDGF, samples of subretinal fluid from eyes with and without PVR, an extract of bovine retinae and media conditioned by cultures of the proliferating test cells.

Fibronectin was chosen as one of the test substances as its importance in ERM formation has often been stressed in the literature (Wiedemann et al, 1988). It is a known chemoattractant for cell types similar to the ones employed in this study. It is known to be present in surgical specimens of ERMs (Hiscott et al, 1985) the vitreous (Wilson-Holt et al, 1992) and samples of subretinal fluid (Immonen, et al, 1989) and it has been shown to be produced by the key cell types in ERM formation (Yamakawa et al, 1987; Hiscott et al, 1992). The general and chemoattractant properties of fibronectin have already been described in sections 1.3.6. and 1.4.2..

The fibronectin employed in the migration studies originated from bovine plasma and was bought as a commercially prepared solution from Sigma Chemical Company Ltd., (St.Louis, USA.). It arrived as a 1 mg/ml sterile solution prepared in 0.5 M NaCl, 0.0 5 M Tris/HCL buffer, pH 7.5. Quality control (Sigma) involved cell culture and toxicity tests on three separate cell lines over a 5 day period and cell attachment properties were assessed by cell binding studies with BHK-21 cells. Its protein content was assessed by the Coomassie blue binding assay to be 1 mg/ml and its purity by immunoelectrophoresis. On arrival it was stored at 4°C then aliquoted (in 50 μl amounts) into siliconised micro tubes and kept at -70°C until required. The tubes needed to be

siliconised because fibronectin, often referred to as the "cellular glue" (Hynes and Yamada, 1982), has a strong affinity for glass and plastic.

The growth factor PDGF was chosen as a positive control to test the effects of fibronectin on the migration of mixed cell populations. PDGF was employed as it is known to be a powerful migratory stimulant for rat retinal glia, human RPE and human fibroblasts (see Fig. 1.16.). and has been implicated as one of the growth factors involved in ERM formation (Burke, 1989). Only one growth factor was used as this study was of cell behaviour rather than an investigation into the effects of growth factors per se.

The PDGF (from human platelets) was again bought as a commercial preparation from Sigma. It was in the form of a sterile lyophilized powder 0.2 μg in quantity, which was a mixture of the isoforms AA and BB and AB. Approximately 70% of the PDGF isolated from platelets is the AB homodimer, the majority of the remainder is BB and a small amount is AA (Ross, 1989). Quality control (Sigma) involved testing for the antibodies to HTLV III/LAV and Hepatitis B surface antigens (negative to both), purity by SDS gel electrophoresis (>90%) and protein content by Coomassie dye binding assay. Its biological performance was based on its ability to stimulate the growth of 3T3 cells in the absence of serum. It was reconstituted on arrival in 100 μl 0.1 M acetic acid at a concentration of 2 μg/ml and stored in 10 μl aliquots in siliconised micro tubes (due to its affinity for glass), at -70°C until required.

The Boyden chamber migration assay, as well as being an effective way to gauge the migratory response of cells to known chemoattractants such as fibronectin and PDGF, is also useful in assessing the potential properties of fluids that may contain any number of unidentified migratory stimuli.

Subretinal fluid (SRF) is the fluid that accumulates between the neurosensory retina and RPE layer during retinal detachment and studies have shown it to contain chemoattractants such as fibronectin. (Immonen et al, 1989) (see also section 1.4.2.). Samples of SRF were collected from patients undergoing surgery for bullous rhegmatogenous retinal detachment (rhegmatogenous indicates the presence of a retinal defect such as a hole or tear) and from those with PVR and proliferative disease undergoing membrane peeling to remove established ERMs (see Fig. 2.8.). SRF was chosen for this investigation in order to test the hypothesis that substances in the surrounding tissue fluid may affect the migration of the test cells.

The SRF samples were collected in the following way. The fluid was drained externally through a needle from a bullous rhegmatogenous retinal detachment and via internal suction during membrane peeling, all samples contaminated with blood were discarded. The SRF was immediately placed in siliconised micro tubes in order to minimise loss of adhesive substances such as fibronectin by adherence to the plastic. It was then stored at -70°C to preserve any temperature labile components for as long as possible. When required, the samples were diluted with serum free MEM media for use in the migration studies. To obtain the dose optimum of migration of the three cell types to SRF, a sample of SRF from a patient with PVR a was diluted to a range of concentrations in serum free MEM (Fig. 2.9.). Subsequently nine samples of SRF; three from patients with retinal detachment, two from those with PVR and four from those with proliferative diabetic retinopathy (Fig. 2.9.) were assessed using the standard micro chemoattraction assay described in section 2.3.1. The migratory ability of the cells was expressed as a percentage of their migration to 10 μg/ml of fibronectin.

In view of the fact that the degenerating retina was likely to produce stimulatory factors (see section 1.4.2) a crude extract of bovine retinae was also tested for its ability to stimulate migration. The extract was prepared from bovine retinae (after the method of Glaser et al, 1980) in sterile PBS containing 0.25% glucose. For every retina, 1 ml of solution was added, for example 30 retinae were placed in 30 ml of solution and left for 2-2.5 hours at room temperature. The retinae were then centrifuged at a speed of 3,000 rpm for 5 minutes (Centaur 1, MSE, Leicester, UK) the pellet discarded and the supernatant centrifuged at 22,000 rpm for 30 minutes. The final supernatant was filter-sterilised through a sterile filter of pore size 0.2 μm (Milipore, UK), aliquoted into sterile siliconised micro tubes and stored at -70°C. The crude extract was used in migration assays at concentrations varying from 50-300 μl/ml, diluted with serum free media.

In an attempt to define some of the active factors that may be produced by a degenerating retina, a preliminary characterisation of the retinal crude extract (RCE) was carried out using treatments employed by Bryan and coworkers (1986) and included heating of aliquots to 56°C for 30 minutes, 100°C for 3 minutes, treatment with acid (pH 3) or alkaline (pH 10) for 30 minutes at room temperature, and molecular weight cut off filtration (Milipore, UK) to exclude substances with a greater molecular weight than 30,000 daltons. The bovine cells were then

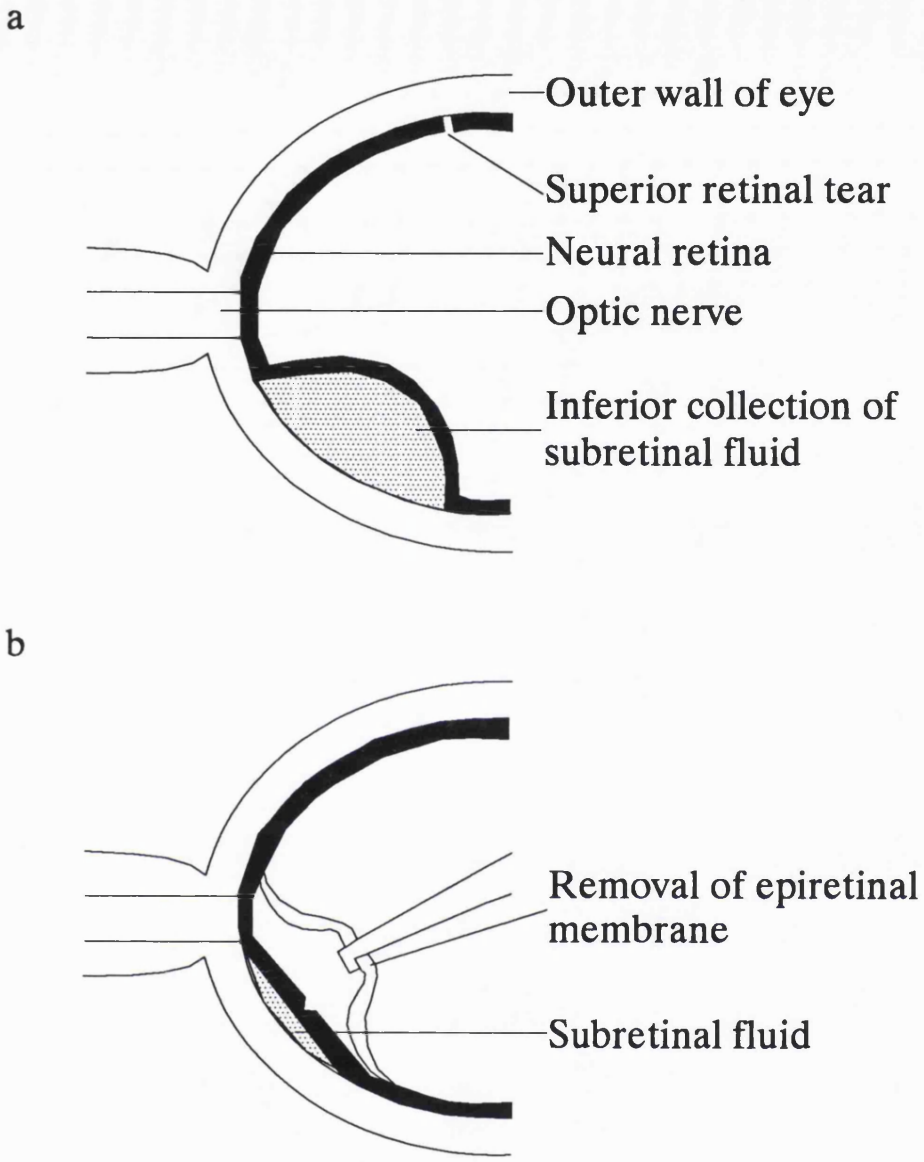


Fig. 2.8. Diagrams to show the sources of subretinal fluid; from an eye with rhegmatogenous retinal detachment (a) and from an eye with proliferative vitreoretinopathy (b; after Tarrant, 1986 and with the kind permission of Dr.P.Hiscott).

Patient Number	Sex	Age	Diagnosis	Sample Number
1	F	64	Longstanding retinal detachment with PVR (Grade C)	- (Dose response curve)
2	M	47	Traumatic longstanding retinal detachment	RD1
3	M	26	Recurrent rhegmatogenous retinal detachment	RD2
4	M	68	Rhegmatogenous retinal detachment	RD3
5	M	45	Longstanding retinal detachment with PVR (Grade C)	PVR1
6	M	55	PVR (Grade C)	PVR2
7	F	21	Proliferative diabetic retinopathy	PDR1
8	M	24	Proliferative diabetic retinopathy	PDR2
9	F	70	Proliferative diabetic retinopathy	PDR3
10	M	40	Proliferative diabetic retinopathy	PDR4

Fig. 2.9. Table of patient and diagnostic details of SRF samples used in the migration studies.

tested for their migration to each of the treated RCE aliquots with untreated RCE as thecontrol.

Finally media conditioned by the three cell types in culture was employed in migration and preliminary characterisation studies as well as settlement and proliferation assays, its preparation and use is described in sections 2.4.1. and 2.4.2. respectively.

2.3.3. Zigmond Hirsch Chequerboard Analysis of the Migratory Response

The cellular migratory response in vitro has several components including chemotactic and chemokinetic movement (see section 1.3.2.). Fibronectin has been shown to stimulate both chemotaxis and chemokinesis of fibroblasts (Postlethwaite et al, 1981; Joseph et al, 1987). To be able in the future to prevent the migration of the cells to the site of ERM formation in PVR, it is necessary to understand fully the types of migratory response of the appropriate cells so that they may be blocked. Therefore in order to characterise the migratory response of the cells Zigmond Hirsch chequerboard analysis was employed (Zigmond et al, 1973; Postlethwaite et al, 1981).

The Zigmond Hirsch chequerboard analysis involves adding different concentrations of chemoattractant above and below the membrane in different wells and counting the number of cells migrated to the lower side of the membrane for each well. In the wells where there is no chemoattractant (Fig. 2.10a.) only a minimal number of cells are found on the lower side of the membrane possibly due to the combined effects of random locomotion and gravity. Where the chemoattractant has been added only to the upper wells (Fig. 2.10b.), the larger proportion of cells will be on the same side of the membrane as the stimulant and there is no concentration differential across the membrane to attract the cells through the pores and so any stimulated random movement taking place in the upper well where the chemoattractant is to be found should not involve movement through the pores. When the chemoattractant is in the lower wells (Fig. 2.10c.) if chemotactic movement is taking place then the cells move up a concentration gradient of stimulus and the cells pass through the pores in the membrane to the underside where the chemoattractant is located. In wells where the concentration of

chemoattractant is the same above and below the membrane (Fig. 2.10d.) there is no gradient and if large numbers of cells pass through the pores it is by stimulated random movement (chemokinesis).

In a 48-well modified Boyden chamber, a series of four different concentrations of stimuli (including zero) were tested. They were run in quadruplicate by using two chambers (two groups on each chamber). The test substances were added to the bottom wells in a vertical direction and the same concentrations were repeated at right angles in the upper wells (see Fig. 2.10e.). This resulted in a diagonal series of wells where the upper and lower concentrations of test substance was equal and there was no gradient across the membrane. The other intervening wells had varying degrees of positive and negative gradients. Each experimental run to assess chemotaxis and chemokinesis using the Zigmond Hirsch chequerboard was run at least three times.

2.3.4. Mixed Labelled Migration of the Three Cell Types

There is little clear information in the literature as to which of the three cell types is the most effective at responding to migratory signals. It would of course be of considerable value to know which of the cell types is the most responsive because it may well further our understanding of the genesis of ERMs. As the chemotactic response is receptor dependent, the migration of the cells seems to be very largely reliant on their condition and treatment during the assay. Slight variations in the culture conditions and state of the cells may alter their migratory response and as the Boyden chamber assay is miniaturised it would be more susceptible to any environmental variations and this may result in noticeable variations in the numbers of migrated cells. In order to eliminate some of these variables the cell types were migrated under as near identical conditions as possible.

It was decided to develop a system which would truly compare the migratory rates of the test cells by migrating them together in the same chemotaxis well. It was felt that as well as being more analogous to the in vivo situation, mixed migration was a natural progression from the classic "one cell type" chemoattraction studies. To migrate the three cell types together it was necessary to label the cells so that they could be easily distinguished from each other during counting

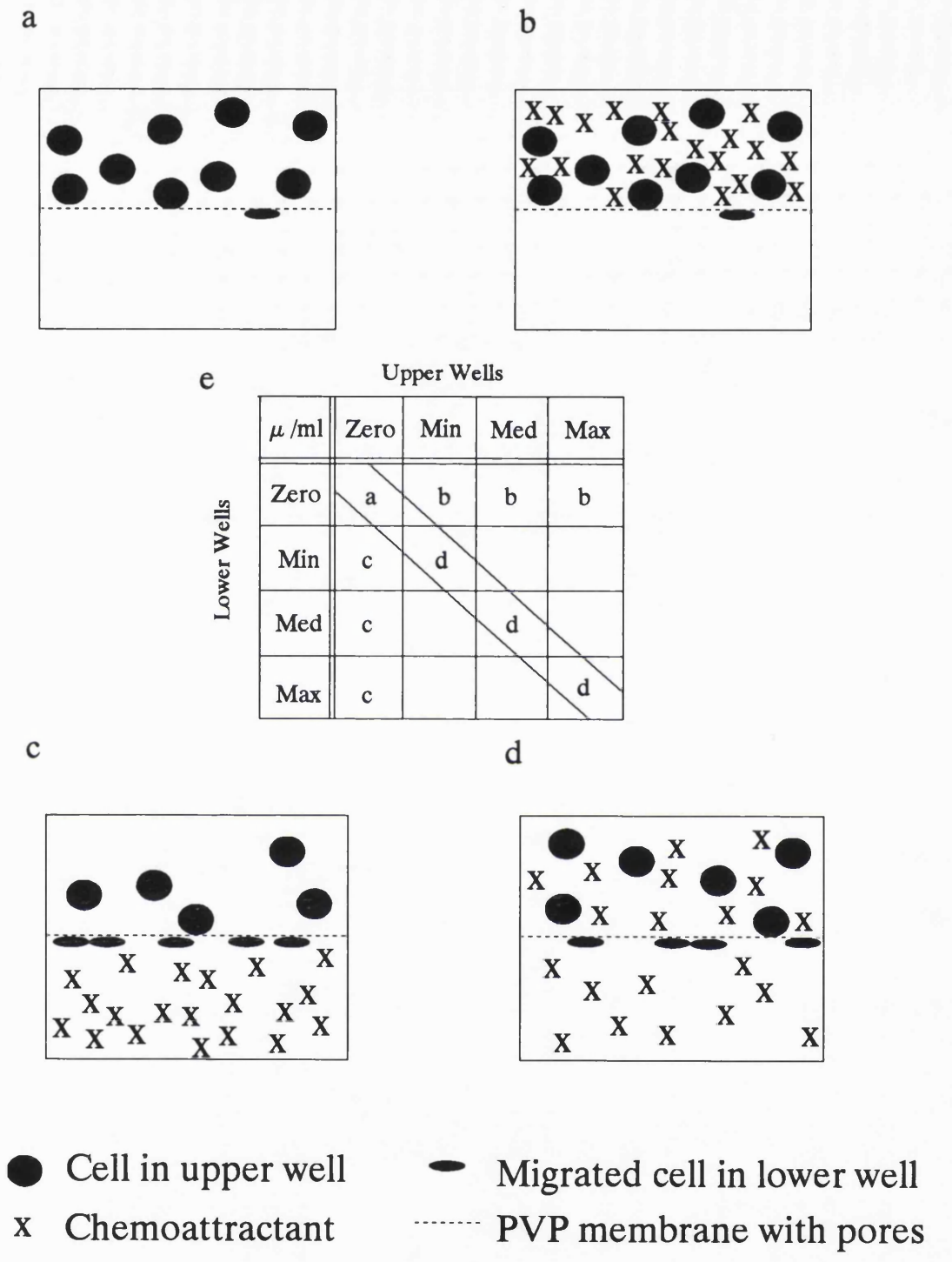


Fig. 2.10. Diagrammatic representations of wells with no concentration gradients (a and d), negative (b) and positive (c) concentration gradients and a Zigmond Hirsch chequerboard.

with a light microscope.

Retinal glia, RPE and fibroblasts are all known to be phagocytic in vivo (Friedenwald and Chan, 1932; Bok and Hall, 1971; Hollyfield, 1976; Wolter and Lichter, 1983; McGaw and Ten-Cate, 1983) and in vitro (Mano and Puro, 1990; Feeney and Mixon, 1976; Albrecht-Buehler, 1977; Schroeder and Kinden, 1983; McKeown et al, 1990) and this activity was exploited in the present study to label the cell types and so distinguish one from another. The cell labels employed in this study had to fulfil stringent criteria, in that they had to be easily taken up and retained by the cells, inert, non-toxic and not so large so as to affect locomotion but large enough to be clearly visible with a light microscope. In addition the incorporated label must not be easily degraded in the cytoplasm by the lysosomal system of the cell.

In the past, studies on the mechanisms of phagocytosis (Wetezel and Korn, 1969) have employed latex microspheres of varying sizes (Toyohara and Inaba, 1989). Grierson and coworkers (Day et al, 1986) showed that microspheres of 0.8 μm were suitable for long term phagocytosis studies in bovine ocular (trabecular meshwork) cells and though the cell settlement rate was increased and proliferation decreased, locomotion was not affected. Burke and coworkers (1985) previously had employed latex microspheres to label rat retinal glia in order to distinguish them from human RPE in co-culture studies. Embryologists have employed latex beads (Bronner-Fraser et al, 1984) to follow the pathways of embryonic cell migration and demonstrated them to be behaviourally inert in these experiments. In addition carmine particles added to cultures of chick and mammalian embryonic myocardium and tendons had no demonstrable effects on morphology, migration, proliferation or contraction of the cells (Marzona et al, 1976).

Other cell labels also used in phagocytosis studies, were considered. These included fixed red blood corpuscles (rbcs), latex microspheres of a different size, other stains such as Janus Green B and natural substances such as melanin. The rbcs were rejected as being too large and in danger of impeding cellular locomotion. Latex microspheres that were small enough to be distinguishable from the 0.8 μm diameter ones chosen, would have been too small to be clearly distinguished with the light microscope and larger ones would have impeded locomotion. Janus Green B is used as a supravital stain for mitochondria (Lillie, 1965) and it would have been considered as a third label if required, also melanin was another possibility as it has been used in phagocytosis studies with cultured RPE (Boulton and

Marshall et al, 1985).

The two cellular labels employed were polystyrene microspheres (also often known as latex beads) 0.797 μm in diameter (Sigma Chemical Company Ltd., USA) and carmine (Carmine "Gurr" Certistain, BDH, UK), a red powder. Both the labels needed to be cleaned before use. As the commercial solution of latex beads contained sodium azide as a preservative which is toxic to cells and the carmine stain powder was not sterile. In order to wash the labels prior to use, weighed amounts of latex beads and carmine were diluted in sterile PBS and centrifuged (10 minutes; 75G) in an ultracentrifuge (Ultrafuge, MSE Ltd., UK). This procedure was repeated five times and they were then suspended in PBS containing 0.07% penicillin and streptomycin and 0.07% Fungizone (Amphotericin B) solutions to prevent bacterial and fungal contamination then stored at 4°C until required (Grierson et al, 1986).

It was necessary to perform titration experiments to find the point where there was a high enough percentage of cells with beads in their cytoplasm at a level which did not influence their migratory behaviour. Latex beads were fed to the test cell type at doses of 1000 (20 μl of bead solution (0.2×10^9 beads) in 5 ml MEM) and 5000 beads per cell (100 μl of bead solution (1×10^9 beads) in 5 ml of MEM) for 16 and 24 hour periods. It was shown that with longer exposure, overloading had an effect and shorter exposure left too few cells with incorporated particles.

Carmine was fed to the three cell types for a 24 hour period at doses of 50 particles per cell (100 μl of 0.1% (w/v) carmine solution ie 4.01×10^7 particles) and to the BSF alone 25 particles per cell. To calculate the percentage labelling efficiency. In each of three 25 cm^2 tissue culture flasks, 300 cells were counted visually on a phase contrast microscope for the presence or absence of label.

In preparation for the migration studies, pre-confluent cultures of retinal BRG and BRPE were fed with 5000 beads per cell and BSF with 1000 beads per cell in 5 ml of MEM and 10% FCS per 25 cm^2 tissue culture flask. The cultures were incubated for 24 hours then employed in the micro chemoattraction assay described in section 2.4.1. The cells were migrated in pairs, 20,000 of each cell type making a total of 40,000 cells per well. To estimate the uptake of label by the cell the number of beads or particles were counted in a small sample of six cells at a magnification of x 1000 and the mean number of beads per cell ±SEM was calculated.

To monitor any effect that labelling may have had on the

migration of the cells a comparison was made of labelled and unlabelled cells (for each cell type used) on their migration to the obtained optimum dose of fibronectin. Similarly it is conceivable that to due cellular interaction that the presence of one cell type may possibly affect the migration of another so to prevent any masking of this when the three cell types were migrated together. The cell types were initially migrated together in pairs. One cell type was labelled and the other unlabelled. As an extra check on any effects the label might have had on the migration of the cells. The identical experiment was run (in the same Boyden chamber) with a cross-over in the labelled cell type.

The final experiments involved all three cell types in the same well, 13,333 of each cell type making a total of 40,000 cells per well. The BRG were unlabelled, the BRPE labelled with latex beads and the BSF with carmine. Also in some of the runs the settled cells were scraped off the upper surface of the polycarbonate chemotaxis membranes. This was to allow for clear photography of the migrated cells so that features such as location of labels and comparative morphology of the labelled cells and unlabelled cells could be carefully scrutinised. Photographs were taken using bright field optics (Polyvar, Reichert-Jung, Austria).

2.4. Cell Migration, Settlement and Proliferation in the Presence of Serum Free Media Conditioned by Cell Cultures

2.4.1. Preparation of Cell Conditioned Media

Cultured cells have long been known to produce factors which have stimulated migration and proliferation of similar and different cell types in culture. Media collected from cultures of retinal cells has also been shown to stimulate migration and proliferation of RPE and glia (See section 1.4.2. and Fig. 1.12.). Media collected from cell cultures after a fixed incubation period is known as conditioned media as it has in effect been conditioned by the cells and contains substances produced by the cells. In studies where the effects of substances

produced by one cell type are being tested to see their influence on the behaviour of another cell type rather than to act purely as a growth enhancer the media employed is serum free to prevent any masking of the stimulatory effects by active products present in the serum.

The media was prepared according to the method of Bryan and coworkers (1986). The cultures were washed three times with PBS to remove any traces of serum and 15 ml of serum free media (MEM for the bovine cells and Ham's F10 for the ERM cultures) was added per 75 cm^2 flask and 5 ml per 25 cm^2 flask. The cultures were incubated (at 37° C in 5% carbon dioxide) for 48 hours. This time period was employed by Bryan and coworkers in order to allow cells sufficient time to divide and to collect as many products as possible without deterioration of the cell cultures.

A time period of 48 hours without serum in the growth media has been shown in the past not to adversely affect the cell types tested (Bryan et al, 1986). However cells vary in vigour in culture and therefore in their susceptibility to prolonged periods without growth factors. So as an added confirmation that the cultures remained healthy during the period of conditioning, they were photographed on a Nikon phase contrast inverted microscope before and after conditioning and checked for obvious signs of cell deterioration. The culture was excluded from the experiment if the cells appeared vacuolate, if there were large gaps present in the monolayer or if there were any dead cells. Deterioration was extremely rare and usually the cells appeared in good condition and there was no obvious cell loss from the monolayer.

It was considered important to have good quality conditioning because although healthy cells are known to produce attachment factors and growth factors, injured and dying cells may also release substances that bias the results (reviewed by Schwartz et al, 1989). The conditioned media was removed, centrifuged at 3,500 rpm for 10 minutes to remove any debris, aliquoted, frozen and stored at -70°C in order to preserve as many of the active constituents as possible, until required. The concentrations of conditioned media used in the investigations ranged from 100 to 1000 μl/ml in serum free media.

2.4.2. Migration, Settlement and Proliferation

Conditioned media was tested for its chemoattractive properties in the Boyden chamber assay as described in section

2.3.1 and diluted for use in serum free MEM to concentrations of 100, 250, 500, 750, 1000 µl/ml. Samples were also collected from cultured ERM cells grown from a membrane removed from a patient with Grade C PVR (sample 3, Fig. 2.5.). Preliminary characterisation of the active factors present in BRG and BRPE conditioned media for the migration of BSF was carried out as for the RCE. This again included heating of aliquots of conditioned media to 56°C for 30 minutes, 100°C for 3 minutes, treatment with acid (pH 3) or alkaline (pH 10) for 30 minutes at room temperature, and molecular weight cut off filtration (Milipore, UK) to exclude substances with a greater molecular weight than 30,000 daltons. BSF were then tested for their migration to each of the treated BRG and BRPE conditioned media aliquots with untreated conditioned as the control.

The settlement of BRG, BRPE and BSF onto plastic in the presence of cell conditioned media was assessed as described in section 2.5.2. Conditioned media was diluted to 100, 250, 500, 1000 µl/ml in serum free MEM. The negative controls were serum free media and the positive controls were solutions of soluble fibronectin. The settled cells were counted on an inverted phase contrast microscope at a magnification of x 100 (x 10 objective and x 10 wide field eyepieces). Percentage intra- and inter-observer errors were calculated as described for the migration assay section 2.3.1.

A proliferation assay was carried out using a two step visual counting method (adapted from Campochiaro et al 1986) to determine any increase in cell numbers in response to substances present in cell conditioned media. Cell cultures in log phase were washed with PBS and trypsinised, centrifuged and suspended in 1 ml of warm MEM containing 10% newborn calf serum (NCS). A sample of the suspension was counted on a Coulter counter and the cells were diluted to a concentration of 1 x 10^6 cells per ml. A one ml volume of culture media was placed in each well of a 24 well plate and the plates were equilibrated at 37°C for 30 minutes. Using a Gilson-Brown micro pipette (Anachem, Luton, UK) 20 µl of the cell suspension (2 x 10^4 cells) was added to each well and allowed to settle for 24 hours at 37°C in 5%CO_2/95% air.

After 24 hours, the control, MEM and 10% NCS and the test media, serum-free MEM, 10% NCS and variable amounts of conditioned media were warmed to 37° C. Four wells to be used as the 24 hour baseline control were washed with PBS, the cells were removed with one ml of 50:50 0.25% trypsin and 0.02% EDTA was added and counted on a Coulter counter. The media was removed from the remaining wells in the plate and one ml of prewarmed

control media (MEM and 10% NCS) was added to four wells and varying concentrations of conditioned media (250, 500, 750 µl/ml) were added to the remaining wells. The plates were incubated for three days and the numbers of cells in each of the wells counted on the Coulter counter (see section 2.4.1.). The results were expressed as percentage increase in cell numbers over the baseline control which was the 24 hour settlement values. Preliminary analysis of the fibronectin content of the conditioned media was carried out by ELISA analysis (see Appendix II).

2.5. Cell Settlement

The retinal Müller cells and astrocytes are thought to be the first cells to migrate through the inner limiting lamina to its vitreal surface (Hjelmeland and Harvey, 1988) and form the scaffold for the other epiretinal cells (Grierson et al, 1987; see section 1.3.3.). One of the ways in which the cells involved in the development of epiretinal membranes appear to influence each others' behaviour is by cell-cell contact, as outlined in section 1.4.2. In order to explore some of the possible effects of the association of the incoming cells with the glial scaffold, the in vitro interactions of the test cells BRPE and BSF with a BRG monolayer were observed and quantified. This was done by employing a series of assays which examined different aspects of the cell-cell contact and cell-substrate behaviour in the following ways:-

1. Static evaluation of settlement
    a. Visual counting
    b. Scintillation counting

2. Longitudinal evaluation of settlement
    a. Video time-lapse microscopy

Initially cell settlement was studied by static methods which involved estimating the numbers of cells settled and rounded, in a fixed time window. This was done in order to obtain background data on the relative settlement speeds of the different cell types and their affinities for a glial monolayer, quickly and easily. The direct method of visual counting was used

to provide baseline figures and morphological information. The indirect method of scintillation counting was subsequently employed to provide corroboration for the preliminary data because much larger sample numbers could be processed in this way.

One of the disadvantages of cross-sectional studies in areas such as cell behaviour is that important events may be missed if they are either side of the time window and no longitudinal information can be obtained about the settlement process. Longitudinal assays such as time-lapse filming of cell settlement have enormous advantages over static assaying systems by allowing the whole process of attachment and adhesion to be viewed for a given cell. However the length and complexity of the experiments allow for fewer runs to be performed, so there is the possibility of greater variability. In the present studies, use was made of both static and longitudinal methods of investigating settlement. The former provided baseline data and the latter gave a behavioural insight into the process.

2.5.1. Static Settlement Evaluation: Cellular Substrates

The relative affinity of BRPE or BSF for a BRG monolayer was measured using two static settlement assays adapted from the methods of de Bono and coworkers (1984). They compared the adhesion of polymorphonuclear leucocytes, lymphocytes, macrophages and smooth muscle cells to cultured bovine aortic endothelium. de Bono used direct and indirect methods for measuring cell settlement. Their first approach involved visual counting of the rounded up adherent cells by light microscopy and the second involved labelling of the adhering cells with tritiated adenine. Adenine was used as it is taken up by a variety of cells and its uptake is independent of cell division, the β emission was estimated in a scintillation counter and the data was presented as counts per minute.

The cells in suspension were referred to by de Bono as the "adhering" cells and the underlying endothelial monolayers as the "target" cells. However to be strictly accurate, cells can only be described as adhering if they remain attached to the underlying or substrate monolayer after the non-adherent cells have been washed away. Therefore in this study the "adhering" cells of de Bono ie those initially in suspension will be

described as the test cells (see Fig. 2.11.). The target layer of cells serves as a substrate for the test cells. The term substrate as used in this study is an underlying substratum (either cellular or plastic). The controls in the assays were wells containing only the test cells and media where the substrate was tissue culture plastic.

The direct method of assessing cell settlement, also known as a "collecting lawn assay" was employed as follows. Bovine retinal glial cells were the target monolayer. They were trypsinised and centrifuged at a speed of 1000 rpm for 10 minutes. The cell numbers were determined on a Coulter counter and the cells were seeded into 24 well plates at a density of 2 x 10^5 cells per well. They were viewed on an inverted phase contrast microscope and were seen to have formed a confluent monolayer within 24 hours. A confluent monolayer was defined as one in which the entire surface of the bottom of the well was covered by a sheet of cells with no spaces between them and little or no overlapping of cell borders.

The test cells (bovine retinal glia, retinal pigment epithelium or bovine scleral fibroblasts) were trypsinised, centrifuged and suspended in MEM with 10% FCS. The cell numbers were determined on a Coulter counter and seeded at a high density of 2 x 10^5 cells per well into the 24 well plates containing an established confluent target monolayer of BRG cells. As the cell numbers were determined indirectly by using the Coulter counter rather than a haemocytometer the percentage viability of the cells was estimated by adding 0.25% trypan blue stain to a sample of the cells and counting the number of stained cells on a Neubaur haemocytometer (Freshney, 1983). The blue dye is absorbed by dead cells but eventually all the cells will become permeable to the stain, so it is important that counts are done immediately after staining.

The incubation time of 30 minutes was chosen because pilot studies following the time course of settled cells showed the majority to have become incorporated by 30 minutes to one hour (data not included). In addition de Bono and coworkers had previously shown this time period to produce the optimum number of settled cells. After 30 minutes incubation at 37° C in 5% CO_2/95% air, the media was removed, the plates gently washed with PBS to remove any non-settled cells and the cells fixed with 2% gluteraldehyde/PBS (vol/vol). The settled cells were still distinguishable from the underlying monolayer as they were rounded up and phase refractile. They were counted in three fields (0.75 mm x 0.75 mm) per well (area 201.10 mm^2) of the 24

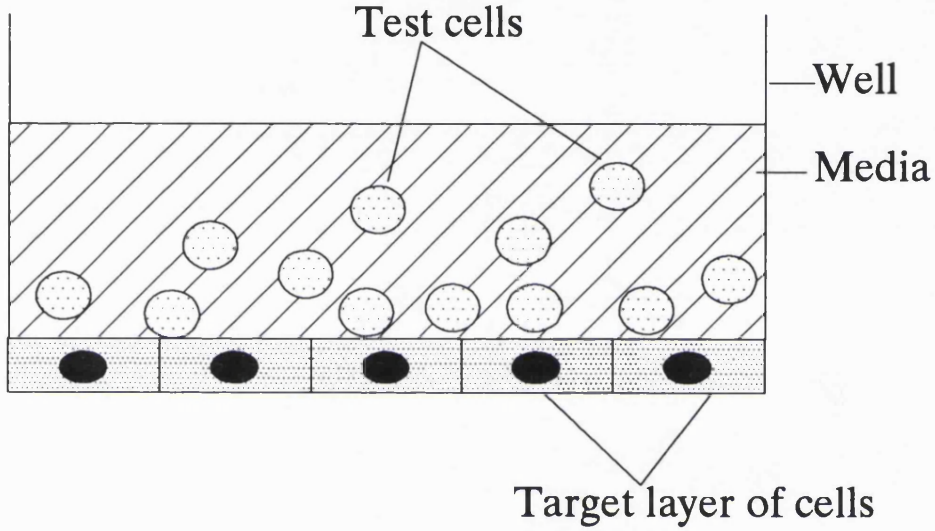


Fig. 2.11. Diagrammatic representation of a cross section of one well of a 24 well plate to illustrate the test cells and the underlying target cell layer.

well plate at a magnification of x 100 (x 10 objective, x 10 wide field eyepieces) on an inverted phase microscope. The x 10 objective was used as it was possible to count the largest number of cells per well accurately at this magnification.

The indirect method for assessing settlement involved labelling of the adhering cells with ^{14}C adenine. The carbon isotope was used in our studies rather than tritium as ^{14}C is known to be more accurately estimated in a scintillation counter. Also its employment enabled the used of "Safe" scintillants being developed at that time by Canberra Packard to replace the xylene based TM 299 necessary then to accurately estimate tritium.

The adhering cells were labelled with 0.1 μCi (3.7 Kbq) of ^{14}C adenine (Amersham International, UK Ltd.) per 10^6 cells, followed by incubation at 37°C in 5%/95% carbon dioxide/air for 16 hours. The cultures were then washed three times with PBS and fed with unlabelled media for 24 hours, after which they were washed again with PBS, trypsinised and suspended in MEM containing an excess (10^{-5} M) of unlabelled adenine. Unlabelled adenine was also used in the final suspension and in the settlement media to avoid the probability that any ^{14}C leakage, due to cell injury and death, might be taken up by the target cell layer. Control experiments to assess any uptake of label by the target monolayer were also run (see Appendix III). The target cell layer was incubated for 16 hours with the filtered media which had contained ^{14}C adenine labelled test cells, to check whether cell to medium leakage of label was a problem. Then the cultures were washed with PBS as before and incubated as above in the presence of excess adenine for 24 hours.

The cell suspensions were centrifuged, counted and pipetted into each well of the 24 well plate at a concentration of 2 x 10^5 cells per well. The plates were incubated for 30 minutes, the media was removed and saved and the cultures were gently washed three times with PBS. All the washes and supernatant fluids were saved and their radioactivity measured in the scintillation counter to monitor any loss of radioactivity due to handling and the washing process. A mixture of 0.5 ml 0.25% trypsin, 0.5 ml 0.02% EDTA and 0.1 ml Triton X (BDH, UK Ltd.) was added to each well to detach and rupture the cells in order to release the radioactivity. Each one ml sample was pipetted into a scintillation vial with 10 ml of scintillant (Scintillant Safe, Canberra Packard, Uk) and the β emission detected using a scintillation counter (CA 1900, Canberra Packard, UK).

The scintillant is a liquid chemical medium capable of converting the kinetic energy of nuclear emissions (β particles)

into photons. The emitted photons are detected by photomultiplier tubes and converted to electrical pulses. To correct for background noise, and reduction in the counting efficiency known as quenching the counter automatically determines the counting efficiency for each sample by the use of spectral analysis. The result is, the detected counts per minute (CPMs) are converted to disintegrations per minute (DPMs). In addition the counter is programmed to automatically make the correct adjustments to detect the ^{14} C adenine isotope.

The relationship between DPMs and cell numbers for each cell type was established from previously constructed calibration curves as follows (see Appendix IV). The cells were firstly labelled with the ^{14} C adenine isotope as above and serial dilutions were performed to give 20, 200, 2000, 20,000 and 200,000 cells/ml. The radioactivity in log mean DPM in each of these samples was plotted against log calculated cell number. The correlation coefficient "r" was calculated for each cell type and the cell numbers read from the regression curves obtained for a given level of radioactivity.

2.5.2. Static Settlement Evaluation: Plastic Substrates

A combination of regulatory factors are known to be important in ERM formation, cell-cell contact (as already mentioned) and, in addition, soluble mediators present in the surrounding tissue fluids, degenerating retina and secreted by the cells themselves (see section 1.4.3.). The attachment factor fibronectin is known to be present in the vicinity of ERM formation; in subretinal fluid, the vitreous and associated with the cells that take part in PVR. In order to investigate the effect of the presence of soluble fibronectin and products produced by the cell themselves on the settlement and spreading of the test cell types. The following experiments were performed.

The settlement of BRG, BRPE and BSF cells onto tissue culture plastic over a fixed time period was assessed by counting the number of cells adherent to the plastic using phase contrast microscopy. The control medium was serum free tissue culture media and the experimental substances were different concentrations of soluble fibronectin and media conditioned by cultures of BRG, BRPE and BSF (see section 2.4.2.).

Fibronectin from bovine plasma (Sigma Chemical Company Ltd, St.Louis, USA.), bought as a one mg/ml sterile solution (commercially prepared in 0.5 M NaCl, 0.0 5 M Tris/HCL buffer, pH 7.5, see also section 2.3.2.), was diluted in serum free MEM to give concentrations of 1, 5, 10, 15 μg/ml. A one ml aliquot was placed in each well of a 24 well plate (the control wells contained serum free MEM) and equilibrated at 37° C in a humidified incubator in 5%/95% carbon dioxide/air for 30 minutes.

Confluent monolayers of BRG, BRPE and BSF were removed from tissue culture flasks by trypsinisation, centrifuged (1000 rpm for 10 minutes), counted and diluted to a concentration of 10^6 cells per ml. Aliquots of 100 μl (containing 10^5 cells) were pipetted into each well. The settlement on to the cellular substrate had been carried out using a high cell density of 2 x 10^5 cells per ml. However subsequent time course studies (data not included) using different cell concentrations of 0.5 x 10^5, 10^5 and 2 x 10^5 per ml showed that for the 30 minute time period there was no significant difference in the number of cells remaining adherent to plastic when 10^5 and 2 x 10^5 cells per ml were compared (ANOVA $p<0.001$). In order to conserve cells the smaller concentration was used. The plates were gently agitated to evenly disperse the cells in each well and then placed in the incubator. After 30 minutes the wells were washed with PBS to remove any non-adherent cells and fixed in 2% gluteraldehyde in PBS. The number of cells settled was counted in three fields (0.75 mm x 0.75 mm) per well (area 201.10 mm^2) of the 24 well plate at a magnification of x 100 (x 10 objective, x 10 eyepieces) on an inverted phase microscope and percentage intra- and inter-observer errors were calculated (see section 2.1.3.)

It was observed during the fibronectin settlement experiments that within the 30 minute period the different cell types appeared to show different degrees of spreading. In order to further investigate this, the degree of spreading of each cell type in the presence of soluble fibronectin was assessed. For each concentration of fibronectin and for each cell type, a random photograph was taken on an inverted phase contrast microscope with a x 10 objective. The negatives were enlarged so that the print magnification was x 100. For each photograph the diameters of 100 cells were measured using a magnifying loupe (x 10) with a graduated scale (Emscope, UK). The amount of spreading was presented as the percentage increase in cell diameter over the negative control which was the diameter of floating spherical cells.

The cells to be settled were fed 24 hours before the experiment to ensure they were in good condition. On the day of the experiment the cells were removed from the flask by trypsinisation, centrifuged, counted and diluted to a concentration of 10^6 cells per ml. Aliquots of 105 cells were pipetted into each well. The plates were gently agitated to evenly distribute the cells and placed in the incubator. After 30 minutes settlement time. The wells were washed with PBS containing Mg^{2+} and Ca^{2+} and fixed in 2% gluteraldehyde in PBS. Again the number of settled were determined by counting the number of adherent cells in three fields (0.75 mm x 0.75 mm) per well (area 201.06 mm^2) of the 24 well plate at a magnification of x 100 (x 10 objective, x 10 eyepieces) on an inverted phase microscope.

2.5.3. Longitudinal Evaluation of Cell Settlement onto Cellular Monolayers: Video Time-Lapse Phase Contrast Microscopy

Time-lapse filming had enormous advantages over static assaying systems by providing a longitudinal follow up of the settlement process for a given cell. In addition time-lapse the observation of much later stages of cellular interaction than was possible with the static settlement assays. The video replay, freeze frame and real time clock functions enabled detailed observations and timing of the cellular interactions thus allowing for a more in depth approach to the analysis of the cell interactions.

The settlement of cells in suspension onto a confluent monolayer was monitored using video time-lapse, phase contrast microscopy. The target monolayer was a 25 cm^2 flask of confluent BRG cells maintained by 4 ml of MEM and 10% FCS. The test cells (either BRPE or BSF) were removed from a confluent culture by trypsinisation, centrifuged, suspended in one ml of MEM with 10% FCS and then counted (see section 2.3.1). The cell suspension was pipetted onto the BRG sheet and the flask allowed to equilibrate in a 5%/95% CO_2/air incubator. Thereafter the flask was tightly sealed with para-film to slow down gas equilibration with the air and placed in a Nikon inverted microscope with phase contrast optics (Nikon Diaphot TMD, Nikon, Japan) fitted with a clear plastic hot box heated to a temperature of 37° C.

The events of cell settlement and incorporation into the target cell layer were filmed with a video camera (Hitachi KP40 Solid State, Hitachi, Japan) and recorded on a time-lapse video cassette recorder (Panasonic SVHS, Matsui Industrial Co. Ltd., Japan) at a speed which expanded a three hour tape to 480 hours. The first part of the experiment involved the pipetting of either BRPE or BSF in suspension onto the BRG monolayer and the events were recorded for 24 hours. This was followed by the pipetting of the test cell type not used in the initial run ie either BSF or BRPE on to the now mixed target layer of either BRPE/BRG or BSF/BRG cells and recording the events for another 24 hours.

The events were observed on a monitor (Hitachi Monochrome) and the videotape (SVHS Professional, Fugi, 180E) was replayed at 160 times normal speed so that 24 hours of real time could be condensed into 9 minutes. Cell loss from the surface of the monolayer was determined by counting the number of cells left on the surface of the glial monolayer at hourly intervals for up to 24 hours using the freeze frame function. The time it took for half the initial number of test cells in the field viewed at time zero to be lost from the target cell layer was designated as the ID_{50} value. Each full cell settlement run was repeated four times and the resultant ID_{50} values were compared by one way ANOVA (see section 2.5.4.).

As well as cell loss, the events involved in the incorporation of individual test cells into the target layer were observed and timed using the fast forward, reverse and freeze frame functions. These were also recorded onto 1 inch tape in a Marconi broadcast machine (Marconi MR-2B One Inch Broadcast Machine, Marconi, USA) and observed in detail in the editing suite of Central Television Services (Liverpool University). The cellular incorporation process was divided into stages and photographic stills of each stage were taken from the monitor screen. Lastly events taking place in the target layer upon contact with the test cells, particularly cell division were observed and settlement runs of either BRPE or BSF onto the BRG monolayer were extended to 72 hours to monitor glial mitotic activity in detail.

2.5.4. Presentation and Statistical Analysis of Data

The raw data from each experiment was presented as the mean of the samples in each group plus or minus the standard error of the sample mean (SEM) as this value gave an idea of the accuracy of the mean. In order to obtain basal and optimal values for cell behaviour, negative controls were run with each experiment and positive controls where suitable. In experiments where different data sets were compared the results were either presented as a percentage or proportional increase over the negative control or as a percentage of the positive control values.

The most suitable type of test to compare the data samples was considered to be one way analysis of variance (ANOVA). It allowed for flexibility in that two or more samples could be compared and multiple comparisons could be made between data sets. In miniaturised biological assays such as micro chemotaxis, variation and unbalanced sample numbers due loss of samples for example by cell death was taken into consideration and ANOVA was robust enough (Altman, 1991) to allow for small sample numbers and unbalanced data sets.

In experiments where multiple comparisons were made, for example between different concentrations of chemoattractant, the error that could arise from multiple significance testing was minimised by using a multiple range test. A suitable test was the Student-Newman-Keuls (S-N-K) interval which divided the groups into homogenous sub sets according to whether there was a significant difference between them or not. Statistical analysis of the data was carried out using the statistics package Unistat (Unistat Version 2.0 for MS Windows, Unistat Ltd., London, UK). Percentage values were compared using the non parametric Mann Whitney U test for very small samples (Siegal, 1956).

Chapter 3.

RESULTS

3.1. Behavioural Comparisons Relating to Relative Migratory Ability

3.1.1. Migration of Single Cell Types: I Dose Response Curves to Fibronectin

The migration of BRG, BRPE, BSF (Fig. 3.1a.) as well as cells grown from excised ERMs (Fig. 3.1b.) was stimulated by the presence of soluble fibronectin in the medium of the lower wells of micro chemotaxis chambers (ANOVA S-N-K intervals showed significant differences between the values for serum free medium and fibronectin for all concentrations). The numbers of migrating cells reached a peak between 5-10 μg/ml fibronectin, with an optimal effect at 10 μg/ml for BRG, BRPE and ERM cells and that for BSF between 10 and 5 μg/ml of fibronectin (significant with ANOVA S-N-K intervals). The optimum migration of BSF appeared to be at 5 μg/ml of fibronectin, however there was no significant difference in numbers of BSF migrated between 5 and 10 μg/ml (ANOVA $p>0.05$) so 10 μg/ml of fibronectin was used as the positive control in comparisons of the migration of the cell types. The BRPE and BSF exhibited a post optimal decrease in migratory response between 5 and 100 μg/ml which was more marked than that for BRG, because the post optimal response for BRG was a protracted plateau up to 80 μg/ml fibronectin whereas the other two went into steep decline after the optimum had been reached.

The ERM cells appeared to be highly active (Fig. 3.1b.) with a mean baseline migration value of 36.7 cells migrated per 20 oil immersion fields (OIF) cells, rising to a mean of 193 cells with 10 μg/ml of fibronectin. The post optimal decrease in migration was initially steep and flattened out at 20 μg/ml of fibronectin and remained above 100 cells per 20 OIF with higher concentrations of the chemoattractant.

The optimum migratory responses between the four cell types were compared using the raw data, by ANOVA S-N-K interval analysis (see section 2.5.4.) and showed that there was no overall significant differences between cell numbers migrated of BRG and BSF and between BSF and BRPE. The BRG migrated in significantly greater numbers than the BRPE and the ERM cells significantly more than the other three cell types. Taking the baseline migration values into account the results were presented as proportional (Fig. 3.2a.) and percentage (Fig. 3.2b.) increase in migration over the baseline controls. The BRG showed proportionally more migration to fibronectin than the others and the ERM cells due to their high baseline value were seen to show proportionally less migration. In order to examine any possible effects of cell settlement on numbers of cells migrated, migration was also expressed as the percentage of the number of cells settled on the membrane (Fig.3.3.). There was no significant difference between the settlement of the test cell types (ANOVA S-N-K) except for the BSF which had a significantly greater settlement than the other three (ANOVA S-N-K). The percentage optimum migration was lower for the BSF and BRPE than BRG but by far the highest percentage migration was associated with the ERM cells (Mann Whitney U test for small samples $p<0.005$).

The experiments to find the correct membrane pore diameter for optimum migration of the test cells had shown that varying the pore diameter affected the migration of BRG and BRPE but not BSF (Fig. 3.4.). The migration of BRG cells through a membrane with pores of 10 μm diameter was 45% greater than that through a membrane with 8 μm pores (ANOVA $p<0.0004$) and that of the BRPE was 448% greater (ANOVA $p<0.0002$) and there was no significant difference for the BSF (ANOVA $p>0.05$). These findings confirmed our need to use the larger pore size of 10 μm for all experimentation. Relative cell sizing of the three cell types in suspension by flow cytometry (see Appendix I) showed that there was no overall difference in cell size between the BRG, BSF and BRPE. Intra- and inter- error counts performed for the migration experiments were 3% and 8% respectively.

3.1.2. Migration of Single Cell Types: II Zigmond Hirsch Analysis of the Migratory Response

Zigmond Hirsch chequerboard analysis of the migration of the test cell types (Fig. 3.5.) demonstrated that the cell types migrated both in the presence and absence of a positive concentration gradient indicating that the cells exhibited chemotaxis (left hand vertical columns) and chemokinesis (diagonal column). Chemotaxis was considered to be the more important component of the migratory responses for the BRG (ANOVA $p < 0.05$) and BSF ($p < 0.005$). However there was no significant differences between the optimum migration values for the BRPE ($p > 0.05$) and the ERM cells ($p > 0.05$). Basal levels of migration took place in the presence of a negative concentration gradient (top horizontal row) and these were minimal. The differences in the amount of chemotactic migration shown by the different cell types were also demonstrated by percentage migration values (Figs. 3.6. and 3.7.).

3.1.3. Migration of the Test Cells in Mixed Populations Labelled with Latex Beads

The results from section 3.1.1. show that all the test cell types including ERM cells migrated to fibronectin and as already stated BRG and BSF responded more than BRPE. In order to make more accurate comparisons between the migratory rates of the test cells, it was decided to migrate the cell types together in the same well of the micro chemotaxis chamber thereby decreasing the number of extraneous variables.

The optimum incubation time for the cultures with the latex labels was found to be 24 hours (Fig. 3.8.). By which time at least 90% of the population was labelled with beads. The beads tended to be located in the perinuclear region of the cell cytoplasm in the BRG and BRPE and more evenly distributed throughout the cytoplasm in the BSF.

The cells were assayed with and without latex microspheres (Fig. 3.9.) and those doses of beads that did not affect migration (5000 beads per cell for BRG and BRPE and 1000 beads per cell for BSF) were chosen for use on subsequent migration runs (Fig. 3.10a.). There was no significant difference between

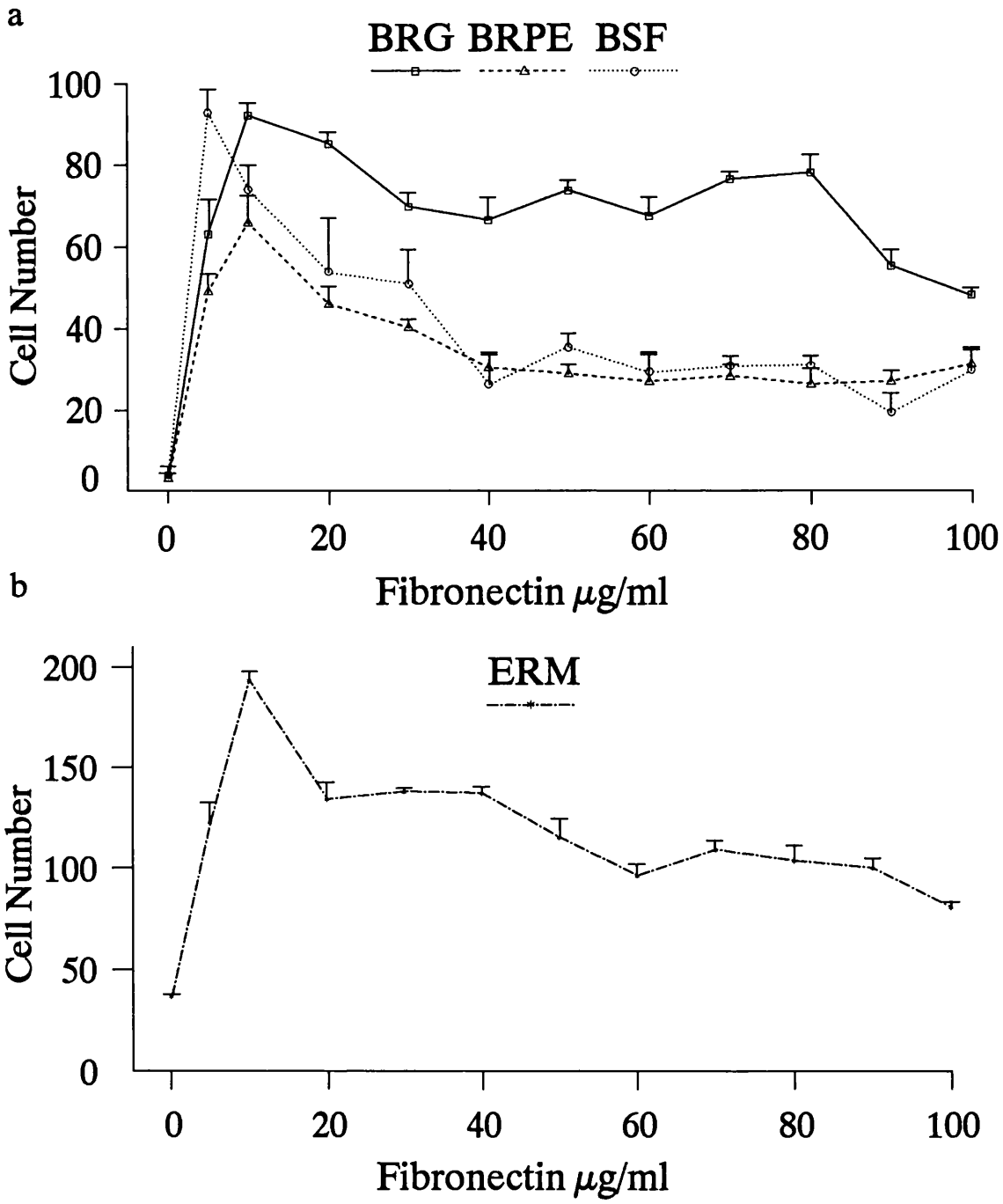


Fig. 3.1. Dose response curves of the migration of BRG, BRPE, BSF (a) and ERM cells (b) to fibronectin. Migration is expressed as the mean number of cells migrated per 20 high power oil immersion fields (OIF). Each point represents the mean ± SEM of four wells and the experiments were repeated three times.

a

Cell Type	Optimum Response ± SEMs	Negative Control ± SEMs	Proportional Increase in Migration
BRG	92.3 ± 2.5	3.3 ± 0.9	28.0
BRPE	65.8 ± 6.6	3.0 ± 0	21.9
BSF	93.0 ± 5.0	4.0 ± 2.0	23.3
ERM	193.3 ± 4.9	36.7 ± 0.9	5.3

b

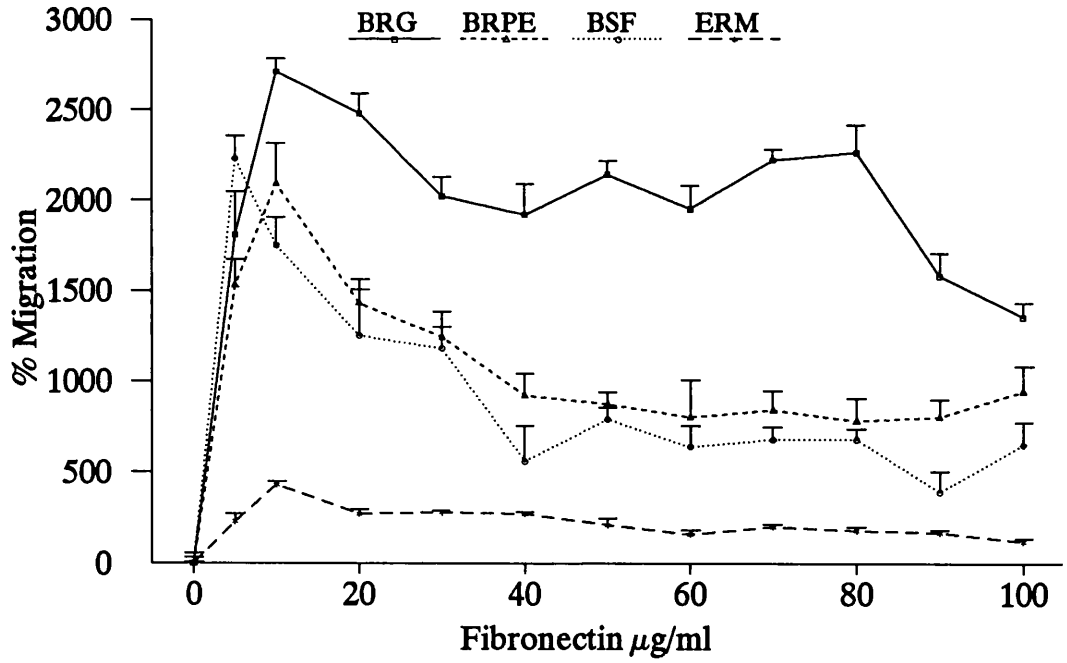
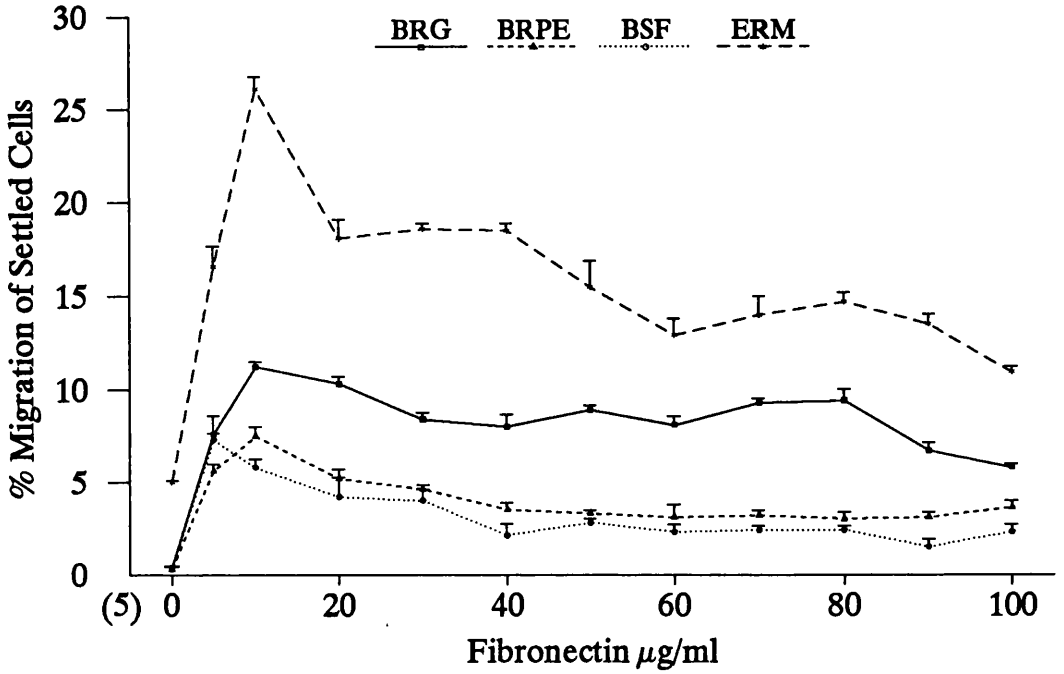


Fig. 3.2. Table (a) of the responses for proportional increase in optimum migration of the test cell types over the negative control (serum free medium) values. Graph (b) of the dose response curves of the percentage increase (± SEM) in migration of BRG, BRPE, BSF and ERM cells over the negative control (serum free medium) values.

a



b

Cell Type	Settlement per 20 OIF ± SEM	% Migrated Optimum Values
BRG	826.7 ± 77.0	11.2
BRPE	880.0 ± 41.7	7.5
BSF	1273.3 ± 78.7	7.3
ERM	740.0 ± 59.3	26.1

Fig. 3.3. Percentage migration of BRG, BRPE, BSF and ERM of the total number of cells settled on the chemotaxis membrane. Dose response curve (a) and table of optimum values (b).

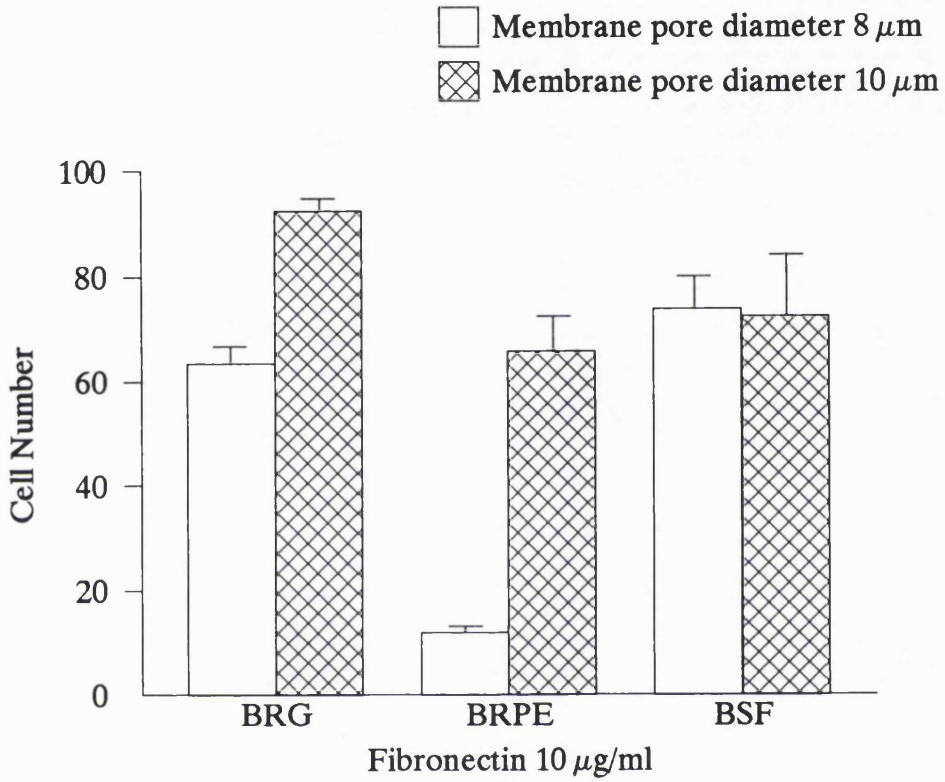


Fig. 3.4. The migration of BRG, BRPE and BSF on membranes of pore size 8 and 10 μm in diameter. The chemoattractant is 10μg/ml of fibronectin. Each point represents the mean ± SEM of six wells and the experiment was repeated twice.

the numbers of cells migrated to fibronectin with or without the label (ANOVA $p>0.05$). The settlement of cells labelled with latex beads was not significantly decreased for BRPE or BSF (ANOVA $p>0.05$). Although there was a significant decrease in the settlement of BRG with beads ($p<0.001$) it appeared to have little effect on the percentage of cells migrated (Fig. 3.10b.).

The doses of latex beads that did not affect the migration of the test cells having been established, the next step in the mixed migration experiments was carried out. Mixed populations of the test cell types were migrated together in the following order:-

1. BRG and BRPE
2. BRPE and BSF
3. BSF and BRG

Of each pair migrated, one cell type was labelled and one not (Fig. 3.11a). The migration of the BRG when mixed with BRPE was significantly greater than the latter (ANOVA $p<0.01$) and the migration of the BSF was greater than the BRPE (ANOVA $p<0.01$). There was no significant difference between the migration of the BRG and BSF (ANOVA $p>0.05$). The cross over experiments (Fig. 3.11b) showed the same migratory trend with significant differences for BRG and BRPE (ANOVA $p<0.02$), BSF and BRPE (ANOVA $p<0.001$) and only a marginal difference for BRG and BSF (ANOVA $p<0.04$). The variations in the numbers of cells migrated were independent of settlement as there were no significant differences in settlement values for any of the pairs (ANOVA $p>0.05$).

A phase contrast light micrograph of the paired migrated cells on the polycarbonate membrane illustrates how clearly labelled and unlabelled cells could be distinguished (Fig. 3.13.). Also that there were no visible differences in morphology when the labelled and the unlabelled cells were compared.

a

Upper Wells

Lower Wells / Fibronectin (μg/ml)	0	2.5	5.0	10.0
0	11.3 ±1.2	15.7± 2.6	14.7± 0.9	20.1± 0.6
2.5	50.3±8.7	38.0 ±1.5	30.3± 3.7	22.3± 3.3
5.0	66.3 ±2.3	51.7±9.8	57.3±6.4	39.7± 2.6
10.0	79.7 ±5.2	84.7±20.4	92.0±6.6	59.5±3.0

b

Upper Wells

Lower Wells / Fibronectin (μg/ml)	0	5.0	10.0	20.0
0	2.3±2.3	2.2±3.7	0.7±0.3	0.7±0.7
5.0	35.0±7.2	8.3±1.9	7.7±1.8	5.3±1.9
10.0	28.0±3.1	20.7±5.8	15.7±3.5	5.7±1.8
20.0	26.3±5.6	21.3±2.9	10.3±2.3	11.0±1.2

c

Upper Wells

Lower Wells / Fibronectin (μg/ml)	0	2.5	5.0	10.0
0	14.0±3.2	9.7 ±2.0	10.3± 3.7	9.0± 3.8
2.5	124.0±5.8	58.7±16.8	54.0±14.7	29.5±4.5
5.0	125.0±10.0	77.7±15.1	66.7±9.3	8.5±1.6
10.0	103.0±7.4	77.3±12.8	64.7±16.0	69.5±2.0

d

Upper Wells

Lower Wells / Fibronectin (μg/ml)	0	5.0	10.0	20.0
0	1.3 ±0.7	1.7 ± 0.9	0.7± 0.3	1.0± 0.6
5.0	5.7±2.7	6.3 ±1.8	5.3±2.2	0.3±0.3
10.0	8.7 ±1.5	4.3±2.9	9.3 ±3.0	0.3± 0.3
20.0	15.0±1.5	6.3± 1.2	4.0± 2.5	2.7 ±1.8

Fig. 3.5. Zigmond Hirsch chequerboard analysis of the migration of BRG (a), BRPE (b), BSF (c) and ERM (d) cells to fibronectin. Migration is expressed as the mean number of cells migrated per 20 high power oil immersion fields (OIF). Each point represents the mean ± SEM of four wells and the experiments were repeated twice.

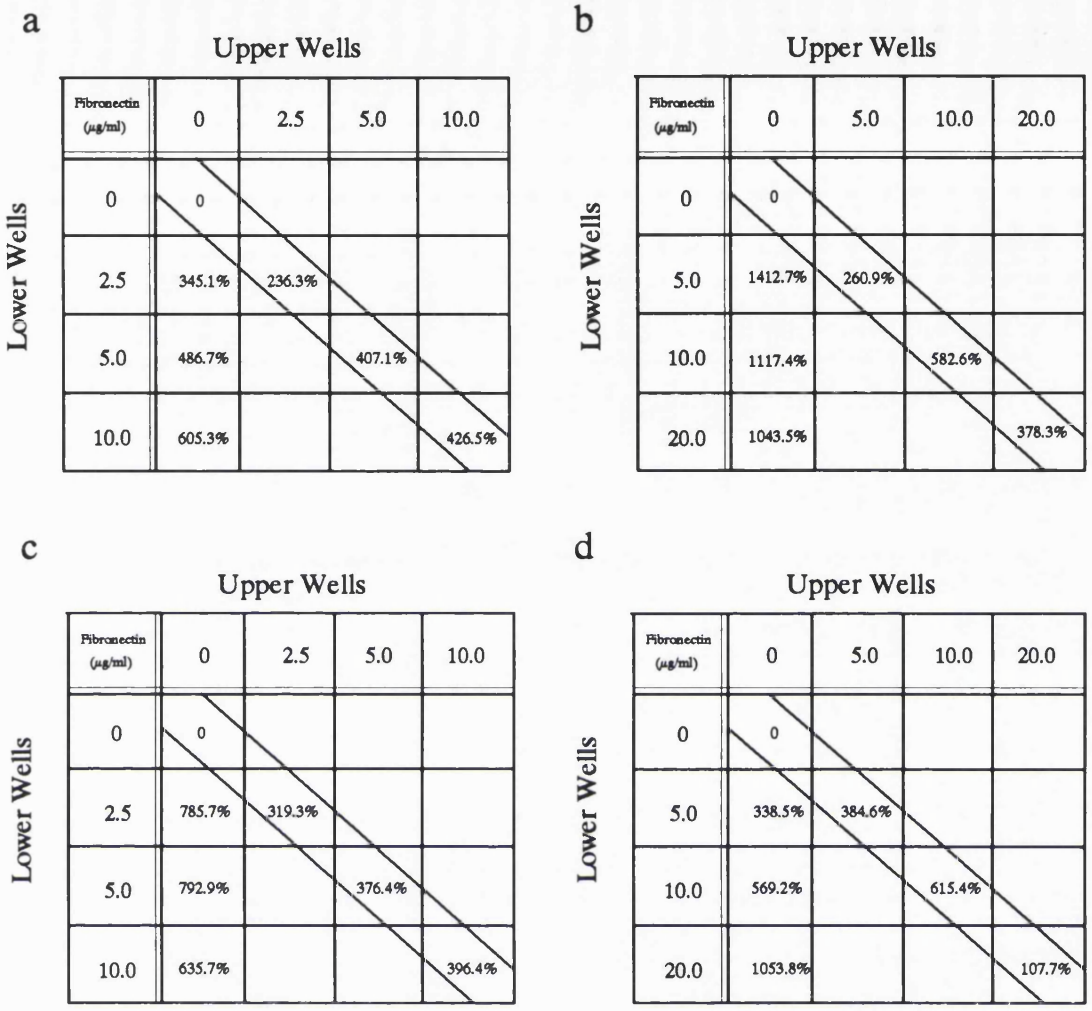


Fig. 3.6. Zigmond Hirsch chequerboard analysis of the migration of BRG (a), BRPE (b), BSF (c) and ERM (d) cells to fibronectin; percentage increase values over the baseline control values ± SEM.

a. Bovine retinal glia				
1	2	3	4	5
[Fn]	%CT ± SEM	%CK ± SEM	% CT/CK	CT/CK
2.5	345.1 ± 77.0	236.3 ± 13.3	46.0	1.5
5.0	486.7 ± 20.4	407.1 ± 56.6	19.6	1.2
10.0	605.3 ± 46.0	426.5 ± 24.8	42.0	1.4

b. Bovine retinal pigment epithelium				
1	2	3	4	5
[Fn]	%CT ± SEM	%CK ± SEM	%CT/CK	CT/CK
5.0	1421.7 ± 313.1	206.9 ± 82.6	445.0	5.4
10.0	1117.4 ± 134.8	582.6 ± 152.2	91.8	1.9
20.0	1043.5 ± 243.5	378.3 ± 52.1	175.8	2.8

c. Bovine scleral fibroblasts				
1	2	3	4	5
[Fn]	%CT ± SEM	%CK ± SEM	%CT/CK	CT/CK
2.5	785.7 ± 112.9	319.3 ± 120.0	146.1	2.5
5.0	729.2 ± 71.4	376.4 ± 66.5	110.7	2.1
10.0	635.7 ± 52.9	396.4 ± 14.3	60.4	1.6

d. Epiretinal membrane cells				
1	2	3	4	5
[Fn]	%CT ± SEM	%CK ± SEM	%CT/CK	CT/CK
5.0	338.5 ± 207.7	384.6 ± 138.5	-12.0	0.9
10.0	569.2 ± 25.4	615.4 ± 230.8	-7.5	0.9
20.0	1053.8 ± 115.4	107.7 ± 138.5	878.5	9.8

Fig. 3.7. Tables of values for Zigmond Hirsch chequerboard analysis of the migration of BRG (a), BRPE (b), BSF (c) and ERM (d) cells to fibronectin.
Abbreviations:-
Column 1:- Concentration of fibronectin μg/ml ([Fn]).
Column 2:- Percentage increase in chemotaxis over negative control (%CT).
Column 3:- Percentage increase in chemokinesis over negative control (%CK).
Column 4:- Percentage increase in chemotaxis over chemokinesis (%CT/CK).
Column 5:- Proportional increase in chemotaxis over chemokinesis (CT/CK).

3.1.4. Migration of the Test Cells in Mixed Populations Labelled with Carmine and Latex Beads

The test cultures of cells were labelled with carmine for 24 hours and the labelling efficiencies (Fig. 3.14.) ranged from 83% and 88% for the BRG and BRPE respectively, to 90% percent for the BSF. The cells were migrated with and without carmine at a dose of 50 particles per cell (Fig. 3.15.) and there were significant differences with and without carmine for all three cell types (ANOVA $p<0.01$) but settlement of all three cell types was not affected by carmine (ANOVA $p>0.05$). When the BSF were labelled with 25 particles per cell they showed no difference in their migration to fibronectin when compared to unlabelled BSF (Fig. 3.16.)(ANOVA $p>0.05$). The settlement and the percentage of migrated BSF was not affected by the presence of carmine (ANOVA $p>0.05$) (Fig. 3.17.). Carmine was clearly visible in the cytoplasm of the BSF both on the settled and migrated sides of the membrane (Fig. 3.18). BRPE were labelled with latex beads and the BRG were left unlabelled.

When all the three test cell types were assayed together in the same chemotaxis well (Fig 3.19a.) the migration of all three cell types were significantly different from each other (ANOVA S-N-K intervals). The BRPE cells migrated significantly less well than the other two cell types (ANOVA BRG/BRPE $p<0.0001$, BSF/BRPE $p<0.0001$). The BRG migrated in larger numbers than the BSF (ANOVA $p<0.0001$).

The cell labels clearly distinguished the cell types and it proved easy to differentiate between them, so that counting migrated cells by light microscope was not found to be a problem (Fig.3.20.). Some of the beads and more occasionally a carmine particle were seen to be loose (Fig.3.20.) and therefore could possibly have been phagocytosed by non-labelled cells. Even less frequently, cells were seen that had ingested both latex beads and carmine. As a precaution, cells with less than three latex beads or particles of carmine or cells with both labels present, were excluded from the counts.

Finally the chemoattractant fibronectin was replaced by the growth factor PDGF (see section 1.4.2). PDGF is a well established chemoattractant for the cell types involved in ERM formation and the use of an alternative chemoattractant like PDGF allowed us to test whether the effects already shown were restricted solely to fibronectin. It was clear that the results with PDGF (Fig 3.21a.) were comparable to those found with

Latex Beads: 5000 beads/cell		
Cell Type	Labelling Time (hours)	% Cells Labelled
BRG	16	80.5
BRPE	16	78.3
BSF	16	83.2
BRG	24	99.7
BRPE	24	90.0
BSF	24	99.3

Fig. 3.8. Labelling times for BRG, BRPE and BSF with latex microspheres.

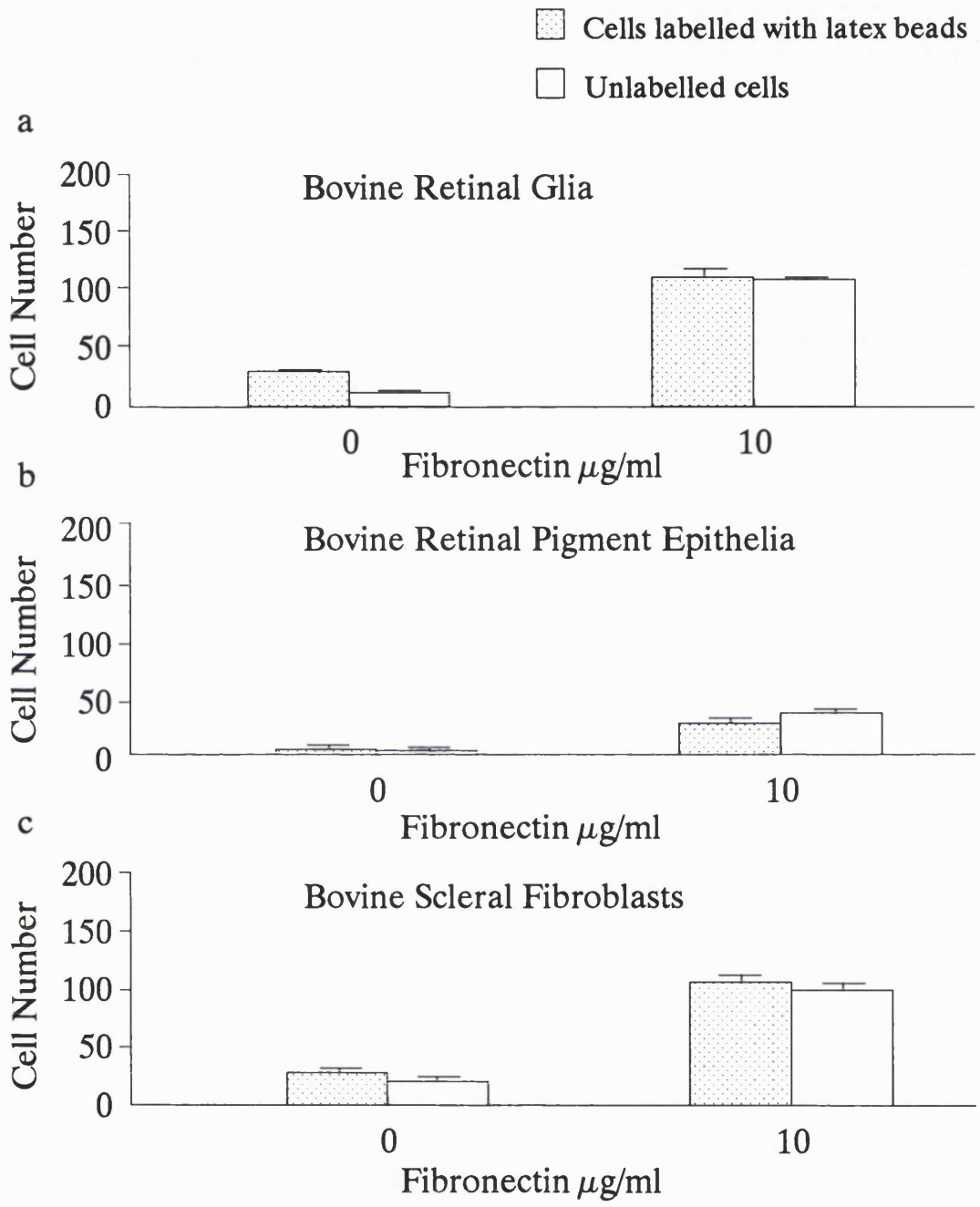


Fig. 3.9. Migration of BRG (a), BRPE (b) and BSF (c) labelled with and without latex beads, to 10 μg/ml soluble fibronectin. Each experiment was run three times and each point represents the mean ± SEM of four wells.

a

Latex Beads			
Cell Type	Dose Beads/Cell	Uptake Beads/Cell ± SEM	Cell Numbers Migrated per 20 OIF ± SEM
BRG	5000	444.7 ± 65.7	+ Beads 111.0 ± 7.4
			- Beads 109.4 ± 2.2
BRPE	5000	318.7 ± 106.3	+ Beads 41.2 ± 2.0
			- Beads 46.3 ± 4.6
BSF	1000	916.7 ± 148.3	+ Beads 106.3 ± 5.3
			- Beads 100.0 ± 5.6

b

Cell Type	Settlement per 20 OIF ± SEM	% Decrease in Settlement +Beads/-Beads	% Cell Numbers Migrated
BRG + beads	1136.0 ± 35.9	26.8	9.8
BRG - beads	1552.7 ± 52.1		7.0
BRPE + beads	565.3 ± 27.4	7.2	7.3
BRPE - beads	609.3 ± 17.4		7.6
BSF + beads	946.7 ± 20.5	11.2	11.3
BSF - beads	1064.0 ± 48.0		9.4

Fig. 3.10. Table (a) of optimum labelling values for the migration of BRG, BRPE and BSF with latex beads. Table (b) of settlement and percentage migration values for the migration of BRG, BRPE and BSF with and without latex beads.

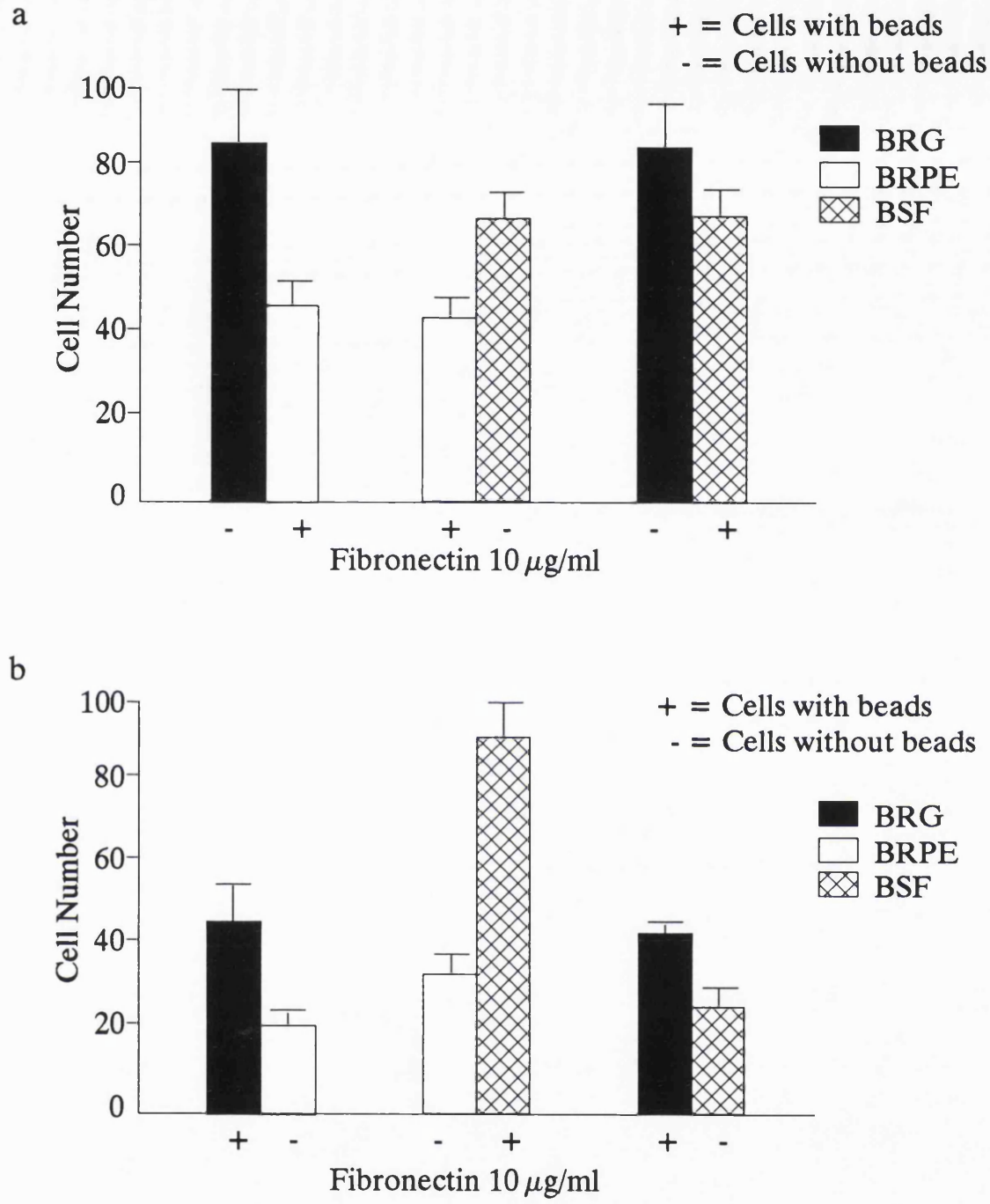


Fig. 3.11. Paired migration of BRG with BRPE, BRPE with BSF and BRG with BSF. Each cell type in the pair was labelled with and without the beads in the same Boyden chamber (a and b). Each point represents the mean ± SEM of six wells and the experiment was run three times.

Cell Type	Settlement per 20 OIF ± SEM	Migration per 20 OIF	% Cell Numbers Migrated/ Settlement
BRG - beads	246.7 ± 66.7	43.3 ± 6.5	17.6
BRPE + beads	246.7 ± 29.1	23.8 ± 2.6	9.6
BRPE + beads	273.3 ± 13.3	22.2 ± 2.6	8.1
BSF - beads	260.0 ± 23.1	34.2 ± 2.9	13.2
BRG - beads	306.7 ± 46.7	42.8 ± 5.1	14.0
BSF + beads	240.0 ± 20.0	34.5 ± 3.0	14.4
BRG + beads	80.0 ± 11.6	23.0 ± 4.4	28.8
BRPE - beads	113.3 ± 33.4	10.5 ± 1.6	9.3
BRPE - beads	246.7 ± 43.8	16.8 ± 2.1	6.8
BSF + beads	286.7 ± 6.7	45.5 ± 4.3	15.8
BRG + beads	246.7 ± 48.1	21.5 ± 1.0	8.7
BSF - beads	226.7 ± 46.7	12.8 ± 2.1	5.6

Fig. 3.12. Table of settlement and percentage migration values for the migration of BRG, BRPE and BSF with and without latex beads.

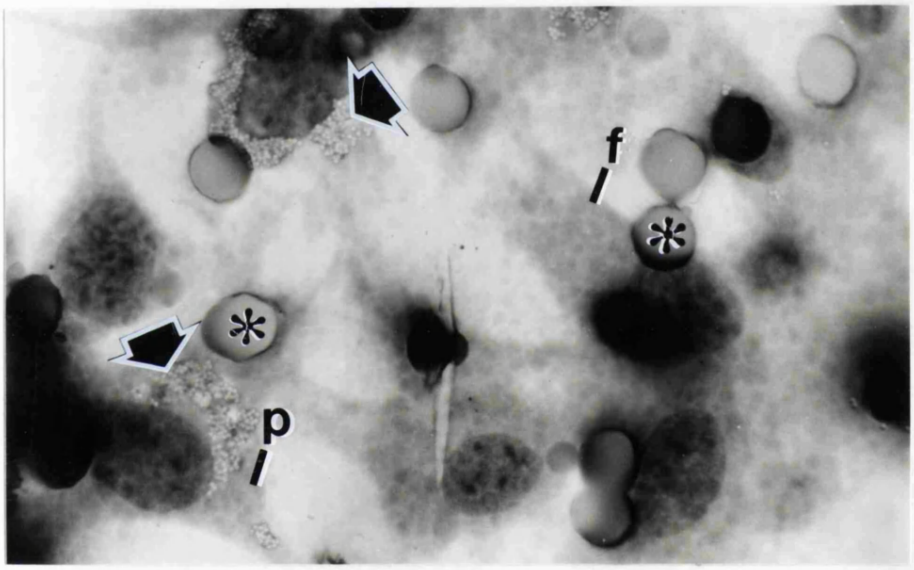


Fig. 3.13. A light micrograph of migrated BRPE and BSF stained with haematoxylin on a polycarbonate chemotaxis membrane with pores (asterisks) of 10 μm in diameter. The bead labelled BRPE (p), with the beads mainly concentrated in the perinuclear area (arrows) are clearly distinguishable from the unlabelled BSF (f). (Magnification:- x 850).

fibronectin (Fig 3.19.). BRPE cells showed a comparatively low level of migration with an optimum response of 40 cells per 20 OIF at 50 ng/ml of PDGF and decreasing again at 100 ng/ml. The BRG produced a good response to the growth factor, rising to a peak response of 200 cells per 20 OIF at 25 ng/ml of PDGF. The BSF also responded well and reached an optimum of 130 cells per 20 OIF between 25 to 50 ng/ml of growth factor. The mean response of BRPE for all the concentrations of PDGF used (apart from 100 ng/ml) was significantly lower than BRG and BSF and the BRG response was significantly higher than the BSF at 10, 25 and 50 ng/ml PDGF (ANOVA S-N-K intervals). Percentage migration over the negative control values produced the same pattern of results (Fig. 3.21b.).

3.2. Behavioural Comparison of the Migration of the Three Cell Types to Samples of Subretinal Fluid and a Retinal Crude Extract

The mixed migration studies showed that in spite of the variation in the single cell type migration to fibronectin the migration rates of BRG and BSF were similar and consistently greater than those of the BRPE. The mixed cell migration model therefore was extremely effective in that it served to vindicate the single cell migration results. However from a purely practical point of view it was a complex and difficult procedure to carry out with possible contact reactions between the cells that have been investigated by other means (see section 3.4.).

Mixed migration was felt to be an eminently suitable method where the chemoattractant was a single known substance such as fibronectin or PDGF and the question to be answered fairly straight forward. However fluids such as SRF and RCE are more complex in character containing variable amounts of potential chemoattractants, not all of which are known. So it was decided that more useful information would be gained from migrating single cell types to SRF and RCE and only if time and number of samples permitted, extend the study to mixed cell migration. In these studies therefore, the cell types were migrated in separate wells and not labelled.

For the results to be comparable, it was necessary to have positive, as well as negative, controls for the migration assays. In the previous sections, fibronectin had been established as a

Carmine				
Cell Type	Labelling Time (hrs)	Dose (Particles/ Cell)	Uptake (Particles/ Cell) ± SEM	% Cells Labelled
BRG	24	50	37.3 ± 6.0	83
BRPE	24	50	36.5 ± 6.3	88
BSF	24	50	46.7 ± 5.8	90
BSF	24	25	18 ± 3.7	90

Fig. 3.14. Table of concentrations and times for labelling BRG, BRPE and BSF with carmine.

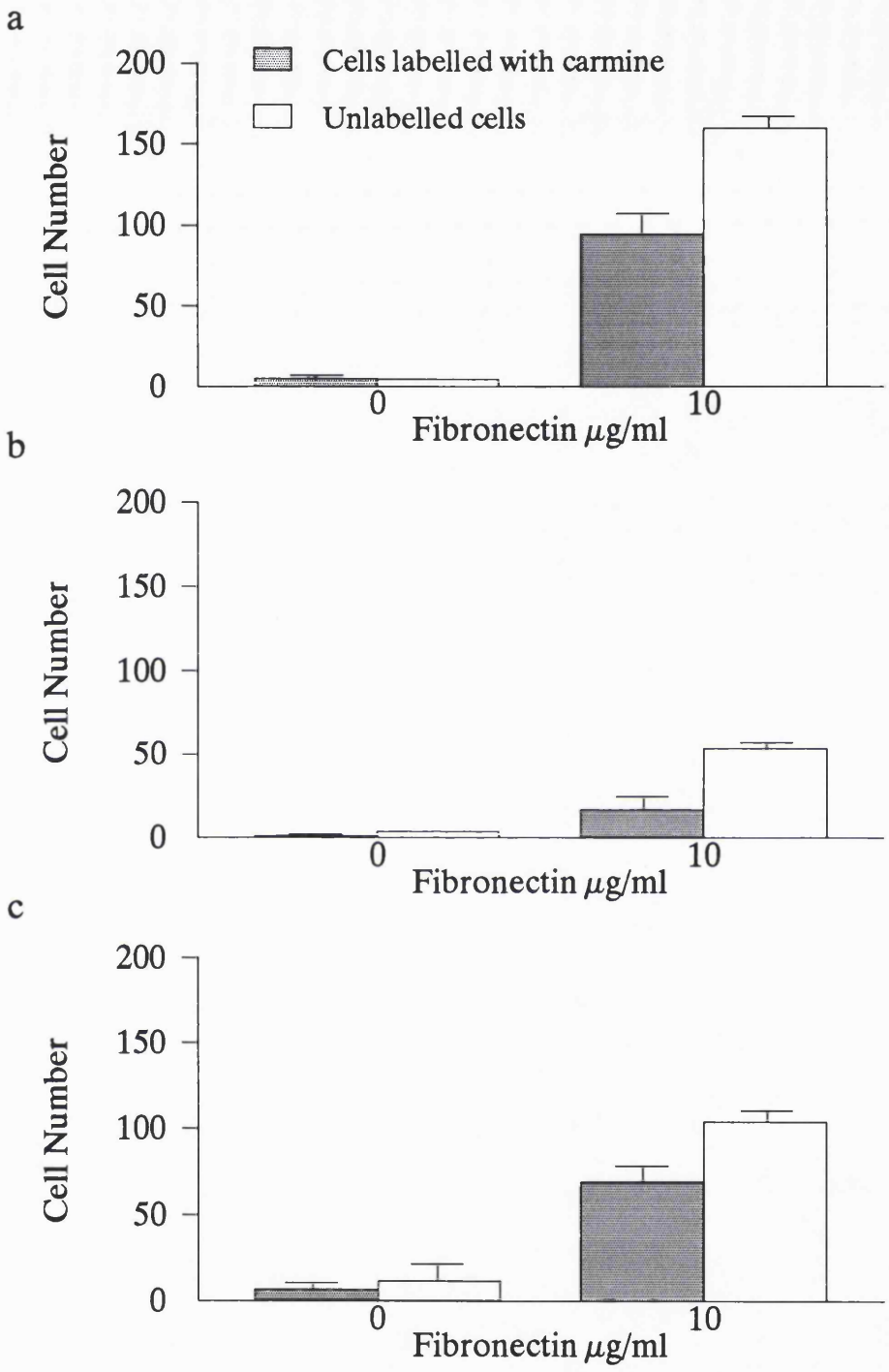


Fig. 3.15. Migration of BRG (a), BRPE (b) and BSF (c) with and without 50 particles/cell of carmine, to 10 μg/ml fibronectin. Each point represents the mean ± SEM of six wells.

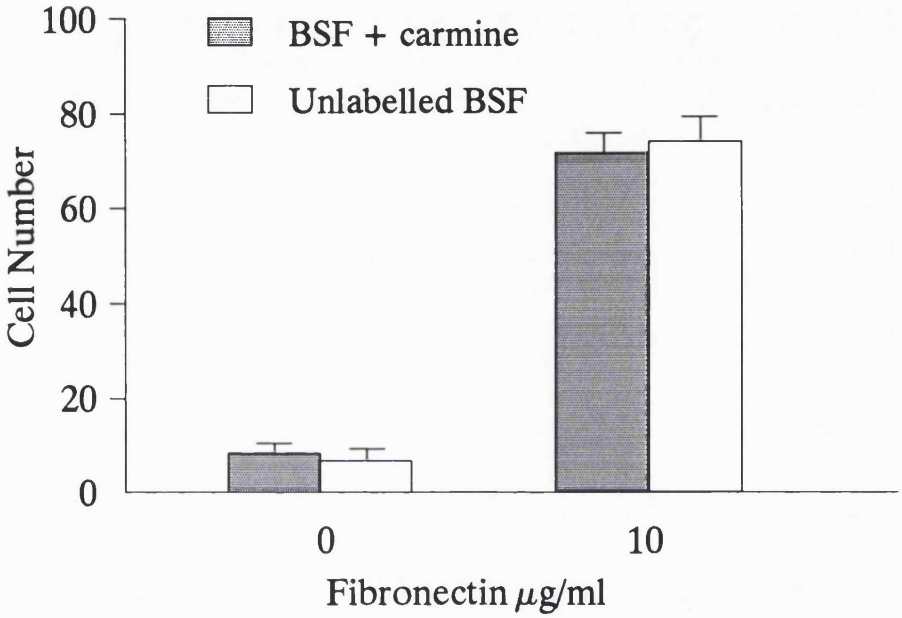


Fig. 3.16. Migration of BSF with and without 25 particles/cell of carmine, to 10 μg/ml fibronectin. Each point represents the mean ± SEM of six wells.

Cell Type	Dose Particles/ Cell	Settlement per 20 OIF ± SEM	% Migrated
BRG + carmine	50	1366.7 ± 135.0	6.9
BRG - carmine	0	1366.7 ± 13.3	11.7
BRPE + carmine	50	626.7 ± 153.5	2.7
BRPE - carmine	0	1013.3 ± 75.2	5.3
BSF + carmine	50	813.3 ± 6.7	8.5
BSF - carmine	0	1486.7 ± 289.3	7.0
BSF + carmine	25	1386.7 ± 150.9	5.2
BSF - carmine	0	1740.0 ± 237.2	4.25

Fig. 3.17. Table of settlement and percentage migration values for the migration of BRG, BRPE and BSF with and without carmine.

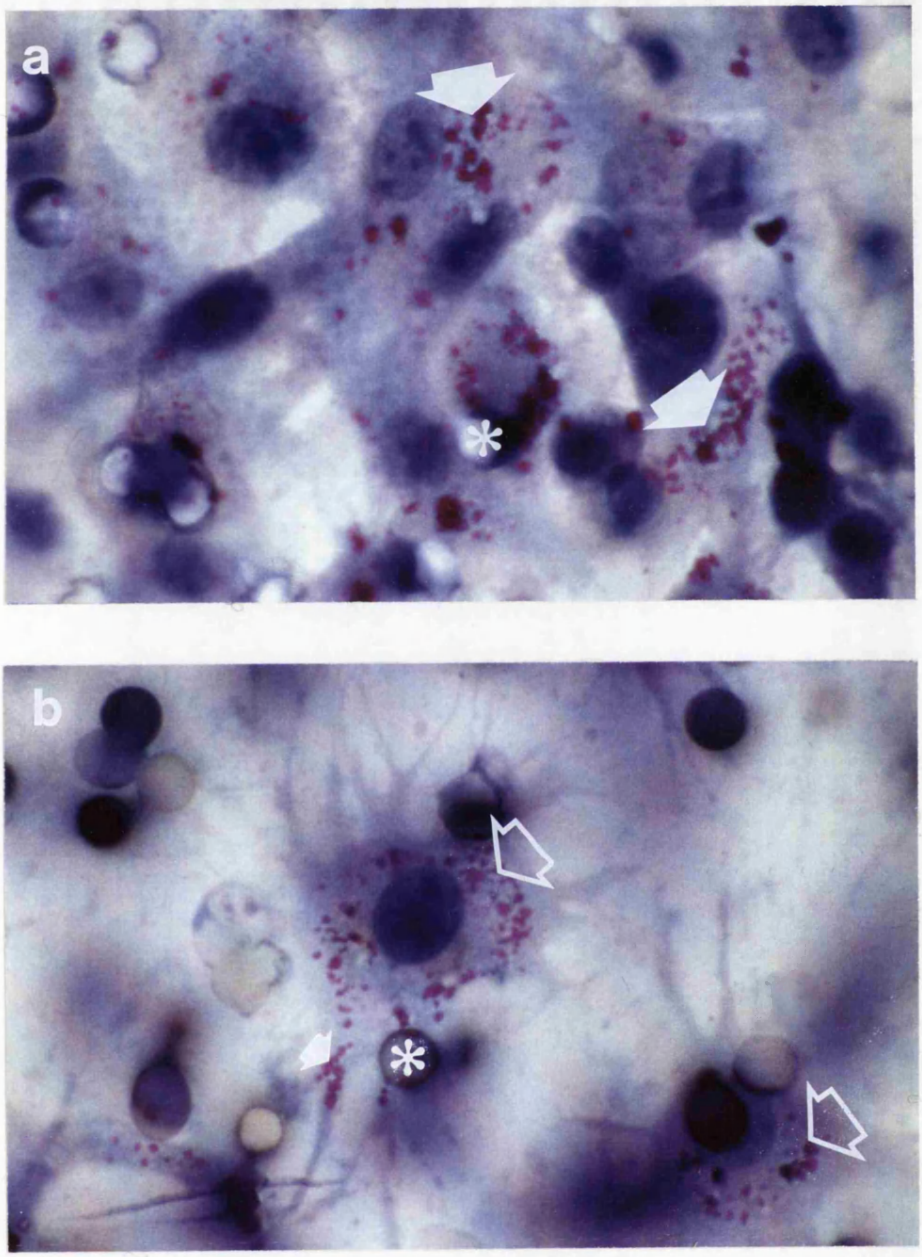


Fig. 3.18. Light micrographs of BSF labelled with carmine and stained with haematoxylin on a polycarbonate membrane with 10 μm diameter pores (asterisks). Cells are shown settled on the upper surface (a) and migrated on the underside (b) of the membrane. The carmine particles are located mainly in the perinuclear regions of the cells (large arrows) and occasionally in the cellular processes (small arrows). (Magnifications:- (a) x 900, (b) x 860).

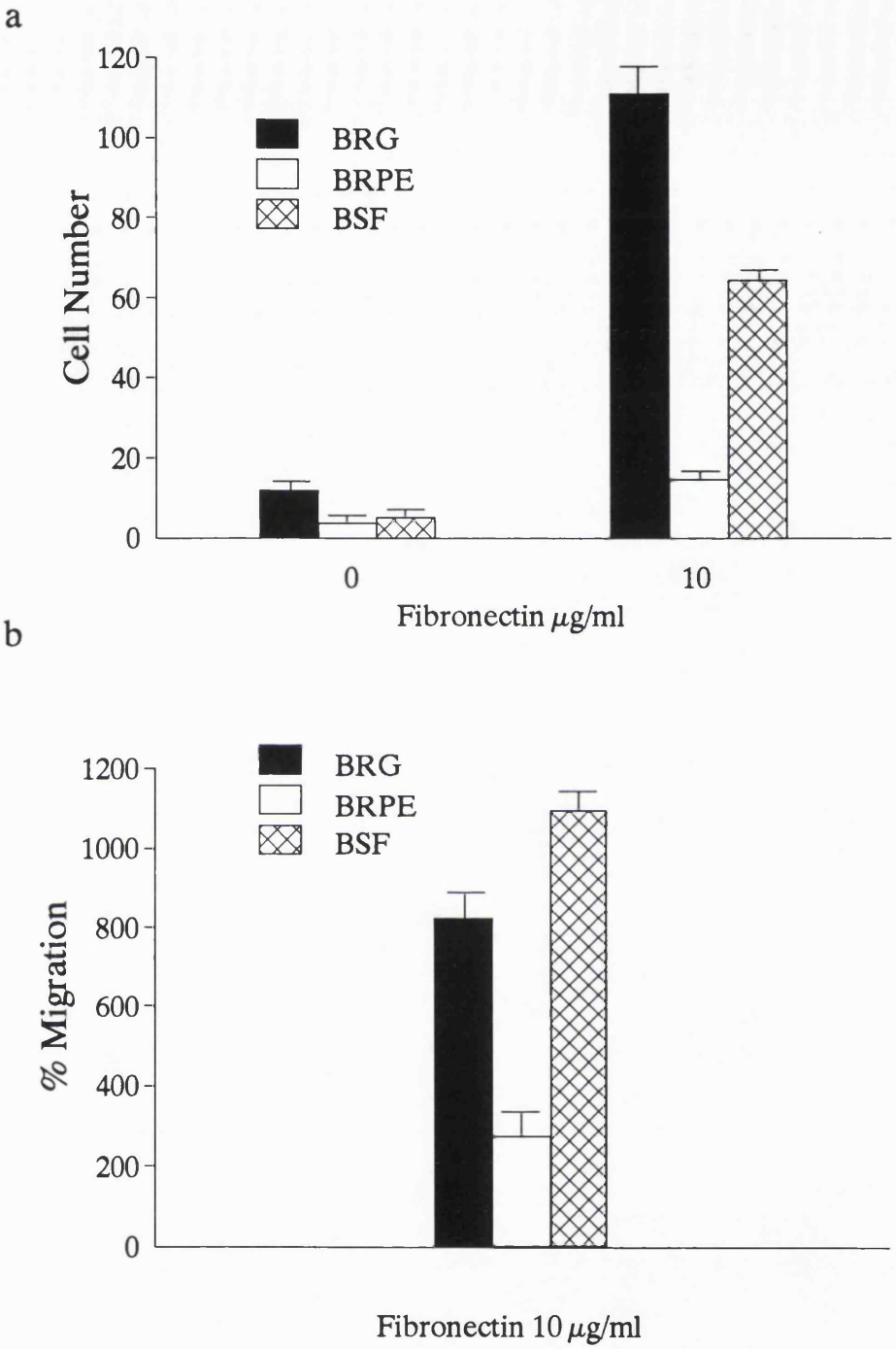


Fig. 3.19. Migration of the three cell types together to 10 μg/ml fibronectin. The BRG are labelled with latex microspheres and the BSF with carmine. The experiment was repeated twice and each point represents the mean ± SEM of twelve wells.

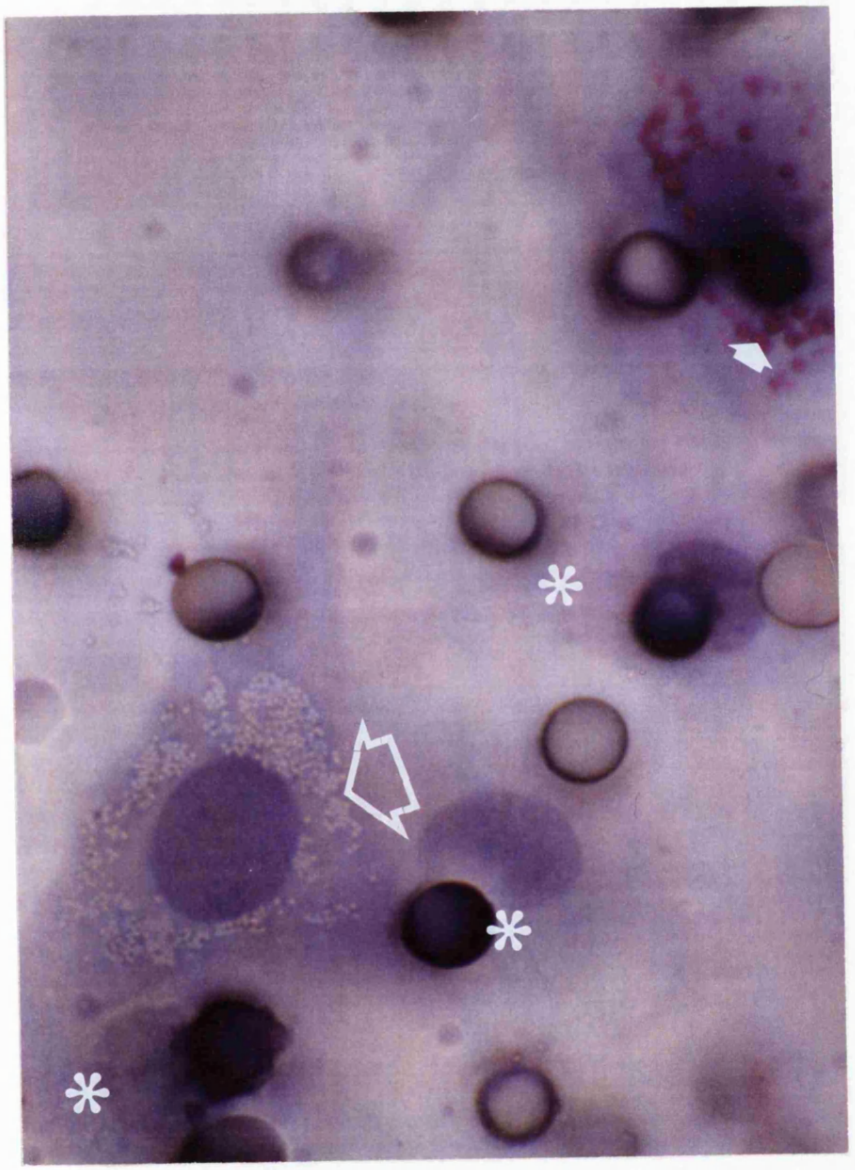


Fig. 3.20. Light micrograph of migrated cells on the lower surface of a polycarbonate chemotaxis membrane, the settled cells on the upper surface have been removed by scraping. The BRG (asterisks) are unlabelled, the BRPE cell is labelled with latex beads (large arrow) and the BSF is distinguished by the presence of carmine (small arrow). (Magnification:- x 1100).

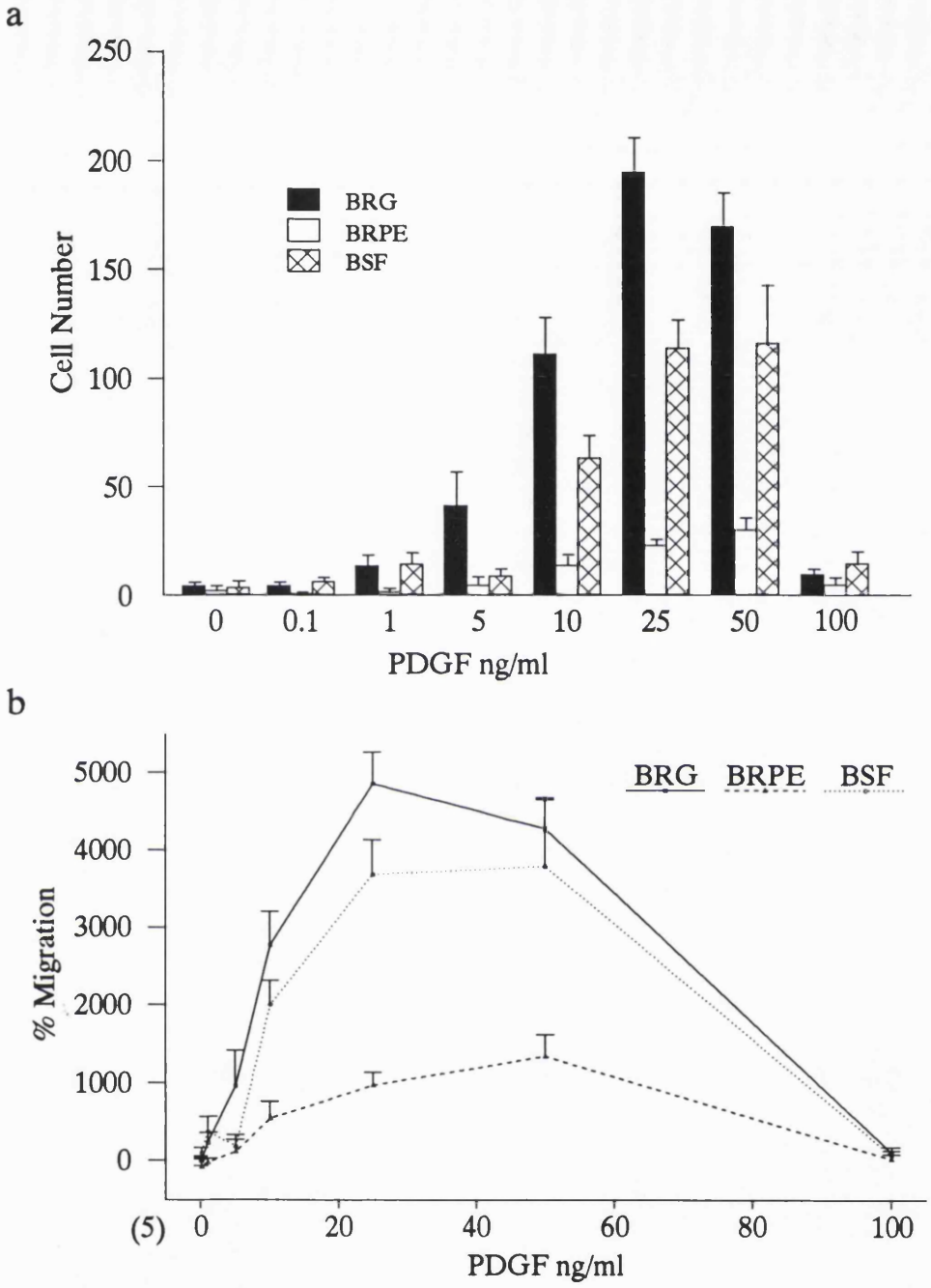


Fig. 3.21. Dose response curves of the migration of the three cell types together to platelet derived growth factor, cell numbers (a) and percentage migration over the negative control (b). Each point represents the mean ± SEM of six wells and the experiment was repeated twice.

reliable stimulant for the migration of the test cells and was consequently used as the positive control in the following studies and the results presented as a percentage value of the same. It was used at a concentration of 10 μg/ml, as this level was found to be the optimal concentration of fibronectin for the migration of the three test cell types.

3.2.1. Migration to Subretinal Fluid

The three test cell types migrated to an SRF fluid sample (Fig. 3.22a.), the BSF cells dominated and the responses from the BRG and BSF were much lower. The SRF was drained from an eye with a longstanding retinal detachment (Fig. 3.22b and Grade C PVR as per the Retinal Society Terminology Committee classification, 1983; see Figure 1.6. (for patient details see Fig. 2.9.). The optimum migration for the BSF was at the concentration of 10 μl/ml SRF or possibly lower (not tested). However the optimum values for BRG and BRPE were at 50 μl/ml and the response from the BSF was extremely high at this concentration, so 50 μl/ml was used as the final dilution for the subsequent SRF migration runs.

The nine SRF samples were drained from the eyes of patients undergoing surgery for a variety of conditions (Fig. 3.23.). Three were from patients with simple rhegmatogenous retinal detachment, two from those with PVR (Grade C) and four had proliferative diabetic retinopathy (PDR) (see also Fig. 2.9.).

A comparison was made of the percentage migration of the three different cell types to all nine SRF samples (Fig. 3.24.). The was overall significantly different amounts of migration between the three cell types (ANOVA S-N-K intervals). Again the BSF displayed the largest amount of migration of the three, ranging from approximately 100% to 550% greater than that to fibronectin (ANOVA $p<0.001$), apart from sample PDR3 where there was no significant difference between the migration of BRG and BSF (ANOVA $p>0.05$) the migration of BRG ranged from 50% to approximately 200% and the BRPE only weakly responded in the range of 2% to 100%. Irrespective of whether the test sample was from a patient with a simple detachment, PVR or PDR, in each case the BSF response was greatest, the BRG response was next and the BRPE consistently exhibited the weakest migrational response of the three cell types (ANOVA $p<0.001$).

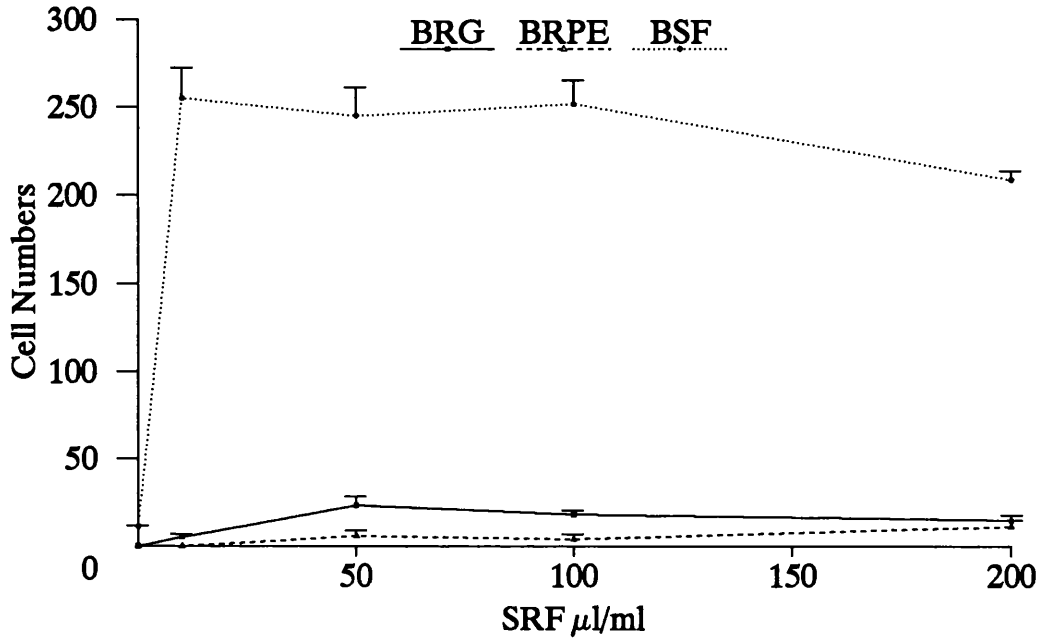
Bearing in mind that the number of samples available for the investigation were extremely restricted, the data was examined to see if there were possible differences between SRF from simple retinal detachments and SRF from proliferative disease (PVR and PDR). For each cell type, migration was more pronounced to the fluid from the eyes with proliferative disease than to the simple detachments. The BRG response was increased by less than 2 fold, BSF by a little more than 2 fold but the BRPE increase was by well over 4 fold. The migratory response of all three cell types was graded for each of the 9 specimens and the fluid from the three simple retinal detachments showed the least amount of chemoattraction. The BRPE were the lowest (Mann Whitney U test for small samples $p<0.05$) and were graded 7th, 8th and 9th. The BRG and BSF were graded 6th, 8th and 9th in each case.

3.2.2. Migration to a Bovine Retinal Crude Extract

There was a noticeable difference in the migratory responses of the three bovine cell types to bovine retinal crude extract (Fig. 3.25.). All elicited some level of response but for BRPE it was minimal being only 10% of the optimal fibronectin response at 30 μl/ml and this was only marginally greater than background (ANOVA $p<0.05$). On the other hand the BRG migration was 50% of the optimal fibronectin response (ANOVA $p<0.01$) and the response of the ERM cells was not significantly different from the BRG (ANOVA $p>0.05$) and the BSF response was massive being over 350% greater than optimal fibronectin and clearly this response was significant (ANOVA $p<0.001$). Extending the concentration of bovine retinal crude extract beyond the 30 μl/ml level did not increase migration any further for any of the three cell types (data not shown).

An attempt was made to determine more clearly the nature of the fibroblast chemoattraction to retinal crude extract using the Zigmond-Hirsch chequerboard analysis (Fig.3.26.). A stepwise increment in migration to an increasing gradient was evident and the difference was marginally significant (ANOVA $p<0.04$) but equally, a stepwise rise in the migration of the fibroblasts to an increasing concentration of attractant without a gradient was also present (ANOVA $p<0.005$) (Fig.3.26a.). Both chemotaxis (left hand vertical line) and chemokinesis (the diagonal line) were identified and these were seen to be similar with no significant

a



b

Patient Number	Diagnosis	Cell numbers Migrated ± SEM to SRF (μl/ml)		
		Cell Type	10	50
1	Longstanding retinal detachment with PVR (Grade C)	BRG	5.3 ± 0.6	23.3 ± 4.0
		BRPE	0	6.0 ± 2.6
		BSF	254.9 ± 18	244.9 ± 15

Fig. 3.22. Dose response curve of the migration of the three cell types to a sample of subretinal fluid from an eye with retinal detachment (a). Each point represents the mean ± SEM of six wells. Table of diagnostic details and optimum migration values (b).

Patient Number	Diagnosis	Sample Number	Percentage Migration ± SEM
2	Traumatic longstanding retinal detachment	RD1	BRG 113.7 ± 10.8 BRPE 2.3 ± 2.3 BSF 156.6 ± 15.9
3	Recurrent rhegmatogenous retinal detachment	RD2	BRG 41.1 ± 5.0 BRPE 7.14 ± 0 BSF 90.1 ± 14.4
4	Rhegmatogenous retinal detachment	RD3	BRG 117.4 ± 13.6 BRPE 42.8 ± 8.2 BSF 245.1 ± 6.63
5	Proliferative vitreo-retinopathy (Grade C)	PVR1	BRG 165.9 ± 5.4 BRPE 109.5 ± 6.4 BSF 415.9 ± 0
6	Proliferative vitreo-retinopathy (Grade C)	PVR2	BRG 137.5 ± 6.1 BRPE 60.0 ± 12.4 BSF 544.1 ± 30.1
7	Proliferative diabetic retinopathy	PDR1	BRG 188.5 ± 13.5 BRPE 112.1 ± 15.7 BSF 246.2 ± 4.4
8	Proliferative diabetic retinopathy	PDR2	BRG 189.7 ± 1.6 BRPE 78.6 ± 10.9 BSF 410.7 ± 8.0
9	Proliferative diabetic retinopathy	PDR3	BRG 111.4 ± 6.4 BRPE 50.0 ± 7.1 BSF 147.3 ± 15.9
10	Proliferative diabetic retinopathy	PDR4	BRG 156.0 ± 10.9 BRPE 45.2 ± 8.6 BSF 333.3 ± 32.3

Fig. 3.23. Diagnostic details and percentage migration values of SRF samples, for patient details see Fig.2.9.

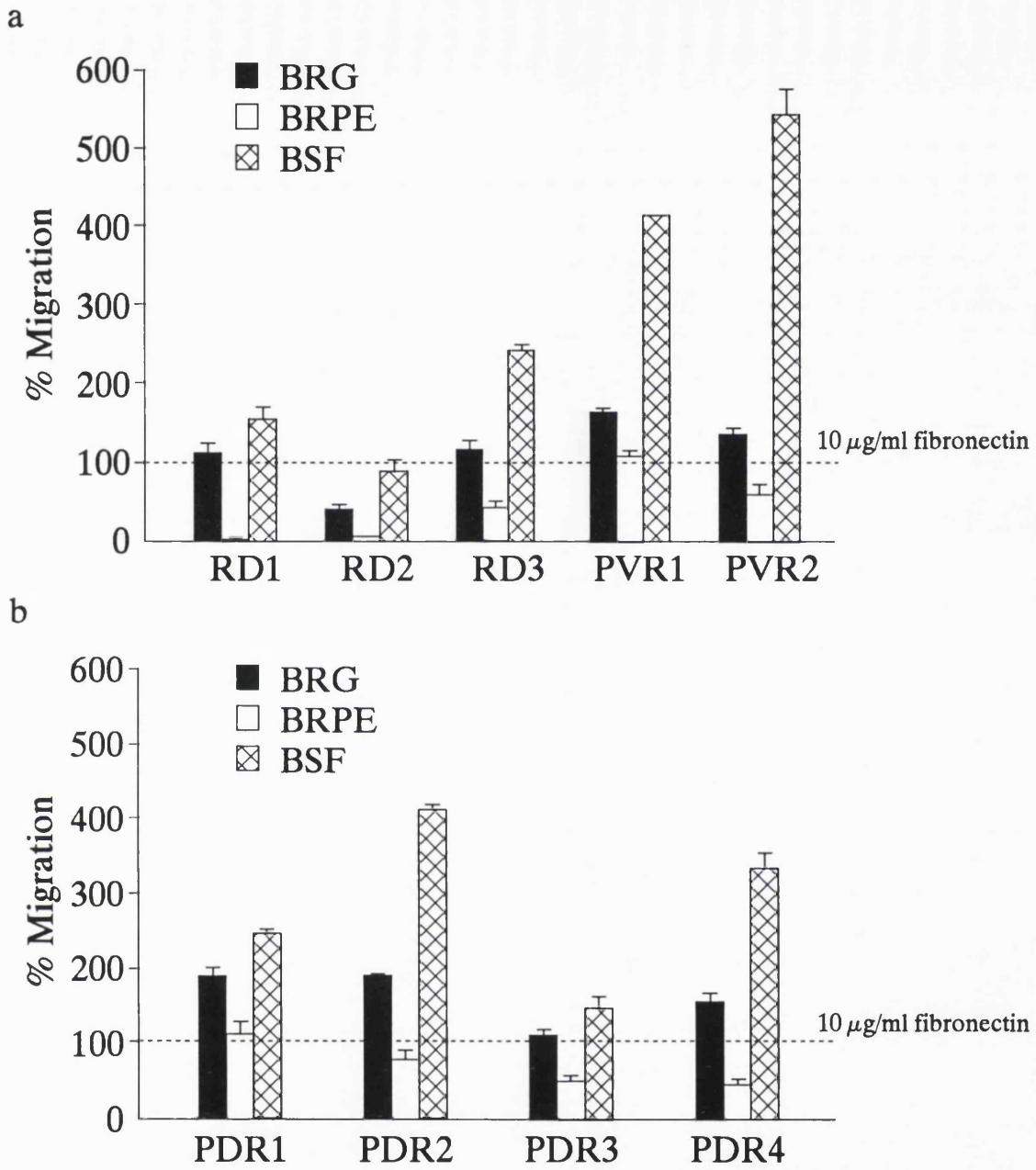


Fig. 3.24. Migration of BRG, BRPE and BSF to 50 μl/ml subretinal fluid in serum free medium. The samples were taken from (a) eyes with simple retinal detachments (RD1-3), proliferative vitreoretinopathy (PVR1-2) and (b) proliferative diabetic retinopathy (PDR1-4). Each point represents the mean ± SEM of three wells. Migration is expressed as percentage of the positive control, 10 μg/ml fibronectin.

difference between the optimal responses of the two types of stimulation (ANOVA $p>0.05$)(Fig. 3.26b.)

Whether or not there was one dominant attractant in retinal crude extract or a series of attractants remained to be established. However we were able to show for BSF, the most responsive cell type, that around 50% of the activity resisted a temperature of 56°C for 30 minutes but little or no chemoattraction resisted boiling for the same time period. Acidic pH removed less than half of the chemoattraction whereas alkaline pH removed about three quarters of the activity. Approximately 60% of the activity seems to be due to a component(s) with a molecular weight above 30,000 daltons (Fig. 3.27.).

3.3. Behavioural Comparisons Relating to Migration, Settlement and Proliferation of BRG, BRPE and BSF in the Presence of Media Harvested from Cultures of the Test Cells

3.3.1. Conditioned Cell Cultures: Serum Deprivation

In order to study the effects of factors produced by cells in culture without any masking effects due to the presence of stimulatory substances in serum, it was necessary to incubate the cells without their normal complement of serum (see section 2.5.1.). Phase contrast light micrographs taken before and after the conditioning period (Fig. 3.28.) illustrated that the appearance and/or confluency of the cell cultures was not affected to any visible degree by the lack of the growth and attachment factors (normally provided by the serum). Indeed the cells after conditioning (Fig. 3.28b.) were observed to retain their pre-conditioning phenotype and appeared more densely packed than the pre-conditioned cells (Fig. 3.28a.) indicating possible increase in cells numbers. Frequently it was possible to identify mitotic figures in the cultures (Fig. 3.28b.) immediately after the conditioning process. Absence of signs of cell stress such as vacuolation or the presence of grossly enlarged cells with large numbers of stress fibres or the presence of dead cells, indicated that the cultures were still healthy. All the other test cultures when conditioned (BRPE, BSF and ERM) behaved

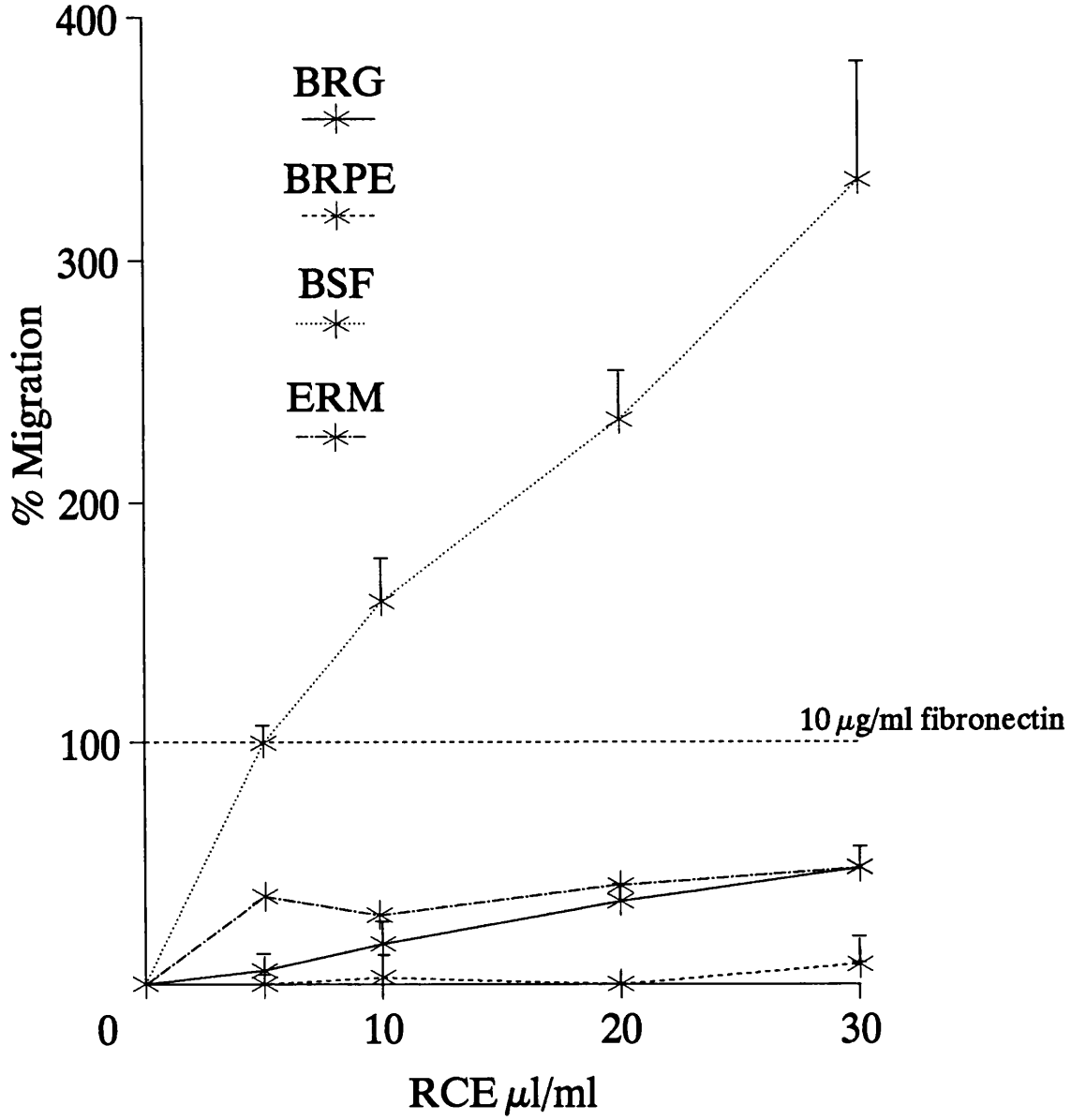
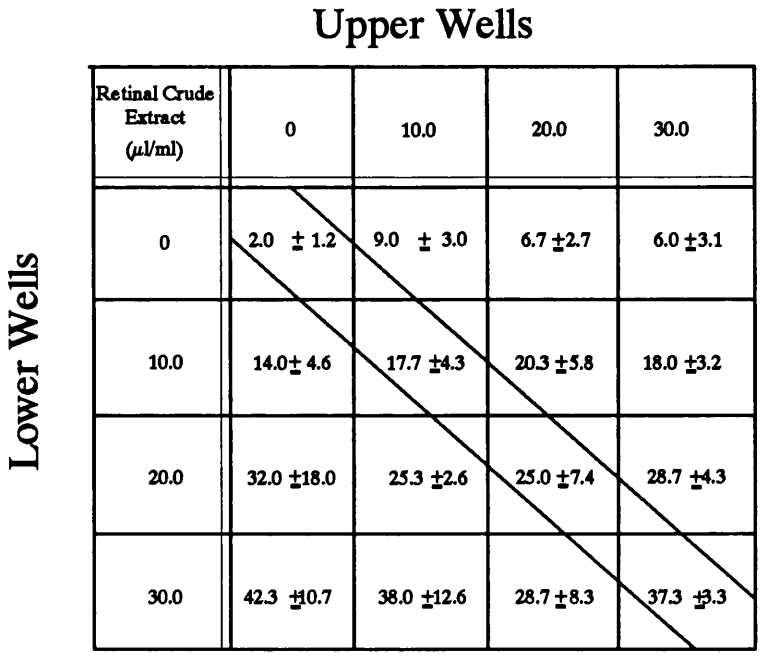


Fig. 3.25. Dose response curve for the migration of BRG, BRPE, BSF and ERM cells to a crude extract of bovine retinae. Each point represents the mean ± SEM of six wells and the experiment was repeated three times. Migration is expressed as a percentage of the response to the positive control, 10 µg/ml fibronectin.

a



b

Bovine scleral fibroblasts				
1	2	3	4	5
[RCE]	%CT ± SEM	%CK ± SEM	%CT/CK	CT/CK
10.0	600.0 ± 230.0	785.0 ± 215.0	-23.6	0.8
20.0	1500.0 ± 900.0	1150.0 ± 370.0	30.4	1.3
30.0	2015.0 ± 35.0	1765.0 ± 165.0	14.2	1.1

Fig. 3.26. Zigmond Hirsch chequerboard (a) and table of values (b) for the migration of BSF to RCE. Each point represents the mean ± SEM of four wells.

Abbreviations for Fig. 3.26b:-
Column 1:- Concentration of retinal crude extract μl/ml ([RCE]).
Column 2:- Percentage increase in chemotaxis over negative control (%CT).
Column 3:- Percentage increase in chemokinesis over negative control (%CK).
Column 4:- Percentage increase in chemotaxis over chemokinesis (%CT/CK).
Column 5:- Proportional increase in chemotaxis over chemokinesis (CT/CK).

Treatment of Retinal Crude Extract	Migration: Remaining Percentage of Optimal RCE Response ± SEM
Optimum response (30 µl/ml, RCE/serum free medium)	100 %
Basal response (serum free medium)	16.5 ± 1.8 %
Temperature of 56°C for 30 minutes	44.4 ± 3.4 %
Temperature of 100°C for 3 minutes	13.7 ± 3.6 %
Change from pH 7 to pH 3 for 30 minutes then back to pH 7	56.0 ± 4.4 %
Change from pH 7 to pH 10 for 30 minutes then back to pH 7	28.6 ± 6.2 %
Filtration, excluding all molecules with a weight above 30,000 daltons	41.5 ± 8.1 %

Fig. 3.27. The preliminary characterisation of RCE. The effect of temperature, pH change and filtration on the migration of BSF to RCE, expressed as a percentage of the migration to 30 µl/ml RCE/serum free medium. Each point represents the mean ± SEM of four wells and each experiment was run three times.

similarly to those shown in Figure 3.28. and to avoid repetition only BRG cells were chosen for illustration.

3.3.2. Migration

The migrational response of the three cell types to media conditioned by the test cells, was expressed as a percentage of the migratory response of the cells to fibronectin (10 μg/ml; see section 3.2.1). The fibronectin value (positive control) was taken as 100% and the serum free media value (negative control) as zero (Fig. 3.29.).

Media conditioned by BRG cell cultures increased the migratory response of all three test cell types in relation to serum free medium and within the limits of the positive control, 10 μg/ml fibronectin (ANOVA $p<0.001$). The BRG response (an autocrine effect) increased in a linear fashion ranging from 38% to 100% of the positive control. BRPE cells reached a maximum of 70% and the chemoattractant effect on BSF increased gradually from 42% to reach a plateau of 90% at 750 μl/ml and 95% at 1000 μl/ml. A comparison of the migrational response between the cell types showed that there was no significant difference between the migratory responses of the BRG and BSF cells (ANOVA $p>0.05$) but the BRPE migrated less effectively with an optimum response which was significantly lower than that of the other two cell types (ANOVA $p<0.01$).

Media collected from cultures of BRPE cells had a limited autocrine effect compared to the baseline controls (ANOVA $p<0.01$) but a marked paracrine influence on the other two cell types (ANOVA $p< 0.001$). Migration of the BRG reached 95% of the optimum fibronectin response whereas the BRPE only reached 10% of the fibronectin response. The BSF cells showed a steadily increasing response, after 250 μl/ml it was on average 50% greater than the BRG response. With 1000 μl/ml (non-dilute) conditioned medium the BSF response was over 50% greater than the optimal fibronectin response and was significantly greater than the BRG migratory effect produced by non-dilute conditioned medium (ANOVA $p<0.001$).

The media collected from cultures of BSF cells elicited only a basal level of activity from cultured BRG cells (an average of 5% of the positive control) and a modest stepwise response from the BSF, to increasing concentrations of conditioned medium, rising to 60% of the optimum fibronectin value showing a clear

autocrine action (ANOVA $p<0.01$). The migration of the BRPE cells rose to 86% at 1000 µl/ml conditioned medium, markedly higher than the other two (ANOVA $p<0.001$). BSF conditioned medium appeared to have a distinct paracrine effect on BRPE migration.

The pronounced migration of BSF to media conditioned by BRPE and BRG was crudely characterised by a series of simple treatments designed to provide clues about the nature of the motogens in the conditioned media (Fig. 3.30.). The results were expressed as the percentage migration remaining of the untreated conditioned medium value (which was taken as 100%). Only BSF were tested for their migratory response to the treated conditioned media of BRPE and BRG because only the BSF cells showed high activity to the conditioned media at or above the level one would expect for fibronectin and varying concentrations of BSF conditioned medium had little effect on the migration of the test cell types. Also introductory characterisation of the motogenic or mitogenic factors in fibroblast conditioned medium had already been carried out to some extent by other groups (Mensing et al, 1983) and had been carried on BSF conditioned medium by Calthorpe and coworkers (1990) for another ocular cell type.

The heating of BRG conditioned medium to 56°C for 30 minutes decreased the migratory activity of BSF by 19% and boiling for 3 minutes decreased the activity by 20%. Exposure to acid pH decreased the activity by 48.6% and all migratory activity ceased after treatment with alkali. Only 5.5% of the migratory activity of the BSF remained after molecules larger than 30,000 Daltons had been excluded.

The characterisation of the migration of BSF cells to BRPE conditioned medium was also carried out. As with the BRG conditioned medium, heating the BRPE conditioned medium to 56°C for 30 minutes only decreased the migratory activity of the BSF cells by 10%, however unlike the former, boiling for 3 minutes destroyed 70% approximately of the activity and subjection to acid decreased the migratory activity by 85%, also unlike the BRG conditioned medium, alkali treatment left 29% of the migratory activity. Finally 97% of the migratory response of the BSF was eliminated by filtration. It may be that there are differences in the types and the proportions of the various motogens which are contained within conditioned medium.

Samples of ERM conditioned medium were available to be tested for their migration stimulating effect (Fig. 3.31.). There was however only a limited supply so it was not possible to run the migration assay with the full range of concentrations chosen for the experiments on bovine cell conditioned media. As a

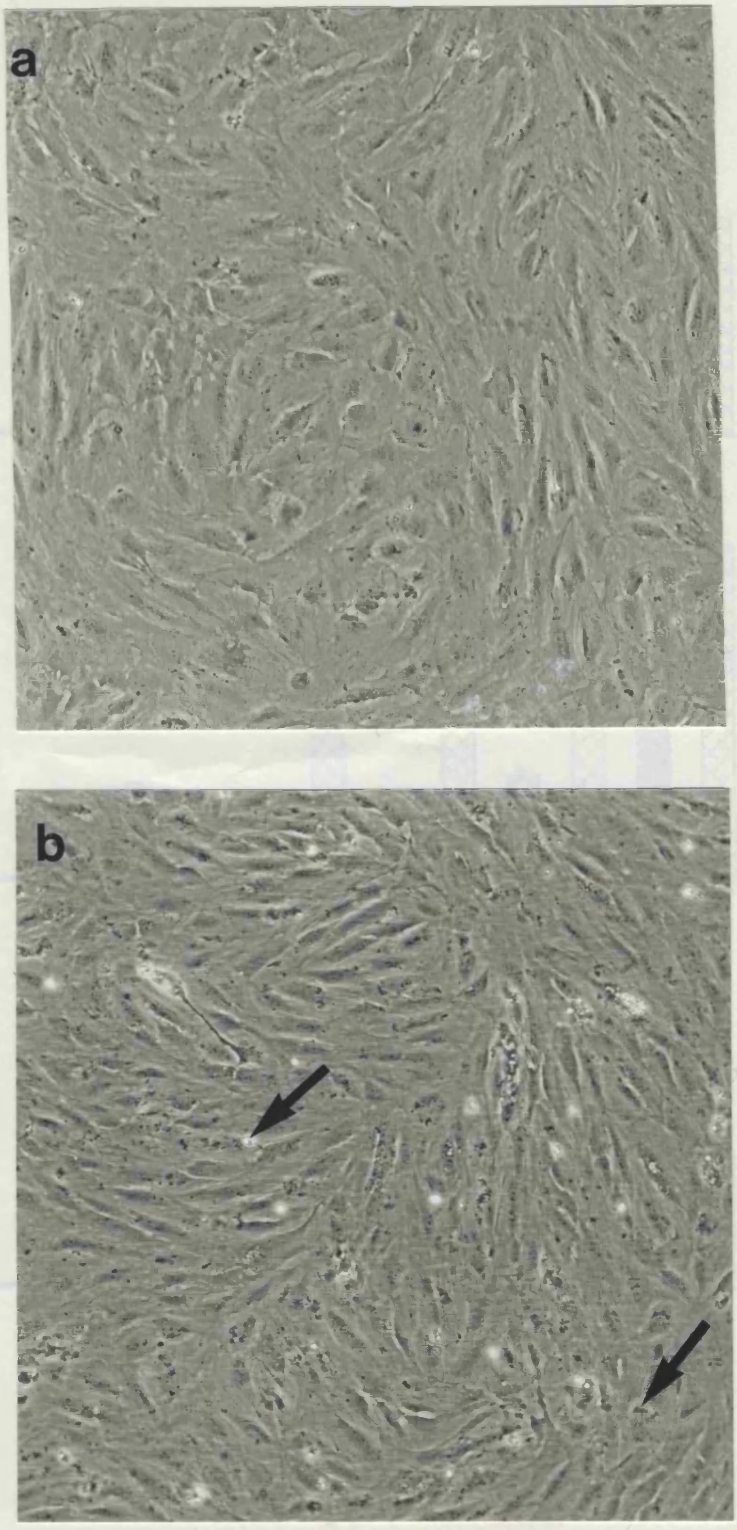


Fig. 3.28. Phase contrast light micrographs showing cultures of fourth passage BRG, (a) pre and (b) post 48 hours conditioning with serum free medium. Cell division (arrows) is still taking place at the end of the conditioning process. (Magnifications:- (a) x 140, (b) x 110).

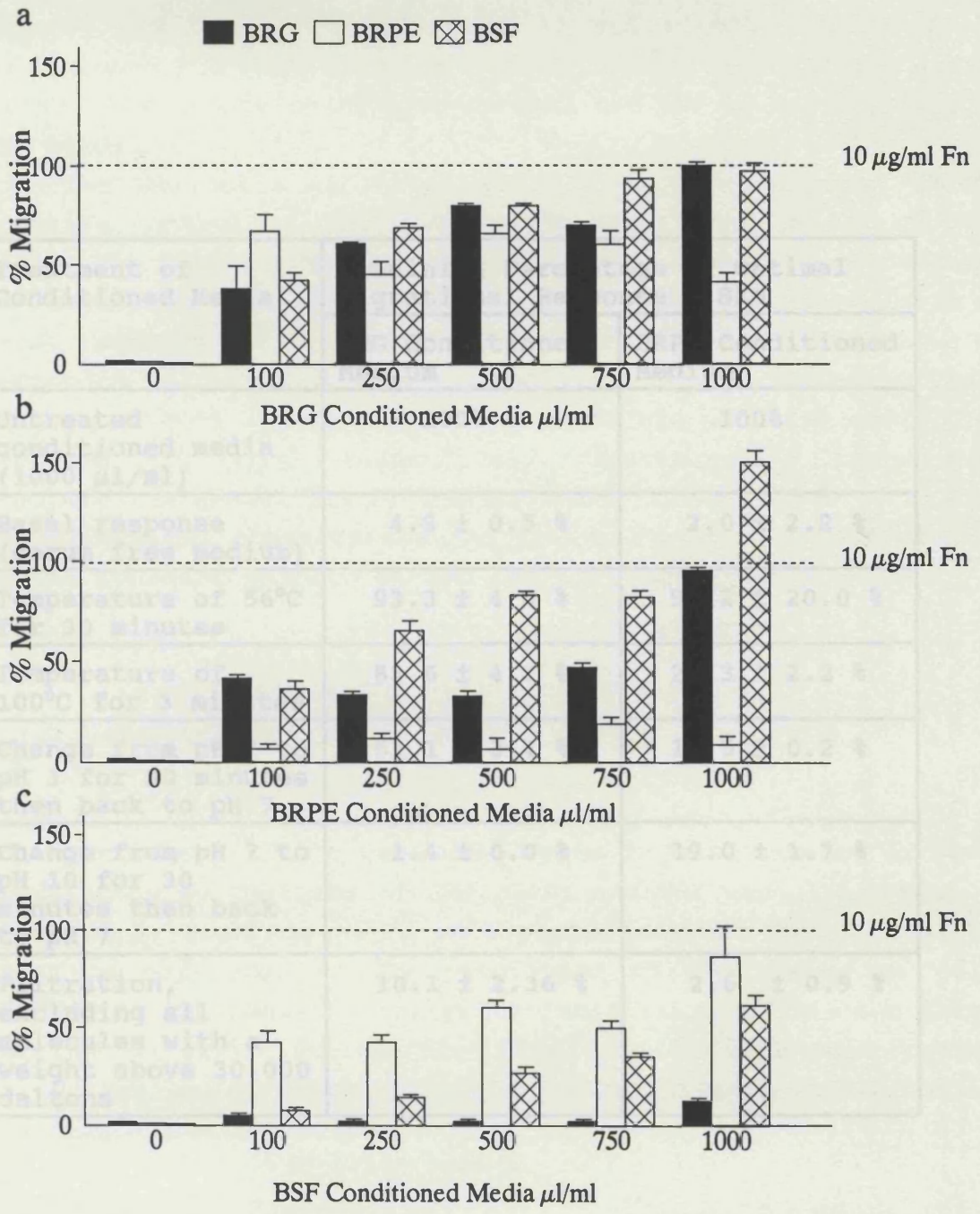


Fig. 3.29. Comparison of the migration of BRG, BRPE and BSF in the presence of BRG (a), BRPE (b) and BSF (c) conditioned media. Each point represents the mean ± SEM of three wells and each experiment was run three times. Migration is expressed as a percentage of the positive control; 10 μg/ml fibronectin. The negative control is serum free medium.

Treatment of Conditioned Media	Remaining Percentage of Optimal Migrational Response ± SEM	
	BRG Conditioned Medium	BRPE Conditioned Medium
Untreated conditioned media (1000 µl/ml)	100%	100%
Basal response (serum free medium)	4.8 ± 0.5 %	2.0 ± 2.2 %
Temperature of 56°C for 30 minutes	93.3 ± 4.0 %	91.2 ± 20.0 %
Temperature of 100°C for 3 minutes	83.6 ± 4.1 %	26.3 ± 2.2 %
Change from pH 7 to pH 3 for 30 minutes then back to pH 7	51.1 ± 3.4 %	15.5 ± 0.2 %
Change from pH 7 to pH 10 for 30 minutes then back to pH 7	1.4 ± 0.0 %	19.0 ± 1.7 %
Filtration, excluding all molecules with a weight above 30,000 daltons	10.1 ± 2.36 %	2.6 ± 0.9 %

Fig. 3.30. The preliminary characterisation of the migration stimulating factors contained in BRG and BRPE conditioned media. The cells tested for their migratory responses were BSF. Migration was expressed as a percentage of the migration to untreated conditioned media. Each point represents the mean ± SEM of four wells and each experiment was run three times.

result, the concentration values chosen were those that had elicited on average the largest responses from the test cells in the experiments using medium conditioned by bovine cells. In general ERM cells did not grow in sufficient numbers to be conditioned and when there was enough cells they did not always survive the conditioning process well and had to be dropped from the study.

The BRG cells exhibited a 100% response compared to the positive control at 250 µl/ml which went down to 42% at 1000 µl/ml. The BRPE characteristically showed a paucity of response in comparison with the other two cell types and their migration never exceeded 30% of the positive control. The BSF cells however responded very strongly to the ERM conditioned medium by being as high as 295% and 326% of the fibronectin positive control at 250 and 500 µl/ml respectively. Unfortunately there were insufficient quantities of the ERM conditioned medium to be able to perform the characterisation treatments carried out earlier on the abundant samples of BRG and BRPE conditioned media.

3.3.3. Settlement

Settlement of the test cell types in the presence of media conditioned by cultures of BRG, BRPE and BSF was assessed by counting cells on a phase contrast microscope. The data was expressed as the percentage of the number of cells settled to the positive control soluble fibronectin (see later; section 3.4.1.). Unlike the migration studies the optimal concentrations of fibronectin for cell settlement varied between the cell types as follows:-

1. BRG 30 µg/ml fibronectin.
2. BRPE 5 µg/ml fibronectin.
3. BSF 10 µg/ml fibronectin.

Media conditioned by cultures of BRG increased the settlement onto tissue culture plastic of the three cell types by varying degrees (Fig. 3.32a.). The settlement of BRG was increased at the higher concentrations of conditioned medium (500 to 1000 µl/ml) ranging from 120% to 150% of the optimum response. The basal level of settlement of BRPE was higher than for the other two cell types (29% of the initial number of cells seeded,

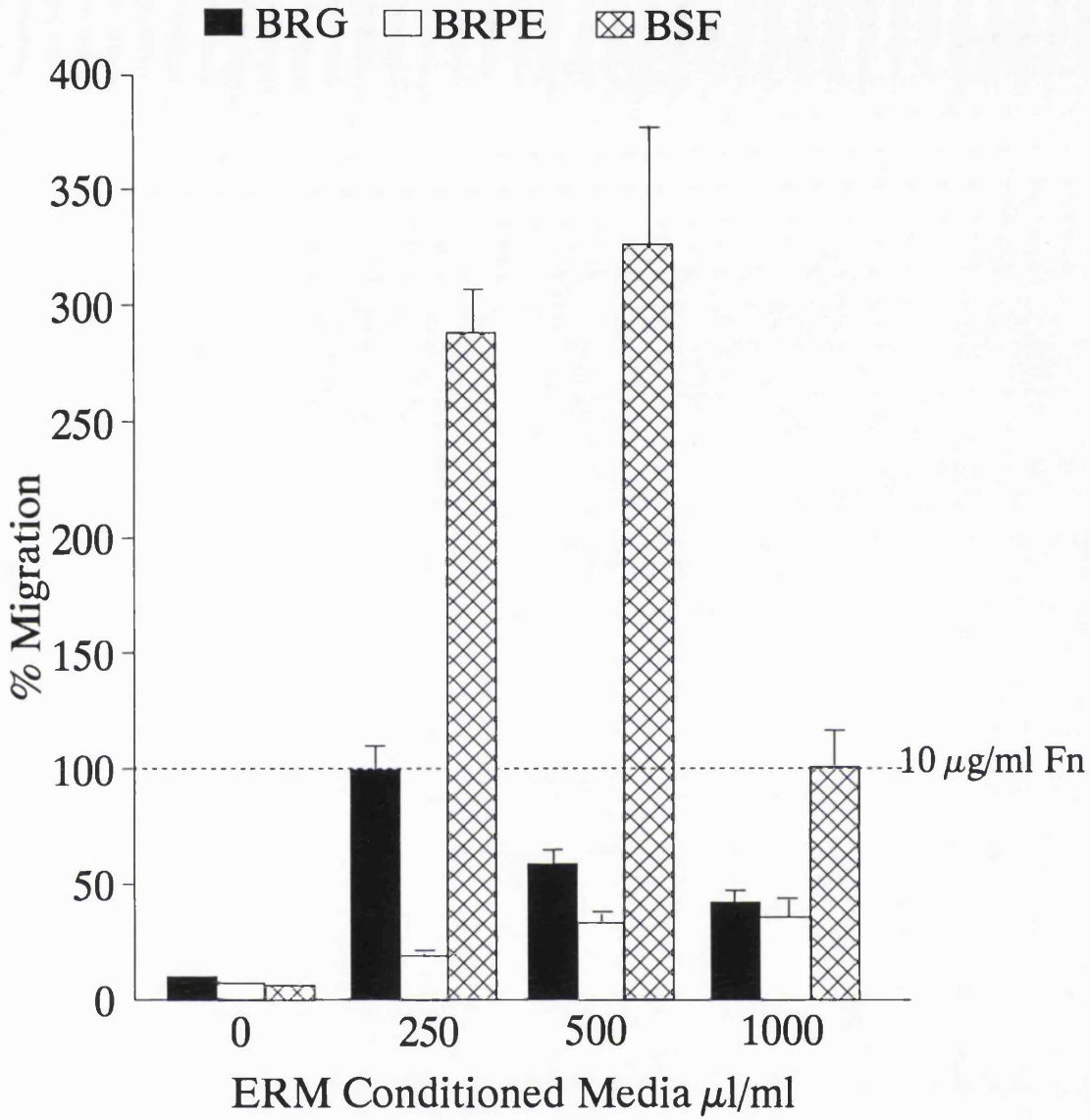


Fig. 3.31. Comparison of the migration of BRG, BRPE and BSF in the presence of medium conditioned by ERM cells. Each point represents the mean ± SEM of three wells. Migration is expressed as a percentage of the positive control 10 μg/ml fibronectin.

settled) and there was a 86% increase in settlement over the zero value at a concentration of 500 µl/ml of conditioned medium (ANOVA $p < 0.001$). The settlement levels of the BSF were comparable to that of the BRG, there was a gradual increase in BSF settlement over the basal level to an optimum of 115% over the negative control at a concentration of 750 µl/ml. The settlement of the BRPE was consistently higher than the other two cell types (except at 1000 µl/ml).

The control settlement levels for all three cell types were relatively high in the studies employing BRPE conditioned medium and the response to fibronectin relatively low (Fig. 3.33b.). The cells showed little increase above the base line control.

The settlement values obtained with BSF conditioned medium were lower than for the other two types of conditioned media tested. The settlement levels of the glia showed little variation from the negative control apart from at the concentrations of 750 µl/ml (97% increase) and 1000 µl/ml (186% increase). The BRPE showed a relatively large increase in settlement gradually going from 25% above the control at 100 µl/ml to a 318% increase at 1000 µl/ml (ANOVA $p<0.001$). BSF conditioned medium did not appear to affect the settlement of BSF themselves the values were all at the basal level apart from 100 µl/ml (27% increase). Comparing the activity of the three cell types, again BRPE showed higher levels of settlement activity in response to the test stimuli than BRG or BSF. Intra- and inter- error counts performed for the settlement experiments were 3.3% and 7.0% respectively.

A preliminary analysis of the fibronectin content of conditioned media samples from the three cell types, using ELISA (see Appendix II) assayed the mean fibronectin concentration in BRPE conditioned medium, to be 18 µg/ml, 15.8 µg/ml for BRG and 5 µg/ml for BSF which may account partly for the increased levels of settlement associated with the various conditioned media.

3.3.4. Proliferation

Media collected from cultures of BRG cells had little obvious effect on BRG and BSF proliferation (ANOVA $p>0.05$) (Fig. 3.33.), the former showed overall lower baseline values (approximately 100% increase) than the latter (approximately 200% increase (ANOVA $p<0.001$). However BRPE proliferation increased threefold with 500 µl/ml of conditioned medium (ANOVA $p<0.005$).

BRPE conditioned medium however appeared to have a slight stimulatory effect (ANOVA $p<0.04$) on the proliferation of BRG (see also time-lapse studies in section 3.3.5.). The BRPE showed little stimulation at a fairly low level (a 60% increase over the baseline control) and BSF no increase in cell numbers. While the numbers of BRPE increased by 212% at 250 µl/ml and 300% at 500 µl/ml over the control value.BSF conditioned medium did not appear to elicit any stimulatory effect and there were no significant increases in cell numbers (ANOVA $p>0.05$).

To summarise this complex study of the different conditioned media and their influences on the test cells. The BRG stimulate the migration of the BSF but not the other way round and to some extent BRPE stimulate BRG but not the other way round. The BRPE have a particularly marked effect on BSF but there is some two way effect. The ERM conditioned medium has little effect on the BRPE and a large influence on the BSF. The ERM conditioned medium although it was only used in the migration studies, appeared to be the most active. The BRPE settle best of the three but their influence on their own settlement is poor. BRG and BSF have no effect on each others' settlement but aid the settlement of the BRPE. The BRPE respond to mitogenic substances secreted by BRPE and BRG but the BSF do not appear to either influence or be influenced in the proliferation experiments, by the conditioned media.

3.4. Behavioural Comparisons Relating to Settlement and Invasion of Retinal Pigment Epithelial Cells and Scleral Fibroblasts into a Retinal Glial Monolayer

3.4.1. Settlement

The relative adhesion of BRG, BRPE and BSF to a BRG monolayer was assessed by the use of static settlement assays (see section 2.4.1.) and comparison was made of the two types of assay commonly used for short term analysis of cell settlement (Fig. 3.34.). Visual counting (collecting lawn assay), showed that 50% of the BSF cells introduced into a serum coated plastic dish settled in 30 minutes but the settlement of BSF cells on a BRG monolayer was only half of that in the same time period. The

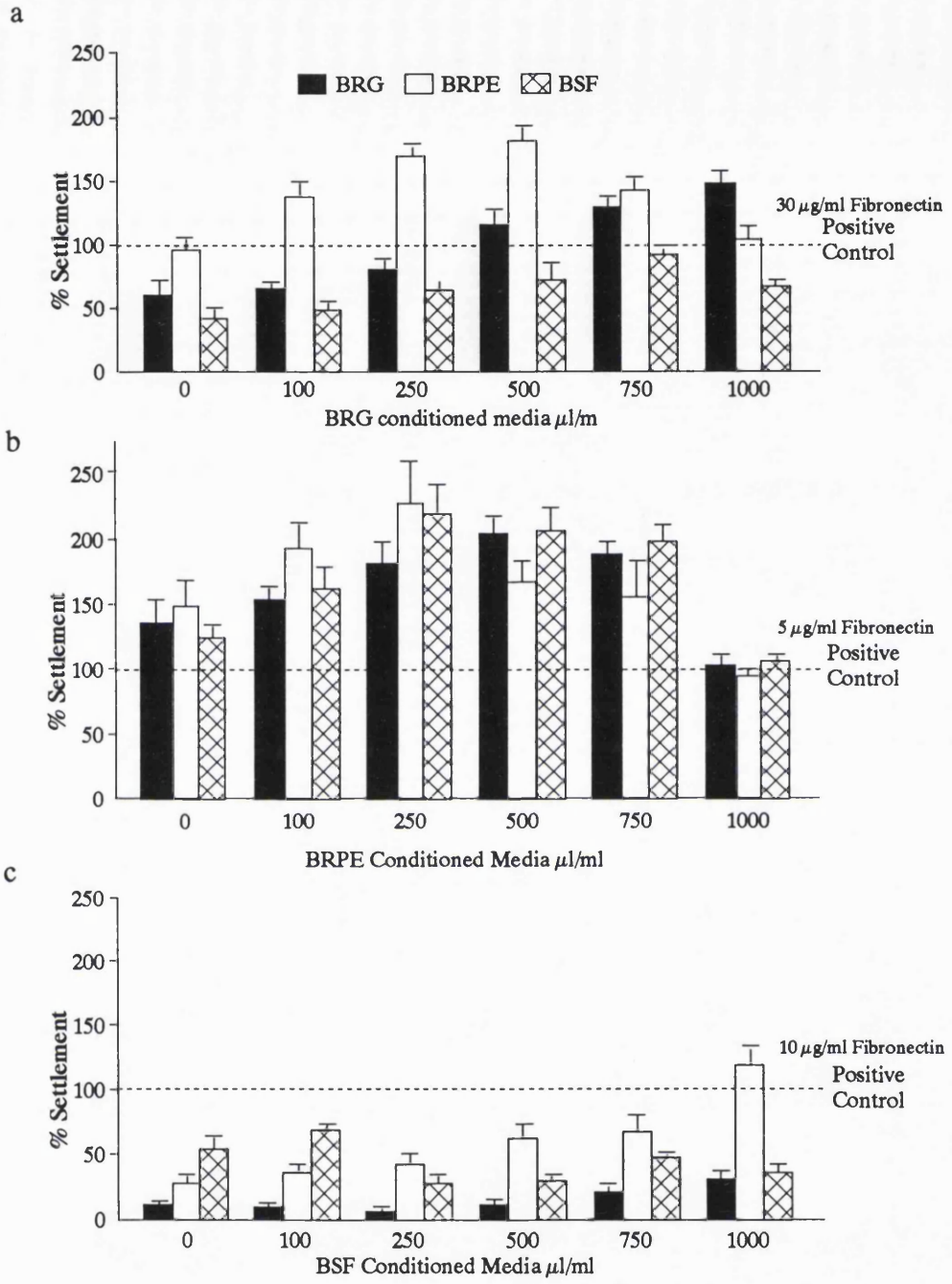


Fig. 3.32. Comparison of the settlement of BRG, BRPE and BSF in the presence of BRG (a), BRPE (b) and BSF (c) conditioned media, for 30 minutes. Each point represents the mean ± SEM of four wells and each experiment was repeated three times. Settlement is expressed a percentage of settlement to the optimum concentration of the positive control fibronectin, for each cell type.

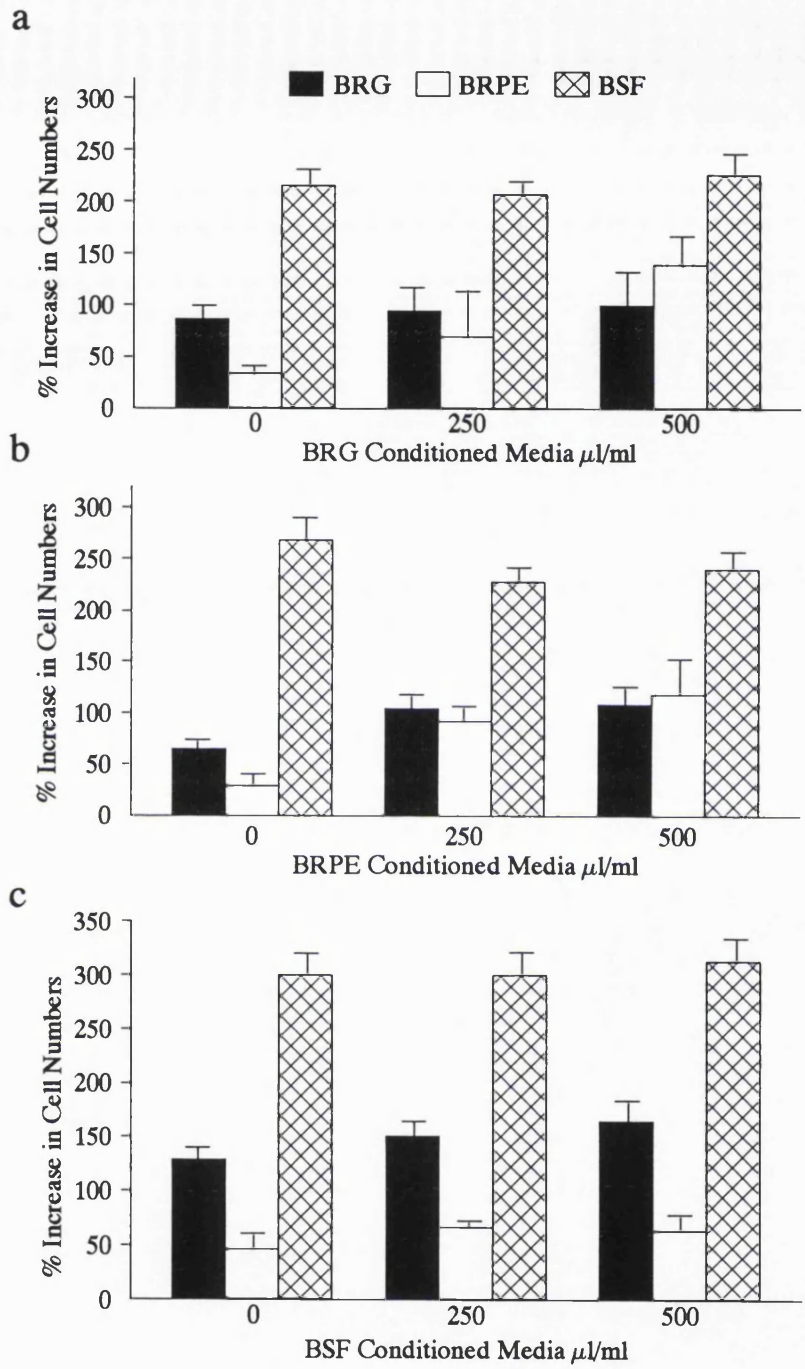


Fig. 3.33. Comparison of the proliferation of BRG, BRPE and BSF in the presence of BRG (a), BRPE (b) and BSF (c) conditioned media, for 30 minutes. Each point represents the mean ± SEM of six wells and each experiment was repeated three times. Proliferation is expressed as the percentage increase in cell numbers over the 24 hour settlement values, after three days incubation with cell conditioned media.

BRG formed a confluent monolayer within 24 hours (6 x 10^5 cells/well). When the assay was repeated using ^{14}C adenine-labelled BSF cells and the subsequent settlement determined by scintillation counting, the findings were virtually identical (ANOVA $p>0.05$). Both assays showed that serum coated plastic was a far more effective substrate than a BRG monolayer for BSF cell adhesion (Fig. 3.34.; ANOVA $p<0.001$).

Collecting lawn analysis was preferred over scintillation counting in all the subsequent experiments to determine cell settlement rates. They both produced similar results and scintillation counting was far less laborious but the latter was indirect. Seeing the settled cells was considered to be a great advantage, as it allowed for evaluation of the condition of the cells as the assay proceeded. In addition, although the collecting lawn technique was slow, changes in cell morphology could be appreciated during the settlement process.

Comparison of BSF with BRPE cells showed that the settlement on plastic of the two cell types was not significantly different (ANOVA $p>0.05$) and that both of these cell types were far more adherent than BRG cells (ANOVA $p<0.001$)(Fig. 3.35.). Fewer BRPE cells (less than a third of the cell numbers) were found to settle and adhere to monolayers of cells when compared to the settlement on serum coated plastic (ANOVA $p<0.01$). In addition, although there was no difference between the settlement of BRPE and BSF (ANOVA $p>0.05$) onto BRG monolayers the settlement of BRG was significantly less effective than that of BSF (ANOVA $p<0.02$) and BRG were extremely poor at settling on BRG monolayers. BSF settlement on BRG was 60% of that on plastic while BRPE was 30%. BRG settlement counts were low and very variable throughout (Fig.3.35a and b.).

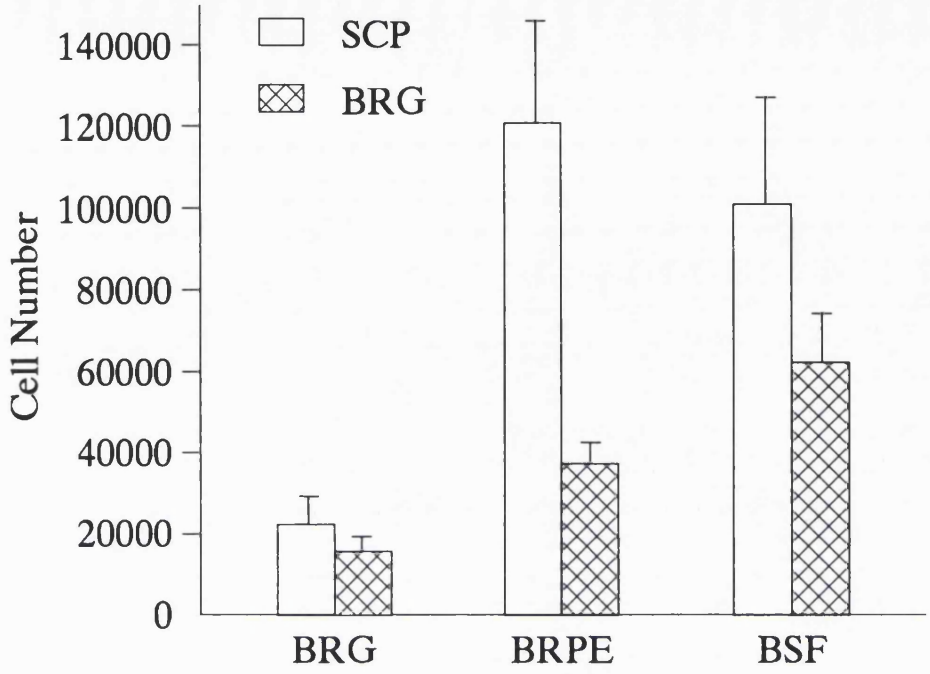
At 30 minutes, the settled cells on top of the glial monolayer varied in shape from spherical to spheroidal and there were clear size differences between these rounded refractile cells on the monolayer (Fig. 3.36.). On the other hand, cells which settled on a plastic substrate for the same time period showed more phenotypic variation (Fig. 3.37 a,c,e.). The increased variation was related to the extent to which the cells spread on the non-biological substrate. Well spread cells lost their rounded refractile appearance, became darker by phase contrast as they thinned out and their area increased.

The influence of soluble fibronectin on cell spreading in serum free medium was evaluated (Fig. 3.37 b,d,f). Increased spreading was shown by all three cell types in response to increased concentrations of fibronectin within the range of 0 to

Cell/Target	Mean Number of Settled Cells	SEM of Settled Cells	% Settlement of Initial Cell Number
1) BSF/SCP	101,178	25,923	50.59
1) BSF/BRG	62,466	11,405	31.23
2) ^{14}C BSF/SCP	107,152	1,983	53.58
2) ^{14}C BSF/BRG	57,544	2,652	28.77

Fig. 3.34. Comparison of the settlement of BSF onto a BRG monolayer and serum coated plastic (SCP); 1) visually counted (n=8 for each category) and 2) estimated by labelling with ^{14}C adenine (n=20 for each category). Each experiment was run three times.

a



b

Cell/Target	Mean Number of Settled Cells	SEM of Settled Cells
BRG/SCP	20,363	± 8,493
BRG/BRG	15,672	± 3,565
BRPE/SCP	121,101	± 25,113
BRPE/BRG	37,336	± 4,763
BSF/SCP	101,178	± 25,923
BSF/BRG	62,466	± 11,405

Fig. 3.35. Comparison of the Settlement of BRG, BRPE and BSF onto serum coated plastic (SCP) and BRG monolayers, (n=8) for each category and each experiment was run three times. Graph (a) and table of values (b).

15 μg/ml (Figs. 3.37 and 3.38.) and the increases were significant (ANOVA S-N-K intervals). As much as a two to three fold increase in mean diameter was produced (Fig. 3.38.). The mean diameter of BRPE was always less than that of the other two cell types (ANOVA $p<0.02$).

Fibronectin was shown, in separate experiments, to substantially increase settlement for all three cell types (Fig. 3.39a.). The enhanced settlement of BRPE cells produced by fibronectin was marked and significantly more pronounced than that of BSF and BRG cells. Optimum settlement of BRPE cells was reached at 5 μg/ml and was twice that seen with fibronectin-free medium. BSF had an optimal increased settlement of 40% with 10 μg/ml of fibronectin and BRG cells had a comparable increase (Fig. 3.39a.) but the optimum response (50%) needed higher concentrations than used in this experiment (Fig. 3.39b.).

3.4.2. Interaction of Retinal Pigment Epithelium and Scleral Fibroblasts with Retinal Glial Monolayers

The interaction of BSF and BRPE cells with BRG monolayers was followed by video time-lapse photography but BRG settlement on BRG monolayers was not studied in this way because of the poor and patchy nature of their initial adhesion (see section 3.4.1.). Spreading of cells was not seen on the surface of BRG monolayers. The settled cells were refractile in appearance and spherical or spheroidal in shape and they projected thin cytoplasmic extensions. The number of refractile settled cells decreased progressively with time and the observations were consistent with settling cells invading the BRG monolayer.

The decrease in the number of rounded cells on the surface of the monolayer followed a decay curve which reflected the rate of loss of settled cells from the surface of the sheet of BRG cells. Cells were seen not to be lost by subsequent detachment into the medium, therefore the cell loss from the surface of the BRG monolayer was reciprocal to the incorporation or invasion of the cells into it (Fig. 3.40.).

Entry into the monolayer started almost immediately for BSF and was virtually over by four hours. A few cells remained on the surface of the monolayer which failed to gain entry but these were never more than 5% of the initial settlement (Fig. 3.40a.). BRPE entry into the monolayer always started by one hour and

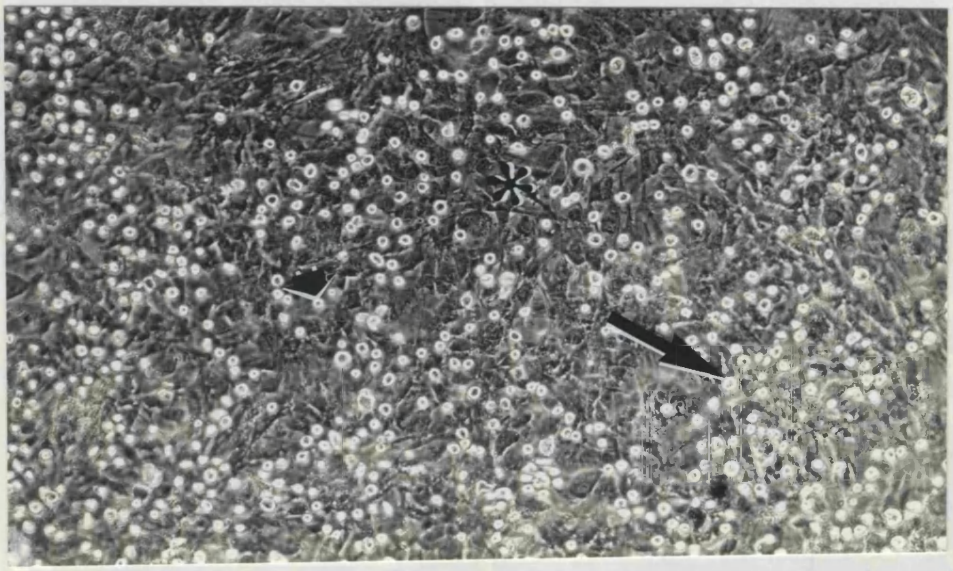


Fig. 3.36. Underlying BRG monolayer (asterisk) with cells settled for 30 minutes. The cells are of varying shapes ranging from spherical (small arrow) to spheroidal (large arrow). (Magnification:- x 100).

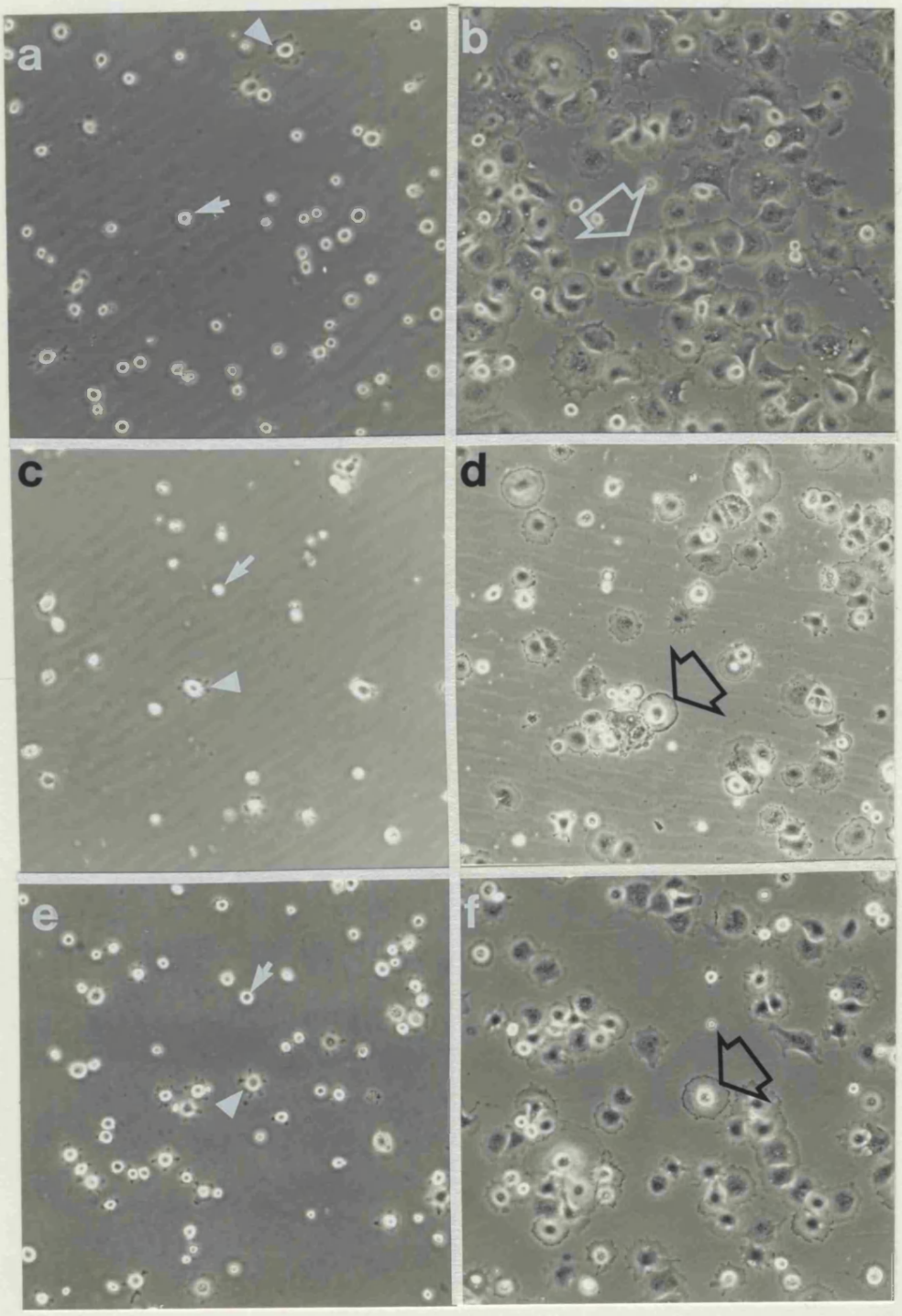


Fig. 3.37. Phase contrast light micrographs showing settlement of BRG, BRPE and BSF onto tissue culture plastic for a 30 minute time period. Firstly in the presence of serum free medium (a,c,e) where the cells are rounded (small arrows) and some have protrusions (arrow heads). Secondly in medium containing 10 μg/ml fibronectin (b,d,f) where most of the cells have adopted a spread configuration (large arrows). (Magnifications:- a-f x 100).

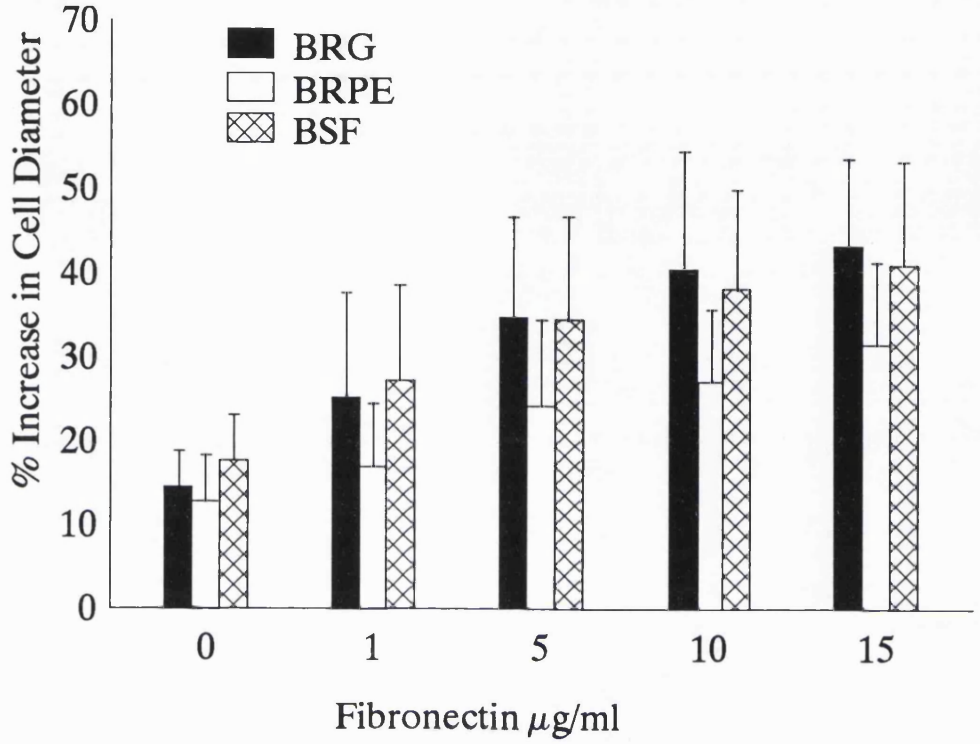


Fig. 3.38. Comparison of the spreading of BRG, BRPE and BSF in the presence of soluble fibronectin. Spreading is expressed as the mean percentage increase in cell diameter (± SEM) over cells settled in serum free medium. For each category n=50.

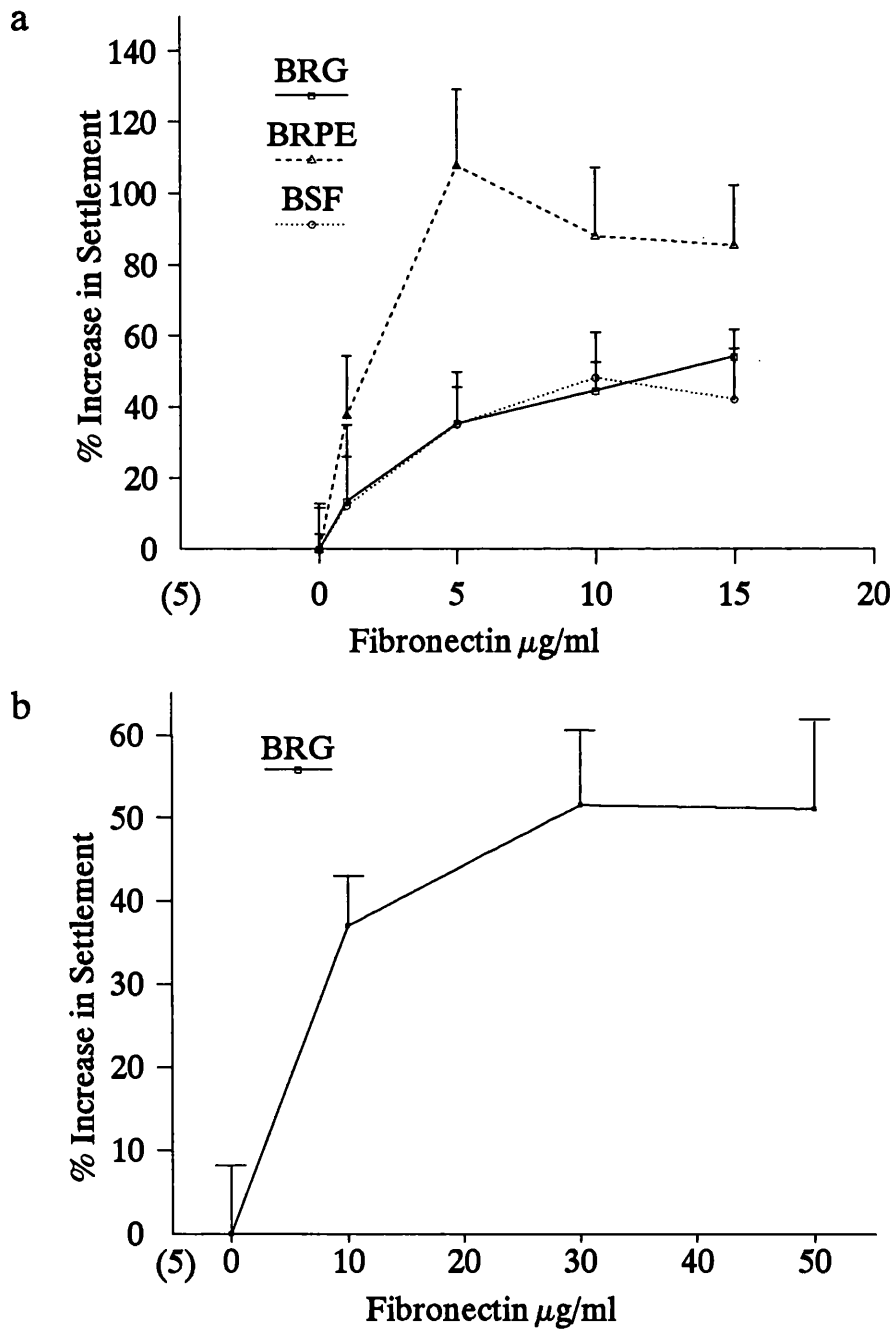


Fig. 3.39. Comparison of the settlement of (a) BRG, BRPE and BSF in the presence of soluble fibronectin and (b) shows full settlement dose response curve for BRG. Settlement is expressed as the percentage increase in settlement (± SEM) over the serum free medium values. Each point represents the mean ± SEM of six wells and each experiment was repeated three times.

could take as long as eight hours to complete. Once again about 5% of the settlement failed to enter and remained on the monolayer surface (Fig. 3.40b.). An ID_{50} was calculated from the curves (Fig. 3.41.) and BSF cells could be seen to enter the monolayers far more rapidly (ID_{50} = 97.5 min) than BRPE cells (ID_{50} = 158.75 min; ANOVA $p<0.001$).

The slow entry of BRPE cells was associated with major disruption of the BRG sheet. After two to three hours, the monolayer had distinct holes in some areas. The BSF cells however slid into the monolayer without causing any obvious alteration to its appearance. When BSF were settled onto the BRPE/BRG mix, the invasion was compete in about one hour which was significantly faster than into an intact glial monolayer (see Fig. 3.41. and 3.42a.; ANOVA $p<0.001$). On the other hand, BRPE incorporation into the heterotypic sheet of BSF and BRG was almost identical to their incorporation rate into a glial sheet (Fig. 3.41. and 3.42b.; ANOVA $p>0.05$).

Despite the differences in their effect on the BRG monolayer, the mechanism of entry, on a morphological basis at least, was very similar and could be separated into a number of stages. Firstly, spherical cells settled on the monolayer and made contact and after a few minutes some were adherent; the adherent cells were not dislodged when their flask was gently agitated under the inverted microscope. Secondly, the adherent cells lost their spherical shape, becoming slightly irregular and were often spheroidal.

Time-lapse showed that spherical adherent cells were immobile whereas the more rugby football-shaped cells were active but did not undergo any directional movement. BRPE probed the intercellular clefts of the BRG monolayer with many small dynamic pseudopodia which were continuously extended and retracted (see Fig. 3.43.). BSF also probed the BRG monolayer with similar but less pronounced pseudopodia (Fig. 3.44.). The cells extended a large cytoplasmic projection into a cleft between adjacent BRG cells in the monolayer and then slowly the refractile body on the surface of the monolayer decreased in size and adjusting the focus of the microscope revealed that the invading cell had taken up a dumb-bell shape as it entered the cleft. Finally, no refractile cytoplasm was seen and at this point the cell was considered to have become incorporated into the sheet. Some cytoplasmic ruffling was seen at this point. The incorporated cell remained associated with the BRG cells it had squeezed between and there was little or no lateral migration of individual cells within the monolayer. Initially at least, the

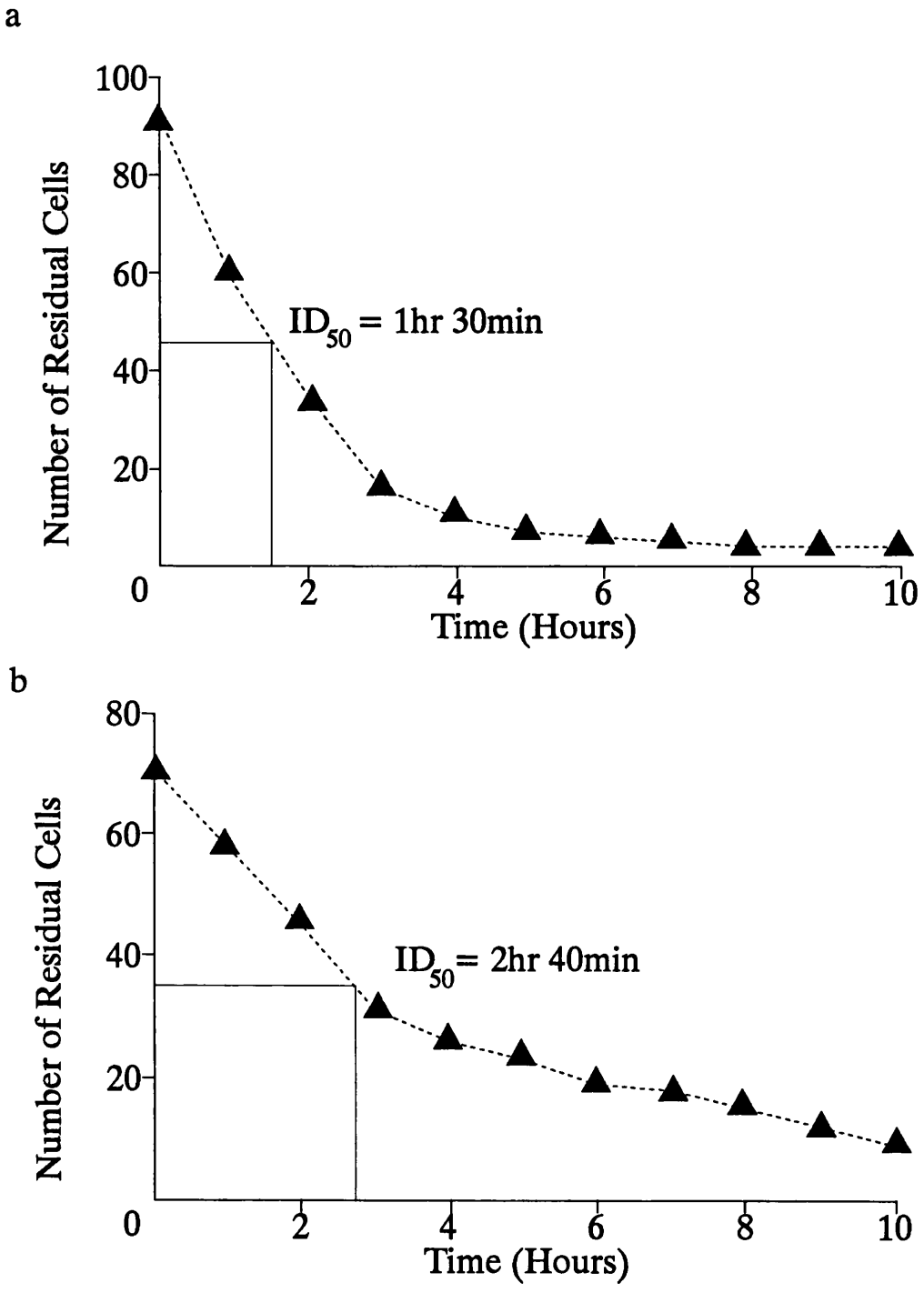


Fig. 3.40. The rate of loss of (a) BSF and (b) BRPE from the surface of BRG monolayers. The experiments were repeated four times each and the ID_{50} values calculated from the decay curves.

Cell	Target	ID_{50} (Minutes)	SEM
BRPE	BRG	158.75	± 9.21
BRPE	BSF/BRG	156.25	± 8.51
BSF	BRG	97.50	± 4.33
BSF	BRPE/BRG	64.75	± 8.47

Fig. 3.41. The mean ID_{50} values (n = 4 for each category) for the rate of loss of BRPE and BSF from the surface of cell layers, as monitored by video time-lapse.

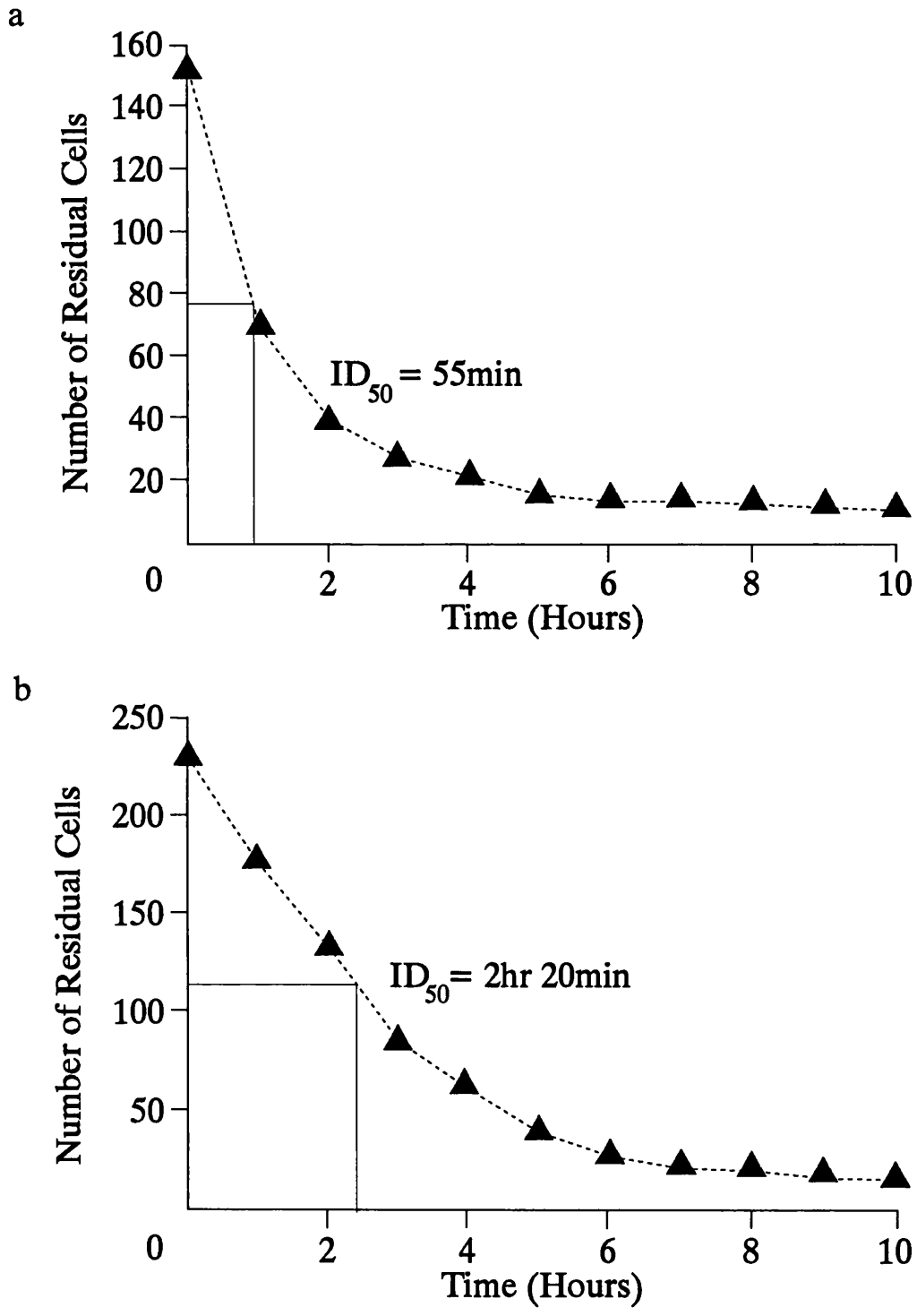


Fig. 3.42. The rate of loss of (a) BSF from the surface of a BRPE/BRG mixed layer and (b) BRPE from the surface of a BSF/BRG mixed layer. The experiments were repeated four times each and the ID_{50} values calculated from the decay curves.

incorporated cells were small and static but later they became extremely active with rapid change of shape and readjustment, surface ruffling was sometimes evident. After a variable period of time the "wriggling" action became slower and then finally stopped when the invading cell had spread out.

The invasion of individual cells could be followed in a longitudinal manner by employing freeze-frame and play-back facilities. Nine key stages were identified (Fig. 3.45.) and these were easily recognisable on video for both BRPE (Fig. 3.46.) and BSF (Fig. 3.47.) invasion into BRG monolayers. In addition the onset time and the duration of each stage in the incorporation process could accordingly be determined from the video recordings (Fig. 3.48.). In general for both BRPE and BSF the first stage when the cells were floating was virtually over in 10 minutes, 85 to 90% of the cells had settled on the glial sheet by this time, for individual cells this could take only a few seconds. On time-lapse no distinction could be made between adherent and less adherent cells (without shaking the flask) but the whole process of contact (stage 2) and adhesion and probing (stage 3) could be completed in 15 minutes and the invasion process (stages 4 to 7) in a similar time period. So the whole mechanism of a round cell on the outside of the monolayer (stage 2) to a round cell in the monolayer (stage 7) could be complete in half an hour. Thirty to forty minutes was the usual time taken for BSF but BRPE tended to take up to one hour to complete the process and the increased time was generally because stages 2 through to 4 were slower. Some cells were held up at stages 2 and 3 for some hours and others which failed to go from stage 4 to stage 5, went back to stage 3 and remained stationary. The latter were for the most part, the cells which made up the 5% of the population which were still present on top of the monolayer at the end of the assay.

Once inside the monolayer, the invading cell remained in a rounded inactive form for not more than half an hour (stage 7) but after this interlude the cell began to spread. The spreading involved periods of steady backwards and forwards motion, short bouts of ruffling and short periods of lively wriggling. The spreading period, stage 8, ranged in length from two to three hours and there was no obvious difference between BSF and BRPE cells in the time spent in this active phase. The BRPE were the more aggressive when in the monolayer and the forces generated by their movement disrupted the glial sheet. At stage 9 the cells were inactive once again.

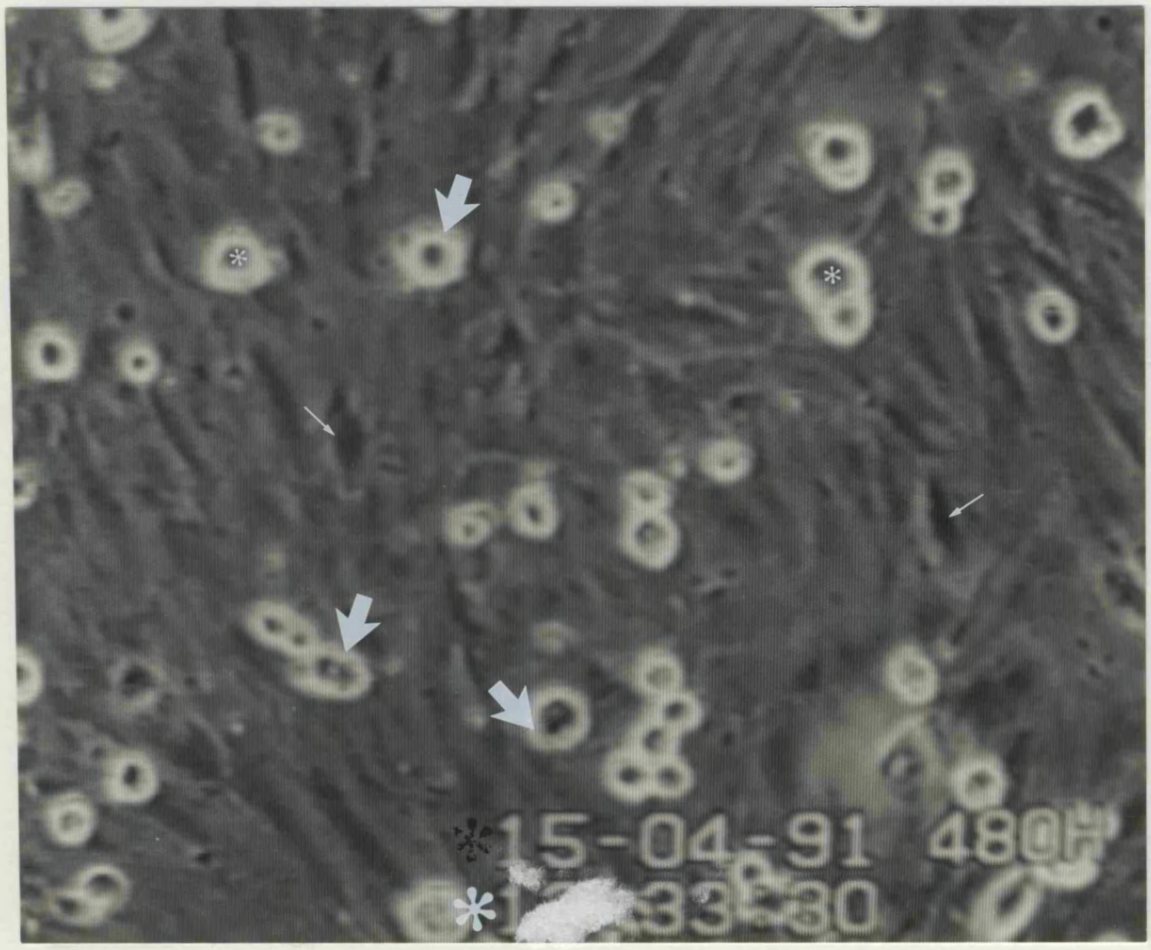


Fig. 3.43. A still photograph taken from video time-lapse filming of BRPE cells (small asterisks) on a BRG monolayer (small arrows) after 1 hour. The BRPE display multiple pseudopodia (large arrows). The date (black asterisk) and time white asterisk) are displayed on the bottom right hand corner of the screen. (Magnification:- x 650)

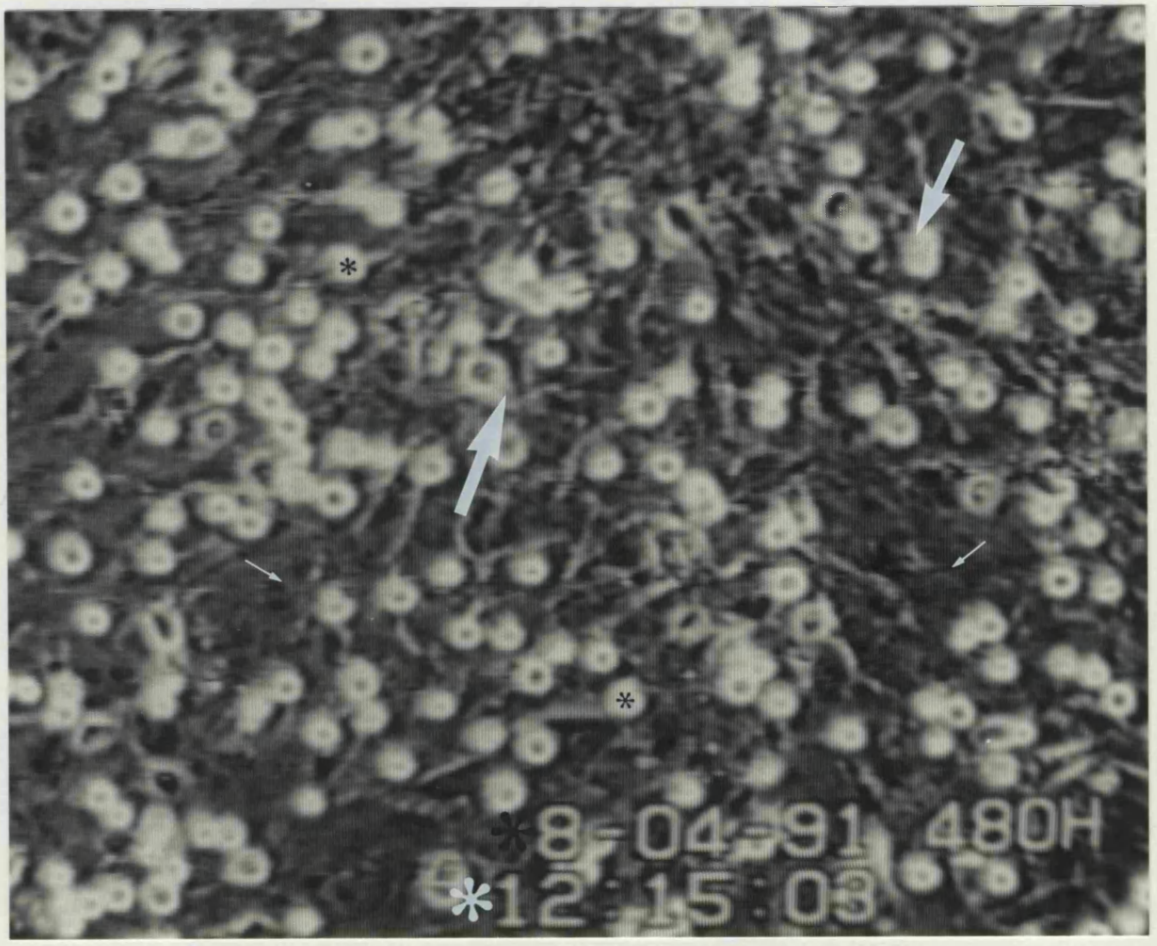


Fig. 3.44. A still photograph taken from video time-lapse filming of BSF cells (small asterisks) on a BRG monolayer (small arrows) after 1 hour. The BSF display few small pseudopodia (large arrows). The date (black asterisk) and time white asterisk) are displayed on the bottom right hand corner of the screen. (Magnification:- x 600)

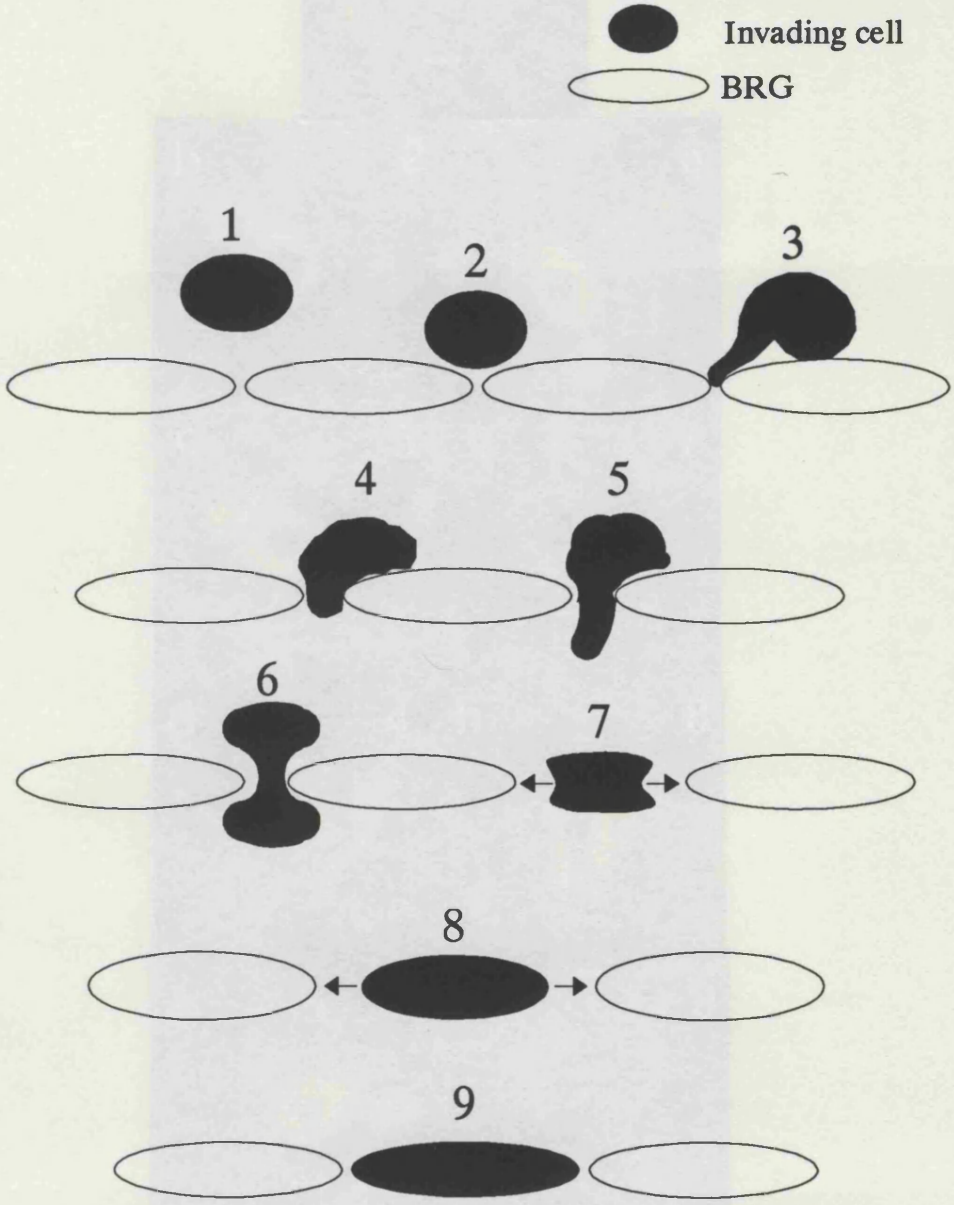


Fig. 3.45. Schematic diagram showing the nine stages of the incorporation of the invading cell (BRPE or BSF) into a BRG monolayer.

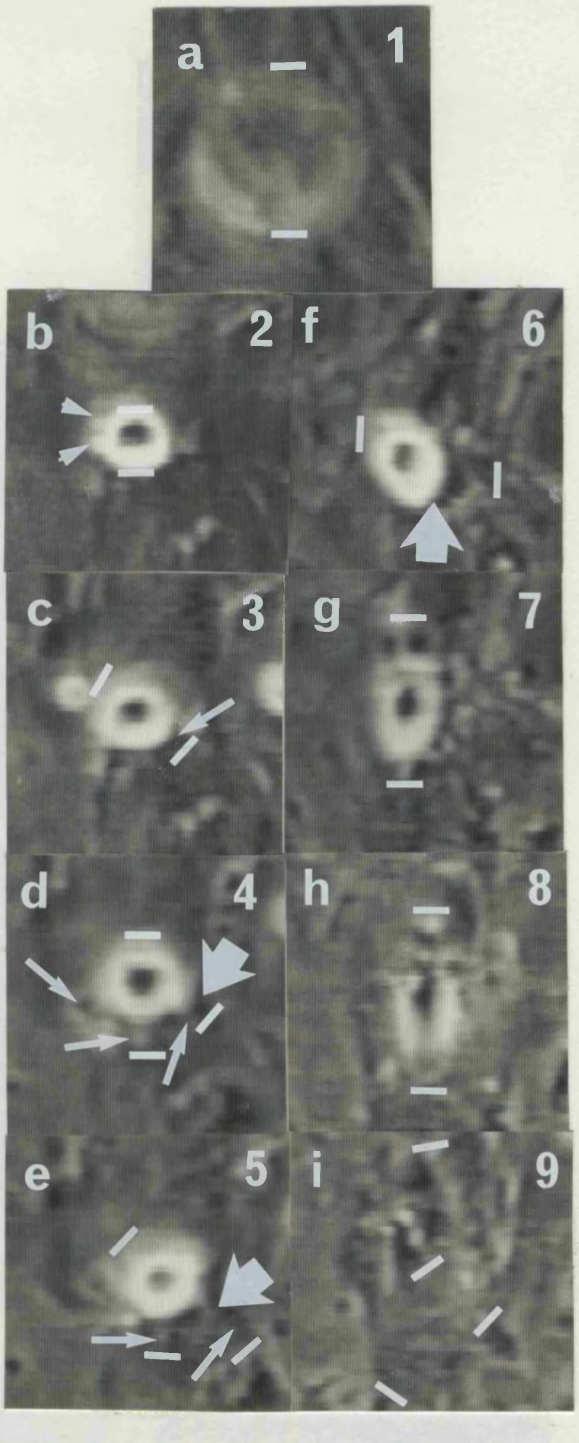


Fig. 3.46. Still photographs taken from a video time-lapse tape showing nine stages of incorporation of BRPE into a BRG monolayer (a-i). The extent of the BRPE cell in each stage is marked (bars). In stage 2 (b) the rounded cell shows small protrusions (arrow heads) and in stages 3-5 (c,d,e) probing pseudopodia (small arrows) extend between the BRG cells. In 6 (f) a large proportion of the cell bulk is under the surface of the monolayer and the point of intersection with the BRG layer is marked by a large arrow. (Magnifications:- a x 750, b-i x 700)

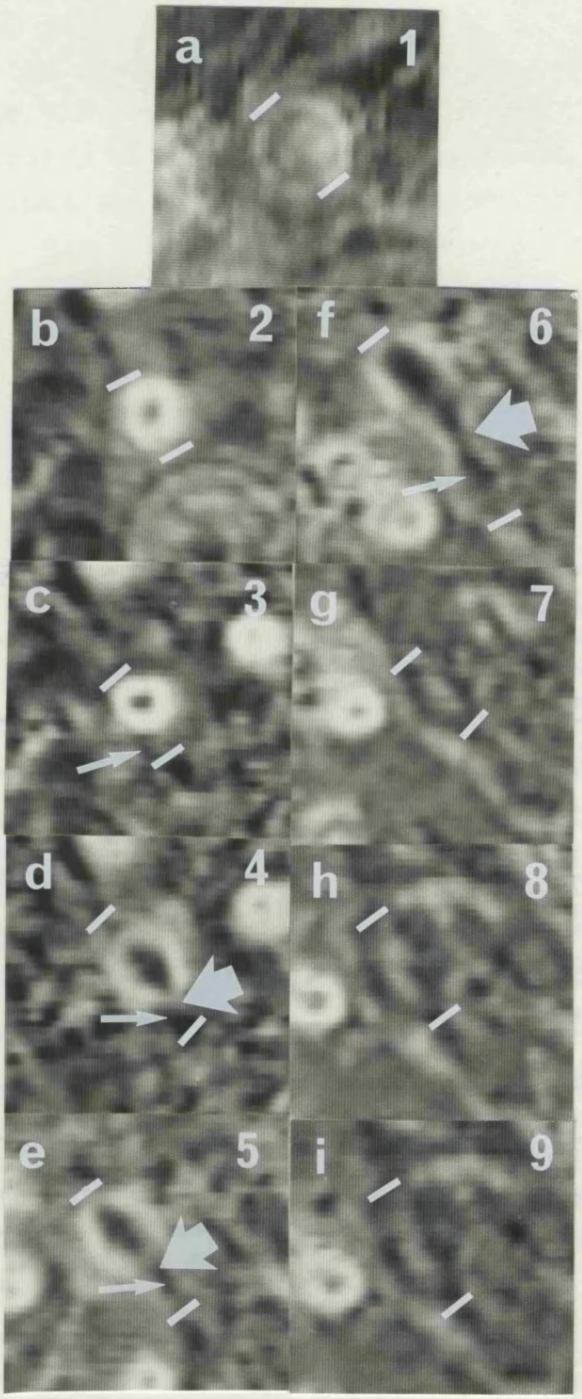


Fig. 3.47. Still photographs taken from a video time-lapse tape showing nine stages of incorporation of BSF into a BRG monolayer (a-i). The extent of the BSF cell in each stage is marked (bars). In stages 3-5 (c,d,e) a single probing pseudopodium (small arrows) extends between the BRG cells and in 6 (f) it expands under the monolayer surface, the area of intersection with the BRG layer is marked by a large arrow. (Magnifications:- a-i x 700)

Stage	Times taken for each stage of BRPE invasion into a BRG monolayer (n=60)	Times taken for each stage of BSF invasion into a BRG monolayer (n=60)
1-2	10 minutes	10 minutes
2-3	15 minutes	7.5 minutes
3-4	15 minutes	7.5 minutes
4-5	10 minutes	5 minutes
5-6	10 minutes	5 minutes
6-7	10 minutes	5 minutes
7-8	30 minutes	30 minutes
8-9	2-3 hours	2-3 hours

Fig. 3.48. Table of the averaged and rounded up times taken by BRPE and BSF to undergo the nine stages of incorporation into the BRG monolayer.

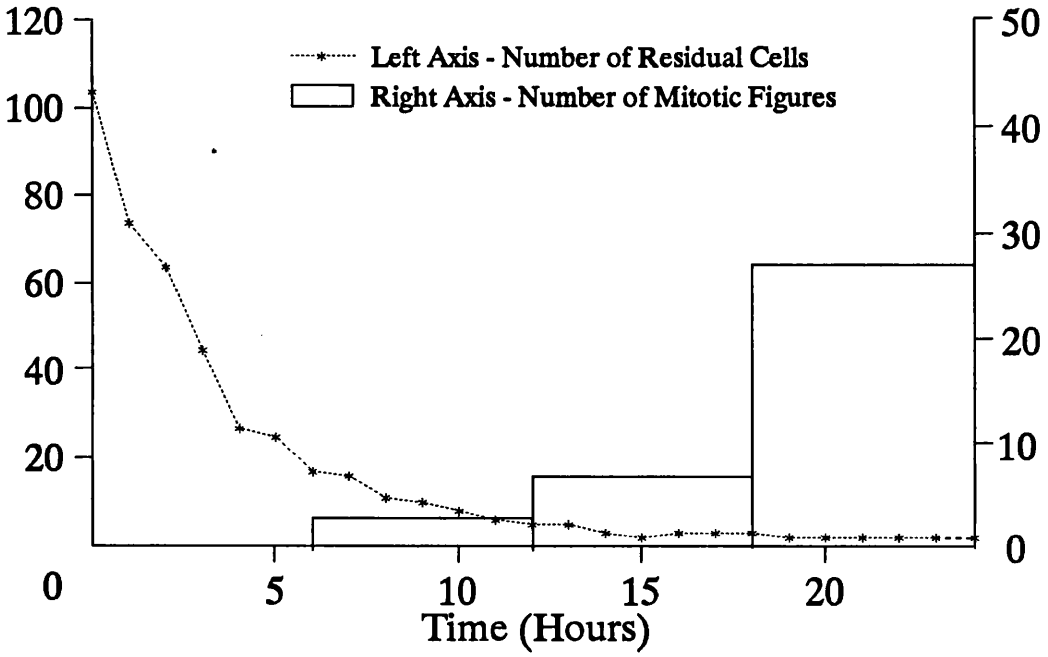
Both BSF and BRPE cell settlement and invasion induced ruffling activity in the BRG monolayer at 8 hours. Up until this time the BRG monolayer had been more or less immobile. Towards the end of the invasion assays, increased mitotic activity was noted in the BRG sheets settled with BRPE but this did not seem to be associated with BSF incorporation.

Time-lapse runs were conducted which were extended up to 24 hours so that the induced cellular division could be investigated further. The BRPE and BSF invasion rates was similar to that shown above, the ID_{50} for BRPE was between two and a half and three hours with the whole process being over by eight hours. For the BRPE mitotic activity in the BRG monolayer was negligible in the first six hours and then progressively increased up to the termination of the assay (Fig. 3.49a.). As the division rate was still increasing at 24 hours, an even longer run of 72 hours was undertaken. Several long term recordings of 24 hours and greater have been done for BSF and they did not have the effect that BRPE did on the BRG monolayer (Fig. 3.49b.).

In the 72 hour BRPE studies, once again the ID_{50} was between two to three hours and the invasion process was over by eight hours. Mitotic figures were not seen in the monolayer in the first six hours, thereafter they were evident and high levels were noted between 13 and 48 hours and then eventually declined (Fig. 3.50.), usually it was all over by 60 hours.

With this system it was difficult to keep recording over 24 hours as the heat box was not a gassed one and acidosis and consequent deterioration in the cells' condition set in. Careful examination of the BRG monolayer showed that it was the BRG cells which were entering mitosis and not the invading BRPE cells (Fig. 3.51.). By using the play-back facility on the video recorder it was possible to deduce that, eg. 10 hours 56 minutes after settlement of BRPE, the BRG cell started to retract from the other monolayer cells (Fig. 3.51a.). After 14 hours 19 minutes the cell was undergoing mitosis (Fig. 3.51b.) and after 14 hours 33 minutes, cytokinesis was observed taking place (Fig. 3.51c.).

a



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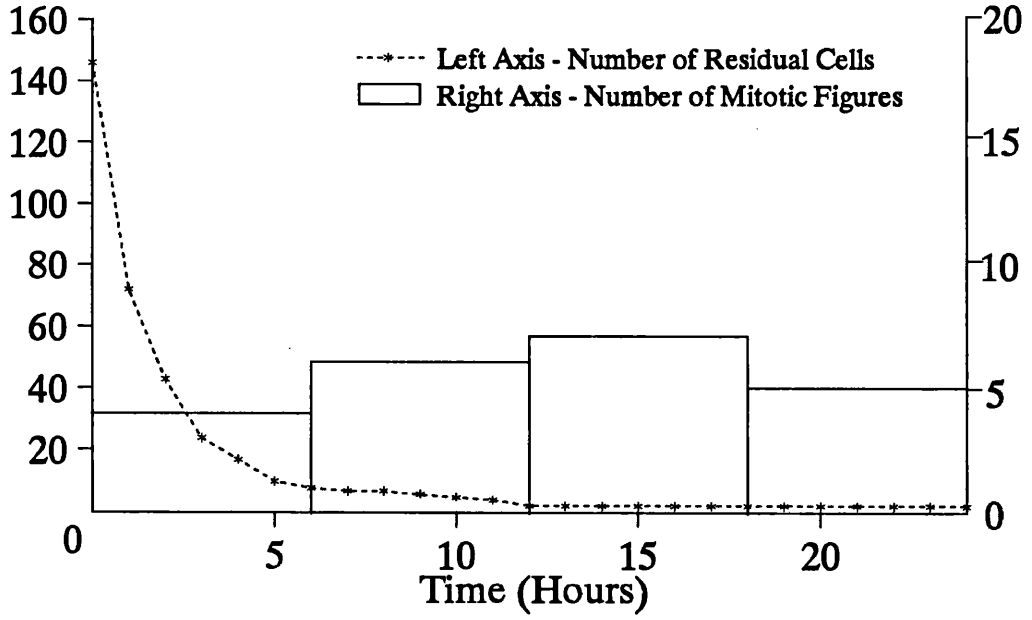


Fig. 3.49. The rate of loss of (a) BRPE and (b) BSF from the surface of a BRG monolayer, showing the number of mitotic figures counted over a 24 hour period.

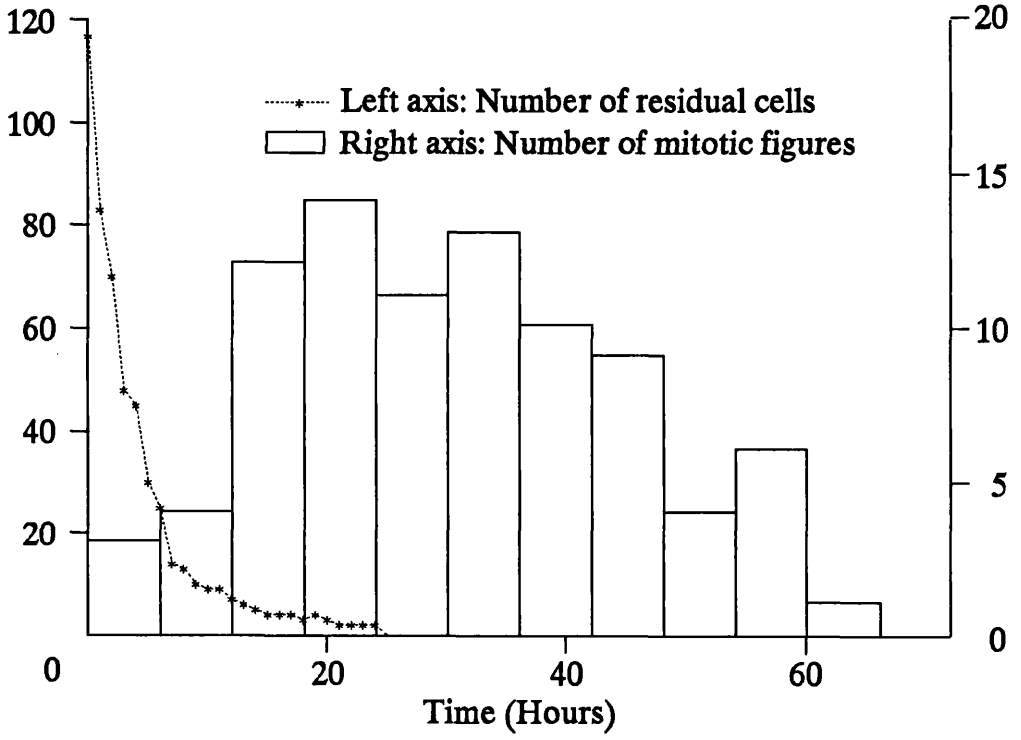


Fig. 3.50.The rate of loss of BRPE from the surface of a BRG monolayer, showing the number of mitotic figures counted over a 72 hour period.

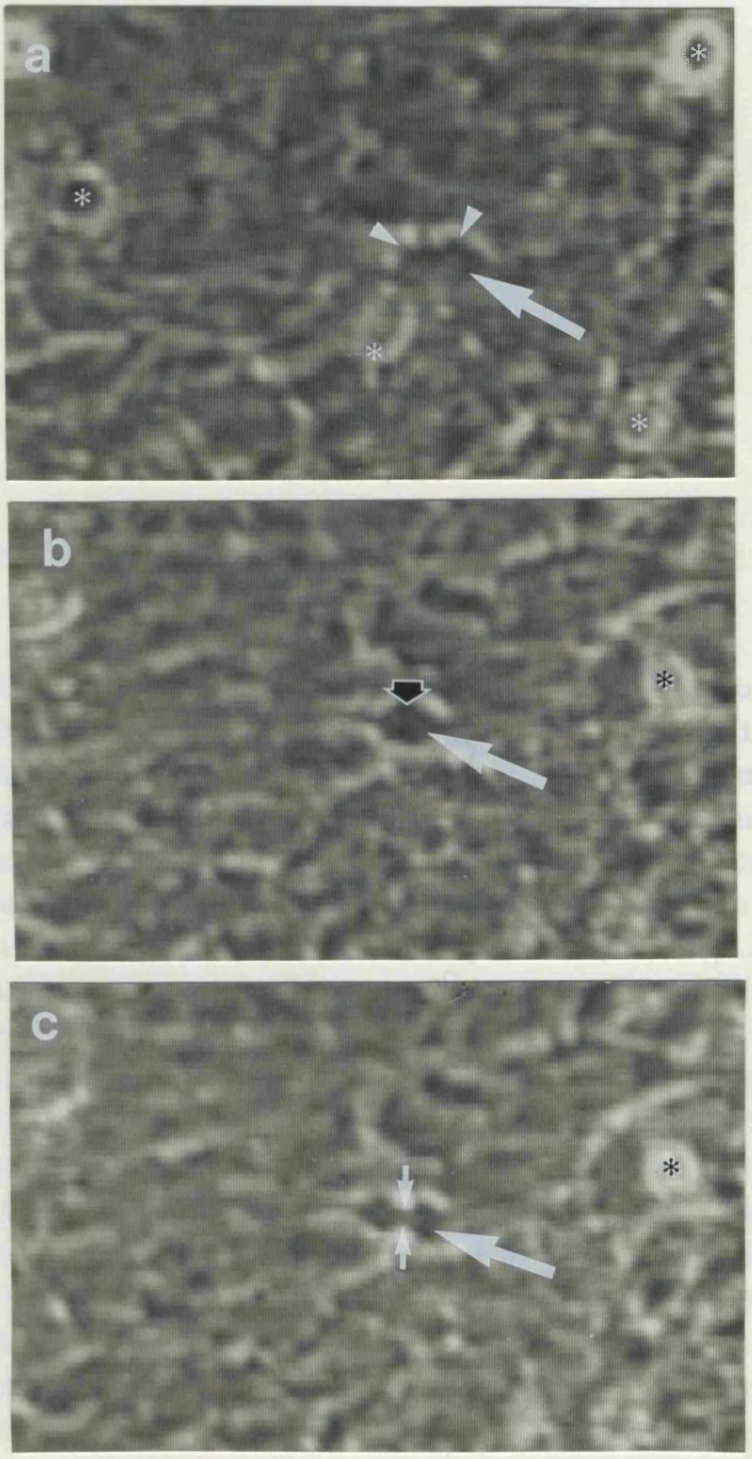


Fig. 3.51. Still photographs taken from a video time-lapse tape of a BRG cell (large arrows) in the monolayer undergoing cell division 10-14 hours after settlement of BRPE. In (a) the cell has started to retract from the other monolayer cells (arrow heads), in (b) the cell is undergoing mitosis (black and white arrow) and in (c) cytokinesis (small arrows). There are still remaining BRPE cells (asterisks) which have not invaded the BRG monolayer. (Magnification:- x 600)

Chapter 4.

DISCUSSION

4.1. Relative Migratory Ability of Cells

In this investigation fibronectin was found to be a potent chemoattractant for BRG, BRPE, BSF and also for cells grown from complex ERMs removed at surgery. The results were consistent with findings from the general wound healing literature in which fibronectin was found to form an integral part of the ECM at the site of healing wounds (Hynes and Yamada, 1982; D'Ardenne and McGee, 1984; Grinnel, 1984; McCarthy et al, 1988) and has been shown to stimulate the migration of cultured cells (Ali and Hynes, 1978). Fibronectin is also known to be a powerful chemoattractant for many cell types including leucocytes (Norris et al, 1982), fibroblasts (Postlethwaite et al, 1976; Gauss-Müller et al, 1980; Tsukamoto et al, 1981), endothelial (Bowersox and Sorgente, 1982) and epithelioid cells (Campochiaro et al, 1984; Watanabe et al, 1988) as well as Schwann cells (Baron-Van Evercooren et al, 1982), C6 glioma cells (Hjelmeland and Harvey, 1988) and tumour cells (McCarthy and Furcht, 1984). The results were also important in the light of investigative studies on ERMs which demonstrated the presence of fibronectin in membranes of short and long duration (Hiscott et al, 1983), in surrounding tissues and fluids such as the vitreous (Campochiaro et al, 1985) and SRF (Immonen et al, 1989) and also to be produced by cell types present in ERMs (Yamakawa et al, 1987; Vaheri et al, 1976; Hiscott et al, 1992; see also Appendix III).

However the results from studies on the effect of fibronectin on the migratory behaviour of cells associated with ERM formation hitherto have been variable and in some instances

inconclusive. Migration studies on retinal glia were a case in point (see Fig. 1.23.). Rat retinal glia had been shown by some to be insensitive (Harvey et al, 1987) and by others to be responsive to fibronectin (de Juan et al, 1988). The present investigation demonstrated that BRG were consistently responsive to fibronectin and these results held true for cells grown from a series of different bovine eyes. Zigmond-Hirsch chequerboard analysis of the migratory response to fibronectin showed that although there was evidence of chemotactic migration, the BRG migration displayed a relatively large chemokinetic component. Evidence from pathological material (Foos, 1978) and in vitro studies (Hjelmeland and Harvey, 1988) indicated that glia reacted to a range of locally acting hormones and autocrine stimuli by migrating from their normal retinal sites to settle and divide on the vitreoretinal surface. If our findings applied to the in vivo situation then fibronectin would be a migratory stimulus for retinal glia to form simple ERMs but the stimulation is only partly gradient and partly direction dependent.

It first became evident during the single cell type migratory studies that BRPE, although responsive to fibronectin were on the whole less effective at migration than the other cell types. Campochiaro and coworkers (1984) showed that human RPE also migrated to fibronectin in similar numbers but with a very high dose optimum of between 50 to 100 μg/ml and the BRPE, like the human RPE in Campochiaro's study, showed a considerable amount of chemotactic migration as well as random stimulated movement. Campochiaro also considered that fibronectin was "a potent chemoattractant for RPE cells" however in comparison to the other two cell types, as shown in this study, the BRPE responded relatively sluggishly to fibronectin.

The migration of BSF compared well with that of the BRG and this was predictable given the results of the numerous fibroblast/fibronectin migration studies in the literature (Postlethwaite et al, 1976; Gauss-Müller et al, 1980; Postlethwaite et al, 1981; Seppä et al, 1981; Tsukamoto et al, 1981; Mensing et al, 1983; Melchiori et al, 1986; Albini et al, 1987; Joseph et al, 1987). However the concentrations of fibronectin required for an optimum response varied markedly from 1.2 μg/ml (Postlethwaite, 1981), 5 μg/ml in our studies and up to 20 μg/ml (Joseph, 1987). Zigmond Hirsch analysis in all the studies demonstrated that a large component of the migratory response was due to chemotaxis.

Though it was apparent that the fibroblasts seemed to differ in their migratory response according to site and species the

response appeared to vary in quantity rather than quality. It was interesting to note that settlement counts showed that although the settlement varied between cell types and between runs, cellular migration was largely independent of it. The findings implied that the numbers of cells introduced into the upper wells of the chemoattraction chamber would have had to be substantially reduced to have an effect on migration.

The dose response curves for the migration of the three cell types tended to vary from run to run. Indeed the variation may have accounted for the disparity between the data of other workers who have looked at the migrational response of retinal cells and fibroblasts (Fig. 1.23.). This study showed by Zigmond Hirsch analysis that it was the chemotactic component of the cells' migration that varied, not the proportion of stimulated random migration. The latter was remarkably constant between the cell types.

The in vitro migratory behaviour of cell types associated with ERMs such as RPE and glia have been extensively investigated but there have been no published micro chemotaxis chamber migration studies to date using cells grown from the ERMs themselves probably because of difficulties culturing these cells. Hiscott and coworkers (1983) observed the migration of cells out of ERMs in culture using time-lapse cine photo microscopy and monitored their locomotory characteristics.

We showed that cells grown from ERMs in culture were stimulated to migrate to fibronectin and this served to reinforce the results obtained with the bovine cells. However the results were obtained employing cells grown from only two surgical specimens which grew particularly well in culture. Also the ERMs were demonstrated to vary in cellular content when viewed by cine photo time-lapse microscopy (Hiscott et al, 1983) and immunocytochemistry (Hiscott et al, 1985) so the results were treated with some degree of caution. It may have been that the two cultures produced atypical outgrowths and it was extremely unlikely that the full diversity of cell types were maintained in later stages of culture. The random activity of the ERM cells was fairly high as demonstrated by the negative control value in the dose response curve and a surprisingly high proportion of the settled ERM cells migrated in comparison to the bovine cell types. This indicated the possible presence of a vigourous subpopulation of cells that migrated well (Laplante and Lemaire, 1990). These results were balanced against lower settlement figures indicating higher rates of cell loss and greater delicacy of the cells. Unfortunately due to the small specimen size and

lack of vigour of the cells in culture and there were insufficient samples of ERM cultured material to be able to draw any definite conclusions.

With these reservations in mind the cells grown from the ERMs were included in the study. They were cultured directly from the pathological material so they were considered to be an extremely useful if somewhat limited yardstick for the behavioural activities of the bovine cells and were incorporated in the study as a support for the main bovine test models, which could be and were repeated many times.

The mixed cell migration model, as well as lessening some of the problems caused by variation in cellular migration between runs, was considered to be a more realistic representation of the migration occurring in the in vivo situation. It was a step nearer to the interdependent migrational behaviour of the ERM cells themselves with the advantage that the cellular types and quantities were already known. Ideally of course human ocular cells would have been preferable to investigate the behavioural responses related to a disease process in the human eye. Human RPE and scleral fibroblasts have been employed in migration studies (Campochiaro et al, 1983, Robey et al, 1992; Calthorpe et al 1990). However human retinal glia though occasionally used (Uchihori et al, 1991) were found to be difficult to culture (Savage et al, 1988; Hjelmeland and Harvey, 1988) and did not grow sufficiently well to produce the large cell numbers required for the assays in these investigations.

It would seem a sensible solution in future to follow the trend of transfecting cells known to be difficult to grow in culture, with SV40 virus, as transformation would extend their capacity to divide in vitro and ensure large numbers of healthy vigourous cells but perhaps with only limited characteristics in common with their counterparts in vivo. Ocular cells in culture such as ciliary epithelium (Coca-Prados and Wax, 1986) and cells grown from human neuroretina (Daya-Grosjean et al, 1984) have already been transformed in this way. Also the rat glioma cell line C6 has been used in numerous studies for example those involving migration to fibronectin, laminin and growth factors (Hjelmeland and Harvey, 1988). In addition spontaneously transformed human RPE cell lines have been used for some time (Dutt et al 1989; Hunt et al, 1994).

The mixed migration studies confirmed the previous experiments that although the BRG tended to be a slightly more responsive to the chemoattractant fibronectin than the BSF but on the whole there was little difference between the migratory

ability of the BRG and BSF to fibronectin whether they were migrated together or separately and that BRG and BSF were both more responsive than the BRPE. The BRG however certainly dominated the mixed cell migration to PDGF, which may be significant as PDGF is present in plasma which is in all likelihood present at the site of ERM formation (Campochiaro et al, 1988). Again the BRPE migrated to PDGF in smaller numbers than the other two cell types. When comparing this result with that of Campochiaro and Glaser (1985) it is possible that they overstated the importance of the migratory response of human RPE to PDGF due to the lack of a reference point on their part. The reference point in this study is the presence of the other two key cell types in ERM formation in the same chemotaxis well as the BRPE. A useful further experiment would be to confirm the result with human cells by repeating the experiment with labelled human RPE, labelled human scleral fibroblasts and human retinal glia (of either wild type or SV40 transformed origin).

The fact that the BRPE were consistently less competent at migration than the other test cell types and that their incompetence in the micro chemoattraction chamber was shown not to be due to complications such as settlement, cell size and membrane pore size was a crucial finding in the light of the emphasis hitherto placed on the importance of RPE migration in the formation of ERMs (Machemer, 1975; Campochiaro and Glaser, 1986; Kirchof and Sorgente, 1990). One of the possible explanations maybe that in the in vivo situation in ERM formation the RPE are "swept" to the vitreal surface through retinal deficiencies and by normal ocular saccadic movements so they may not have to undergo too much active migration (Machemer, 1975). When they do undergo substrate dependent migration to the site of ERM formation then their response to soluble stimulants is sluggish and it should be remembered that many complex ERMs have only a small population of RPE. Histological evidence (Hiscott et al, 1985) from surgical specimens shows that although RPE, as would be expected, are prominent in subretinal membranes but far less prevalent in ERMs (often less than 25% of the total cell population).

It is also possible that the products of inflammation and tissue damage could induce the RPE still present in the monolayer to secrete stimulatory substances which may in turn diffuse through the compromised neural retina as part of the subretinal fluid and attract other cell types. In such a situation there need be little movement of the RPE themselves. Their role in ERM formation would perhaps be more concerned with the production of

biologically active substances than to be the essential building blocks of ERM development. Some support for the theory might come again from the surprisingly low numbers of RPE reported in many ERMs (Hiscott et al, 1985).

The labelling system developed for the mixed cell migratory model as well as being an effective way of comparing the migratory rates of different cell types has interesting applications in the fields of longer term co-culture of retinal cells and in the identification of cells in the video time-lapse invasion assays. It would now be possible to replace carmine with integrally dyed latex microspheres in a range of different colours (Polysciences, USA). Also other labelling possibilities lie in the field of aliphatic reporter molecules with fluorescent tags (PHK2 and PHK26, Sigma Chemical Company, USA) which are incorporated into the lipid bilayer of the cell membrane. They were originally developed by Horan and Slezak (1989) for use in cell tracking and their advantages are that due to their localisation in the membrane lipid bilayer they are stable and are distributed evenly on cell division (Samlowski et al, 1991). It is also feasible that they could be used in micro chemoattraction chamber assays and detected with an epifluoresence microscope linked to image analysis equipment.

4.2. Migration to Subretinal Fluid and Retinal Crude Extract

From the results of present study, it was clear that the BSF, and to a lesser extent the BRG, were stimulated to migrate to SRF and this could be extrapolated to the in vivo situation to mean that these cell types could be responsive to stimuli likely to surround them during retinal detachment and ERM formation. SRF had also previously been shown to induce migratory and proliferative responses in human RPE (Hackett et al, 1988) and bovine retinal microvascular endothelial cells (Boulton et al, 1992). The motogens which were present in SRF did not appear to be a particularly potent stimulus for the migration of BRPE. It is possible that this was again a function of the relatively poor migratory response of the BRPE when compared with the other two cell types. On the other hand the BRPE migration was substantially increased to the SRF samples from patients with proliferative disease although it never reached the levels

associated with BRG and BSF.

Immonen and coworkers (1987) immunoassayed specimens of SRF, from eyes with retinal detachments and from those with retinal detachments complicated by PVR. They found fibronectin to be present in all the samples at a mean concentration of 20.9 μg/ml and an extremely wide range of 0.9 to 111.1 μg/ml, so that their SEM was large (± 7.4 μg/ml). The fibronectin concentration was found to be increased with the duration of retinal detachment from 7.2 ± 2.8 μg/ml at or below 2 weeks detachment to 42.6 ± 16.0 μg/ml between 2 and 6 weeks but the mean concentration in samples from eyes with PVR was only 26.0 ± 10.7 μg/ml. In the present investigation the level of BRPE migration was at or below the optimum fibronectin value indicating that the migratory response of the BRPE possibly could have been due to fibronectin alone. However the results from the migration of BRG and BSF were over and above that which would be expected from the fibronectin content alone and indicated the possible presence of some other stimulatory factor(s). Unfortunately the small size of our SRF samples (20-30 μl) precluded biochemical analysis as well as migration studies but techniques such as antibody neutralisation procedures may be important in the future.

In the past, biochemical and immunochemical investigations of the protein content of SRF showed it to contain constituents both from the vitreous (Akhmeteli et al 1975) and some serum components from the blood (Chignell et al, 1971). In instances where there is a retinal tear and/or retinal damage or degeneration, it is probable that this would allow for the passage of soluble molecules between the vitreoretinal region and the subretinal space. Given this it is possible that the SRF could contain many of the stimulatory factors present in the vitreous for example the FGFs (Gaudric et al, 1988), IGF I (Grant et al, 1990 and TGFβ (Gaudric et al, 1988). Recent studies employing ELISA have shown the growth factor bFGF to be present in SRF samples and the levels of bFGF increased with severity of PVR (Xu et al, 1994). Boulton and coworkers (1992) demonstrated that the chemoattractive and mitogenic stimulating activities of specimens of SRF and vitreous were similar and the activity produced by SRF was less than that of vitreous samples. Serum components also maybe present in the SRF as a result of the breakdown of the blood retina barrier due to disruption to the integrity of the RPE cell layer and exudation from the leaky fenestrated choriocapillaris (Chignell et al, 1971).

It is unfortunate that in this study there were too few specimens for effective evaluation of the differences in

motogenic stimulation between the SRF from patients with retinal detachment, PVR and PDR. Burke and Foster (1882) demonstrated that injured vitreous stimulated DNA synthesis in RPE cells in vivo and in vitro and Campochiaro and coworkers (1985) showed increased migration of human RPE cells to vitreous from eyes with PVR in comparison to vitreous samples from eyes with macular pucker and simple retinal detachments. It should be mentioned however that Campochiaro also used small numbers of samples.

Light and electron microscopical studies of the cellular components of SRF samples from eyes with rhegmatogenous retinal detachment by Feeney and coworkers (1975) indicated the presence of fragments of neural retina, macrophages and RPE. The macrophages could either be microglia from the retina, hyalocytes from the vitreous or they could originate from the blood. The RPE were present both in their cuboidal polarised form and what was described as their "migratory" form. The migratory phenotype displayed filopodia and had large phagocytic vacuoles containing rod outer segments. The importance of the presence of macrophages and migratory RPE in SRF is that they are both known to be a source of stimulatory factors (Campochiaro et al, 1988; Nathan, 1987).

Our results demonstrate that the BSF respond most strongly of the three cell types to SRF samples, which would support the hypothesis that in the in vivo situation the fluid under the retinal break contains bioactive substances that attract fibroblast-like cells into the area. At first glance the BSF and BRG appeared to respond more strongly to samples of fluid from eyes with proliferative disease than from those with retinal detachment. The number of SRF samples was very small and analysis by the Mann Whitney U test for very small samples (Siegal, 1956) showed a significant increase in migration by the BRPE cells. The conclusions that can be drawn from these results are limited by the number and size of the samples but there is potential for expansion of the investigations with adequate numbers of specimens to possibly include video time-lapse settlement studies. There is also scope for further analysis of the SRF, not only to include the detection ECM proteins and growth factors but also motility factors such as scatter factors known to effect the mobility of epithelial cells in vitro (Stoker, 1989).

One of the sources of the stimulatory factors present in the SRF and also the vitreous is likely to be the retina itself, as the vitreous only has a sparse cellular population (Hogan et al, 1972) and the amount of plasma leakage in non vascularised ERMs is relatively small (Chignell et al, 1971). Also the retina is

known to be a source of stimulatory growth factors (Arruti and Courtois, 1978; Barritault et al, 1981; Baird et al, 1985). In addition the products of injured neural cells in particular in the damaged CNS are known to influence cell behaviour due to the release of active factors (Finklestein et al, 1987; reviewed by Schwartz et al, 1989). Experimental models of retinal detachment in the owl monkey demonstrated degeneration of photoreceptor outer segments in detached portions of the retina with underlying focal proliferations of RPE (Kroll and Machemer, 1968) and periretinal focal proliferations of retinal glial cells (Laqua and Machemer, 1975).

In the past crude retinal extracts have been shown to stimulate proliferation in several cell types including retinal vascular endothelial cells (D'Amore et al, 1981; Glaser et al, 1980) human RPE, fibroblasts and rat brain astrocytes (Campochiaro et al 1986). Peptide growth factors present in the retina are the fibroblast growth factors (acidic and basic, originally known as eye derived growth factors I and II; Barritault et al, 1980; Courty et al, 1985; Baird et al, 1985), PDGF (D'Amore et al, 1981), IGF I, TNFα (reviews by Gospodarowicz, 1983 and Hicks et al, 1991) and TGFβ (Gaudric et al, 1988).

Interestingly the migratory response of the bovine test cells to a retinal crude extract (RCE) followed the same trend as that to the SRF samples. Fibroblasts which responded very well to RCE and far better than the other two cell types were known to migrate in response to acidic (Senior et al, 1986) and basic (Diegleman et al, 1986; Buckley-Sturrock et al, 1989) FGF. BRPE barely responded to RCE and it is interesting that Campochiaro and coworkers showed that human RPE were did not respond to basic FGF. However Harvey and coworkers (1987) demonstrated that although rat retinal glia (unlike brain astrocytes) were insensitive to basic FGF they were stimulated to migrate by RCE so it is possible therefore that there was some other stimulatory factor present in the retinal extract preparations such as PDGF (Glaser et al, 1980; D'Amore et al, 1981) or it may have been due to species difference between the response of rat and bovine retinal glia. Or alternatively the meagre response of the BRG to RCE may reflect the presence in retinal glial cultures of a mixed population of responsive retinal astrocytes and unresponsive Müller cells.

Approximately half (41%) of the activity of the bovine retinal extract remained after filtration. The finding suggested that there was more than one stimulatory factor present in RCE

and at least one having a molecular weight greater than 30,000 daltons and one with less. Amongst the larger molecular weight substances would be included glycoproteins like fibronectin. The molecular weight range for PDGF (35-38,000 daltons) is close to the cut off but other cytokines such as acidic and basic FGF and TNFα with weights in the range 15-17,000 and IGF I 7-8,000 are small molecules which would pass through the filter. Indeed as 44% of the activity was destroyed by heating to 57°C this indicated that the active site on at least one of the molecules was sensitive to temperature change. There may have been irreversible denaturation of the protein. Also all the stimulatory factors present were very susceptible to boiling, indicating that at 100°C there may have been sufficient destruction of the molecule to give loss of biological activity and the molecule possibly had a tertiary or quaternary structure which underwent irreversible conformational changes on heating.

The loss of activity after acid and alkali treatment pointed to the fact that one of the factors may have been dependent for its structure and biological activity on a large number of amino acid residues and a change in pH caused them to alter their polarity so affecting their ability to form cross-links with other residues. There was a greater loss in migratory activity with an alkali than an acid pH and this suggested that the amino acids were mainly positively charged. The activity was retained on change of pH, which may have indicated the presence of a large number of non-polar amino acids in one of the stimulatory factors or at least an ability of the native structure to be reformed when the pH was restored to neutral.

In conclusion RCE appeared to contain at least two (and possibly more) migration stimulating factors probably of different amino acid compositions; potential factors suggested from the work of others have been the FGFs (Baird et al, 1985; Mascarelli et al, 1987) and PDGF (Glaser et al, 1980). The bioactive site on PDGF is known to be heat stable at 100°C (Antoniades and Scher, 1974) whilst the activity of FGF is destroyed by heating to 70oC for five minutes (Gospodarowicz, 1983) and most of the activity of the RCE is destroyed by heating to 100°C. One possibility for future study would be to assay for the growth factors using techniques such as ELISA. Changing the environment of a fluid such as RCE then assessing the activity it elicits from the test cells is a crude but effective way to initially reveal basic characteristics of the active molecules so that later detailed and more specific analyses can be carried out.

One of the cautions that has to be exercised when analysing the results from RCE and extrapolating to the situation in PVR is that RCE is made from normal intact retina and the products of the degenerating retina may differ markedly due to additional or altered cellular components for example the presence of inflammatory cells. Also RCE is harvested from the neural retina only and excludes the RPE which are known to be an important source of stimulatory factors in PVR (Bryan and Campochiaro, 1986) and these are discussed in section 4.3.

4.3. Behavioural Responses to Products Harvested from Cultured Cells

In order to simplify the complicated interactions of the cell types, schematic diagrams and charts were drawn up to illustrate the relative effects of the different cell types on each others' behaviour (Figs. 4.1., 4.2 and 4.3.) The bovine retinal glia in this study produced factors which stimulated the migration of all three cell types (Fig. 4.1.). Burke and coworkers (1989) demonstrated that rabbit retinal glia secreted chemoattractants as well as mitogens for bovine RPE. In the current investigation it is possible that a likely component of the test cells' migratory response is due to fibronectin as it is known to be a present in conditioned medium (see Appendix III). Glial cells are known to produce fibronectin in vitro (Vaheri et al, 1986) and mRNA for fibronectin has also been identified associated with epiretinal glia, by in situ hybridisation (Hiscott et al, 1992). In addition heating the BRG conditioned medium to 56°C for 30 minutes which is a treatment known to denature glycoproteins such as fibronectin, only removed 10% of the migratory activity of the BSF, so it is likely fibronectin is responsible for some of the BSF migration.

The remainder of the treatments indicated that the molecule(s) were larger than 30,000 daltons and that the conditioned medium may contain more than one active factor as 50% of the activity remained after acid treatment. All the migratory activity was destroyed by increasing the pH to 10 possibly signifying a structure with a large number of positively charged ions. A substantial proportion of the activity (80%) remained after heating to 100°C suggesting that either a tertiary or quaternary configuration is not essential for the activity of the

molecule(s) or that the cross links that for these can easily reform on cooling. PDGF as mentioned previously (section 4.2.) stable at 100°C (reviewed by Ross and Vogel, 1978) so is a likely candidate.

Soluble substances produced by the BRPE had little effect on their own migration (Fig. 4.1b.), this was in contrast to the literature on the effect of RPE conditioned medium on the proliferation of RPE (Bryan et al, 1986) where RPE migration was substantially increased. The results in our study indicated that BRPE did not have an autocrine stimulatory effect on migration in vitro. They did however produce substances that were stimulatory for BRG.

The BRPE in culture appeared to produce a powerful chemoattractant for the BSF (Fig. 4.1b.) which begged the question, in the in vivo situation would the RPE provide motogenic stimuli to attract neighbouring fibroblasts or fibroblast-like cells? Boiling the BRPE conditioned medium left 27% of the migratory activity of the cells implying that an active molecule was heat stable. From the evidence it was possible to conclude that BRPE conditioned medium, unlike that produced by the BRG contained only one migration stimulating factor, as the majority of its activity was lost on all the treatments, apart from heating to 56°C. Basic FGF is heat labile, has a molecular weight under 30 000 daltons (Hicks et al, 1991) and is known to be produced by human RPE (Shipley et al, 1987; Schweigerer et al, 1987; Sternfield et al, 1989). The small amounts of activity retained on heating may either have been due to parts of the molecule that were untouched by the short time at 100°C or fragments which retained biological activity. The results from the treatments of BRPE in this investigation differed from those described in the literature for proliferation experiments (Morse et al, 1989, Bryan et al, 1986), where alkaline and acid conditions and heat treatment (100°C for three minutes) appeared to enhance the effect of RPE conditioned medium.

Medium conditioned by BSF cells appeared to reverse the migratory trend hitherto followed by the three cell types. The BRG were barely stimulated to migrate above control levels and the migration values for BRPE were consistently higher than the other two cell types (Fig. 4.1.). BSF conditioned medium had a selective effect and the migratory pattern was different to the results produced with the other conditioned media, which may indicate that there was possibly some inhibitory factor present. These results agree to some extent with the literature that BSF

conditioned medium though it produces some migratory response (Calthorpe et al, 1990) it is less than epithelial cell types such as bovine corneal endothelium (BCE) or BRPE.

Introductory characterisation of the active factors in BSF conditioned medium was carried out by Calthorpe and coworkers (1990) and the active factor was found to be heat sensitive as most of the activity was lost on boiling. About two thirds of the activity remained after acid treatment, indicating that as for BRG conditioned medium there may have been more than one active factor. Most of the activity was lost with the 30,000 dalton filter indicating that the molecule was fairly large or possibly existed as a polymeric form or bound to a carrier (Mensing et al, 1983).

Medium conditioned by a ERM proved to be a very effective migratory stimulant for the BSF (Fig. 4.1.). Implying that a mixture of products in ERM medium was acting synergistically on the BSF or possibly that as BRPE conditioned medium had greatly enhanced the migration of fibroblasts this particular ERM culture was predominantly RPE in content.

The enhanced settlement of the BRPE over the other two cell types in response to the conditioned media from BRG and BSF corresponded well with the relatively high settlement response of BRPE to soluble fibronectin (see Fig. 3.5b). The higher fibronectin content in BRPE conditioned medium may partly have accounted for the increased levels of settlement (see Appendix III).

The BRG and the BSF produced factors which had a positive influence on the settlement of the BRPE (Fig. 4.2.). In the in vivo situation if proliferating glial sheets secreted substances which promoted the settlement of RPE which either made their own surfaces and/or the surrounding substrates (such as the ILL and vitreous) more attractive for RPE to settle on and thereafter migrate and divide, then this would enhance complex ERM formation. Also later on in the process fibroblastic cells may well continue to attract RPE to the ectopic site as ERMs retain their cellularity well into the late stages of PVR. Curiously the BRPE did not settle as well as one might expect to substances produced by themselves, especially as RPE are known to produce fibronectin in culture (Appendix III and Yamakawa et al, 1987). Although BSF conditioned medium increased the settlement of BRPE it had little effect on BRG or BSF themselves. In wound healing, fibroblasts are the cells that are receptive to factors produced by other cell types and their secretory function would seem to be more the production of extracellular matrix materials such as

collagen (Albini et al, 1985) which results in fibrosis and the eventual decrease in cellularity of the membrane.

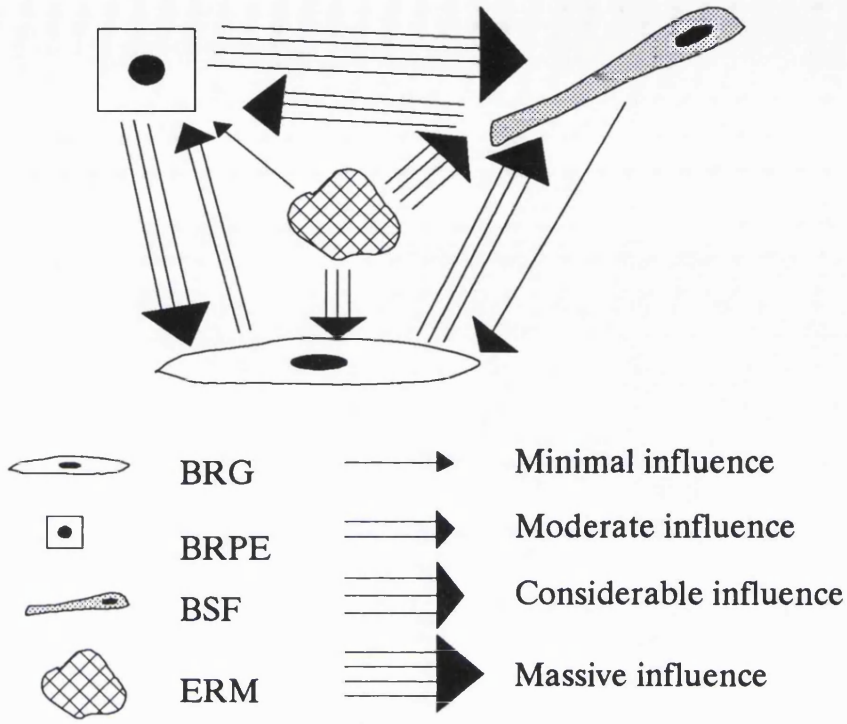
The results from the two step proliferation assays were rather inconclusive in that there appeared to be little increase in cells numbers over the three day period in relation to the control cultures (Fig. 4.3.). It would have been expected that the conditioned medium of one cell type would affect the migration and proliferation of each cell type in a similar fashion.

The controls produced the most interesting information in these proliferation assays; that BSF appeared to grow faster than the BRG and the BRG faster than BRPE which reflects their normal in vivo functions. The BRPE responded to BRPE conditioned medium and this agreed with the study by Bryan and coworkers (1986) in which conditioned medium collected from human RPE stimulated an increase in tritiated thymidine incorporation and cell numbers of RPE. The BSF in this study showed little increase in cell numbers and it is possible that the results were affected by the limits of sensitivity of the assay.

Due to the discouraging initial results, the effect of conditioned media on the proliferation of the test cells was not pursued beyond the preliminary stages. However in the future, further investigations altering the cell numbers initially seeded and the length of time allowed for cell proliferation, together with concurrent flow cytometric assays employing markers of proliferation such as bromodeoxyuridine, may produce more conclusive answers.

Employing media conditioned by cultured cells, to assess the influence that one cell type may well exert on another is a technique routinely used in behavioural studies. Its advantages over using natural fluids such as SRF are that conditioned media can be collected in much larger quantities and produced under controlled conditions. The limitation is that the cells are adapted to culture conditions. The range of substances produced are not under the influence of the complex environmental stimuli present in the eye during the development of PVR. Also this particular technique, although it is flexible in the number and types of behavioural studies in which it can be employed, lacks the reciprocity of signalling between cells which would be achieved in assays where they are grown in co-culture.

a



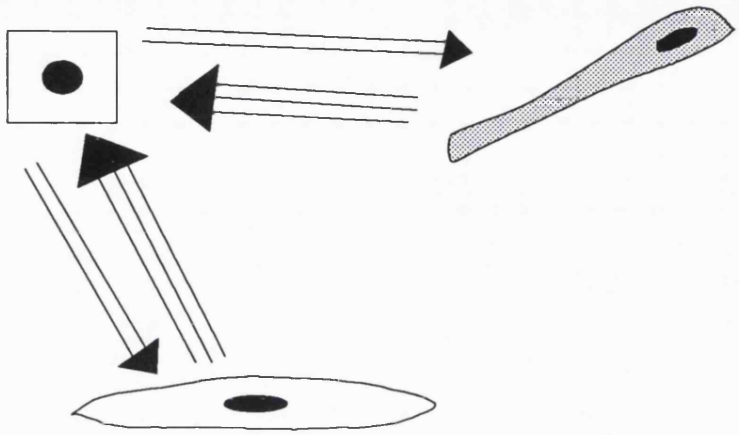
b

Migration			
Target Cell / Conditioned Medium	BRG	BRPE	BSF
BRG	3	2	3
BRPE	3	1	4
BSF	1	3	2
ERM	3	1	4

Fig. 4.1. Schematic diagram (a) and table (b) of the relative effects of the conditioned media from the test cell types on the migration of the test cells.
Table of proportional increase in settlement

0 = No influence
1 = Minimal
2 = Moderate
3 = Considerable
4 = Massive

a

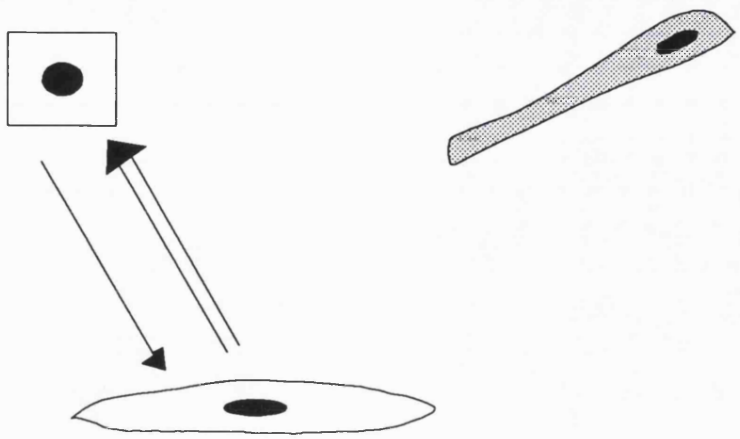


b

Settlement			
Target Cell / Conditioned Medium	BRG	BRPE	BSF
BRG	1	3	0
BRPE	2	2	2
BSF	0	3	2

Fig. 4.2. Schematic diagram (a) and table (b) of the relative effects of the conditioned media from the test cell types on the settlement of the test cells.

a



b

Proliferation			
Target Cell / Conditioned Medium	BRG	BRPE	BSF
BRG	0	2	0
BRPE	1	2	0
BSF	0	0	0

Fig. 4.3. Schematic diagram (a) and table (b) of the relative effects of the conditioned media from the test cell types on the proliferation the test cells.

4.4. Mechanisms of Cell Invasion

The settlement of BRPE and BSF cells on a BRG monolayer did not compare favourably with their settlement on serum coated plastic. So it would seem, from the static settlement assays that a BRG sheet per se, is not a particularly good substrate for adhesion. In other words, the static settlement assay provided scant support for the notion that a simple epiretinal sheet of glia served in vivo as biological Velcro for fibroblasts and RPE floating in the vitreous. It was intended to compare settlement on a BRG substrate with settlement on various extracellular components of the inner limiting lamina such as type II collagen and type IV collagen but following the poor performance of a BRG substrate in the initial studies, this line of investigation seemed relatively fruitless.

It is easy to over interpret simple in vitro results when applying them to the complex pathological events which take place in vivo and the previous study takes no account of the possible effects of settlement enhancement agents in the surrounding fluids. In PVR there are large quantities of biologically active substances, such as glycoproteins and growth factors, in the surrounding periretinal fluids which are likely to modulate settlement and other behavioural activities during the formation of complex ERMs. The glycoprotein, fibronectin is abundant in the vitreous in PVR (Campochiaro et al, 1986; Wilson-Holt et al, 1992) and as well as being a powerful chemoattractant is known to mediate the attachment of many cell types (Yamada et al, 1983). The present study showed that soluble fibronectin encouraged spreading of all three cell types under investigation and promoted their adhesion to plastic. Furthermore the fibronectin enhancement of adhesion of BRPE was far higher than for the other two cell types.

It may at first seem surprising that fibronectin has more effect on the settlement of BRPE than on BSF, since the glycoprotein is usually associated with mesenchymal attachment. On the other hand, epithelial cells produce copious amounts of fibronectin during wound healing and RPE cells are no exception (Yamakawa et al, 1987). Epithelioid cells in culture are known to spread optimally in a time frame of around 30 to 45 minutes to a solution of 5 to 10 μg/ml of fibronectin (Hughes et al, 1979). The present findings indicate that the BRPE optimal response of 5 μg/ml is very much in line with what has been shown previously for epithelium. Glial cell production of fibronectin

is well established (Vaheri et al, 1976) as is their behavioural responsiveness to the glycoprotein in development (Bunge and Bunge, 1983; Bunge, 1987), neural injury (Aguayo et al, 1981) and culture conditions (Vaheri et al, 1976).

Hiscott and coworkers (1992) emphasised the possible importance of fibronectin in the genesis of PVR membranes. The findings in the present thesis provide support for the idea that adhesion and spreading of the three key cell types involved in membrane formation are behavioural activities which are influenced by this glycoprotein. It may be worth noting that the optimal effective concentrations which are seen in this study are reached and often bettered in the vitreal (Weller et al, 1988; Wilson-Holt et al, 1992) and subretinal (Immonen et al, 1989) fluids of patients with PVR. Campochiaro et al (1985) found extremely high levels of fibronectin in the vitreous of PVR patients (mean = 178.5 μg/ml) but this was not confirmed by others who calculated more modest values from their ELISA assays of 4 to 7.5 μg/ml (Weller et al, 1988) and 6.5 to 25 μg/ml (Wilson-Holt et al, 1992).

Approximately 10% of fibronectin in solution becomes adherent to tissue culture plastic (Hughes et al, 1979). Cells adhering to plastic and other surfaces produce small processes and these tend to be immunoreactive for fibronectin antibodies. It is suspected that appropriate receptor molecules are concentrated initially at the tips of the protruding processes (Aplin, 1993). Integrin receptors of the beta-1 family are involved in the adhesion and perhaps also the spreading of cells on extracellular matrix materials including fibronectin (Weller, 1991). Fibroblasts and glia are known to have these receptors and recently they have been identified in RPE cells (Anderson et al, 1990). Furthermore it has been shown that fibroblast and RPE adhesion to, and spreading on, fibronectin (Avery and Glaser, 1986) is impaired if a monoclonal antibody to the beta-1 subunit is present (Chu and Grunwald, 1991).

The use of video time-lapse recording to monitor the interactions between the ERM cell types has not been previously reported in the literature. The technique has been previously employed in studies of the interaction of granulocytes and monocytes with endothelial cells in culture (Grünwald et al, 1989). Video time-lapse revealed the interactive process to be one of invasion and incorporation of the test cells into the underlying monolayer, rather than a layering of the test cells on the monolayer, as described by De Bono and Green (1983) for the interaction of vascular endothelial cells with "vascular

intimal spindle-shaped fibroblast-like cells". Or alternatively a layering of cells under the monolayer as found by Grünwald and coworkers (1989) from their observations of the behaviour of the interaction of monocytes with the endothelial cell layer.

Studies of the locomotory behaviour of substrate dependent cells in culture on a flat surface (reviewed by Abercrombie, 1970) have shown that when cells on a flat substrate come into contact with each other they form monolayers on the substrate in preference to crawling onto the dorsal surface of their neighbours and the cessation of forward movement is often accompanied by halting of the normal pseudopodial activity of the cells. The phenomenon is termed "contact inhibition of movement". Abercrombie distinguishes between type I and type II inhibition. In the former (type I) there is complete cessation of movement and in the latter (type II) pseudopodial movement continues. In general truly invasive cells are distinguished by their lack of contact inhibition of locomotion in culture. The invasive behaviour of the BRPE and BSF may fall into the category of type II contact inhibition of movement and be linked with their preference for a serum coated surface rather than a cellular substrate. The former would contain settlement enhancing substances (Tucker et al, 1981) like fibronectin (Grinnel, 1978) as would the plastic under the basal surfaces of glial cells in culture (Vaheri et al, 1976).

The invasiveness of cells is a much more complex process than merely the lack of contact inhibition of movement (Eagle and Levine, 1967). Studies of the adhesion and invasion of endothelial monolayers by neutrophils have show that invasiveness is thought to be linked to the absence and presence and possibly the up- and down- regulation of receptors for adhesive proteins such as fibronectin (reviewed by Weissmann, 1989; Oppenheimer-Marks et al, 1991). In addition experiments in the field of tumour cell metastasis (Parish et al, 1987), have shown the presence of specific proteins on the surface of tumour cells linked with invasive ability. More recently the increase in migratory behaviour of choriocarcinoma cells on a fibronectin substrate has been demonstrated to be related to changes in integrin expression (Aplin et al, 1991).

The consistently faster rate of incorporation of the BSF into the BRG cell layer may be linked with differences in the amount, the location and/or the status of their receptors for adhesion (Tooney et al, 1993). Chinese hamster ovary fibroblasts in culture have been shown to have a large pool of fibronectin receptors which only appear to bind weakly (Brown and Juliano,

1987), which might give fibroblasts greater flexibility in locomotion and account for their faster incorporation into the monolayer. Whereas we have shown BRPE to require less fibronectin for optimum settlement than the BSF and show greater amount of spreading in its presence in culture. In addition the time-lapse studies have shown that the delay in the BRPE infiltration is due to the extra time they spend undergoing intense pseudopodial activity on the surface of the BRG and that the BRPE were more disruptive to the structure of the monolayer than the BSF when invasion took place. So it is possible that the BRPE formed stronger attachments on the upper surface of the BRG and that the comparatively small amount of attractants present in the serum on the upper surface of the BRG delayed the infiltration of the BRPE between the BRG cells.

The fact that the rate of BSF incorporation into a mixed BRPE/BRG cell layer was faster than BSF into a pure BRG layer, may have been due to the fact that the previously invading BRPE had already disrupted the BRG sheet and consequently physically facilitated the subsequent entry of the BSF. On the other hand we have shown earlier from the conditioned media studies that the BRPE produced substances which promoted the settlement and migration of BSF with a small stimulatory effect on BRG proliferation and these may, if produced in sufficient quantities, have facilitated BSF incorporation in the present assay. A logical series of experiments to follow this aspect of the behavioural investigations would be to assess the settlement of BRG and BRPE on to BRG monolayers in the presence of conditioned medium from cultures of the three cell types and ERM cells. In order to gain some idea of the active substances the conditioned media could be subjected to a series of treatments as for the migration studies, coupled with ELISA analysis and antibodies to substances such as fibronectin.

Human glia in vitro are known to have well regulated growth control mechanisms resulting in the formation of stable cellular monolayers (Pontén et al, 1969). The efficient intercellular signalling that would be necessary to maintain the status quo possibly accounts for the relative asymptomatic behaviour of simple ectopic glial membranes in vivo. Growth control is density dependent and once disruptive influences come into play such as the introduction of another cell type (Nakamura et al, 1983), for example RPE, this may well modulate the signals transmitted between the glia so that they are stimulated to divide.

In our investigation the contact of bovine RPE with a BRG monolayer has been demonstrated to stimulate BRG cell division

over 24 and 72 hour time periods. Burke and coworkers (1985) demonstrated that co-culture of human RPE with rabbit retinal glia for 48 hours gave rise to an increase in DNA synthesis in both cell types detected by ^{3}H thymidine labelling but in contrast, co-culture of rabbit dermal fibroblasts with glia only resulted in a rise in ^{3}H thymidine levels of the fibroblasts.

It is possible that in vivo there is a similar effect in ERM formation when mobilised RPE come into contact with the glial cell layer lining the vitreal surface of the retina. This in turn may cause the glia to proliferate and produce substances attracting other less well regulated cell types such as fibroblastic cells. The studies could be continued to follow the exact pattern of cell interactions using the cell labelling techniques adapted from the migration studies as short term labels (see section 4.1) and to see if there was any reciprocal mitogenic effect on the BRPE or the BSF.

4.5. Final Comments

The genesis of simple ERMs involves glia, particularly Müller cell glia, entering the cell cycle and pushing their way actively through the inner limiting lamina (Hitchins and Grierson, 1988; Hjelmeland and Harvey, 1988) to form non-contractile sheets on the surface of the retina. The stimulating factors remain unknown but inflammatory cytokines are likely candidates (Gilbert et al, 1988; Weller et al, 1989) as are blood, blood breakdown products and serum (Lean and Ryan, 1989; Grierson and Forester, 1980) to be involved in the provocation and modulation of early events. Indeed inflammatory cells are always components of ERM's (Wiedemann and Weller, 1988). Simple membrane formation occurs in the ageing eye (Foos, 1978) but retinal detachment probably accelerates the process and with it comes the constituents for potential catastrophe, i.e. the maturation of simple membranes into complex contractile, fibrous membranes whose tractional activity fixes the detached retina into permanent folds. Surgical removal of the contractile ERMs can lead to anatomical reattachment but reattachment does not mean visual restoration (Chignell, 1988). Indeed vision is usually poor after ERM removal, far worse than can be expected if simple detachments are effectively managed (Wong et al, 1992; Gartry et al, 1993).

The development of complex detachments requires the coming together of fibroblasts, or cells which adopt the behavioural characteristics of fibroblasts, with the preformed epiretinal glial sheets (Grierson et al, 1987). The process involves a plethora of signalling stimuli and behavioural responses which are hard to evaluate in vivo but can be reduced to simplistic one step events in vitro. Determining one step signalling and behavioural responses of the key players in ERM formation has been the essence of this thesis. It was hoped to be able to find clues from these studies which would aid our understanding of the biology of membrane formation and verify some of the assumptions which have never been tested.

We depend heavily on the work of Robert Machemer (Laqua and Machemer, 1975) for our working hypothesis of the mechanism by which RPE become a component of complex ERMs. Under an experimental rhegmatogenous or tractional detachment, the RPE lose their contact inhibition and adhesion to Bruch's membrane. The RPE proliferate and migrate through the detached neural retina and the retinal hole to reach the vitreal cavity (Laqua and Machemer, 1975; Hitchins and Grierson, 1988). There the RPE can be seen clinically and have been described as being like "tobacco dust". The tobacco dust then comes in contact with the glia by unknown guidance mechanisms and the RPE make their contribution to ERM formation by adopting a fibroblastic or even macrophagic role. The origin of true fibroblasts is likely to be from the coats of the eye when ERMs form after perforating injury but when ERMs complicate rhegmatogenous retinal detachment their origin is speculative. Irrespective of their origin, they can be expected perhaps to become part of the tobacco dust and also to move on available surfaces like the inner limiting membrane and coalesce with the glia and RPE at the site of forming ERMs.

If we examine mechanisms by which fibroblasts and RPE accumulate at the site of a sheet of proliferating glia, there are two obvious but not mutually exclusive models to examine. The first might be called the velcro model (Fig. 4.4.). Cells are wafted on currents in the vitreal and subretinal fluids, to accumulate in the vitreal cavity (tobacco dust). At this site cells make occasional random contact with the detached retina but adhere readily only where simple glial membranes are present. Thus the velcro action of simple glial aggregates would pick cells out preferentially so creating mixed cell accumulations on a physical rather than an attractional basis. In the velcro model there would be little need for the local release of chemoattractants but local production of settlement factors would

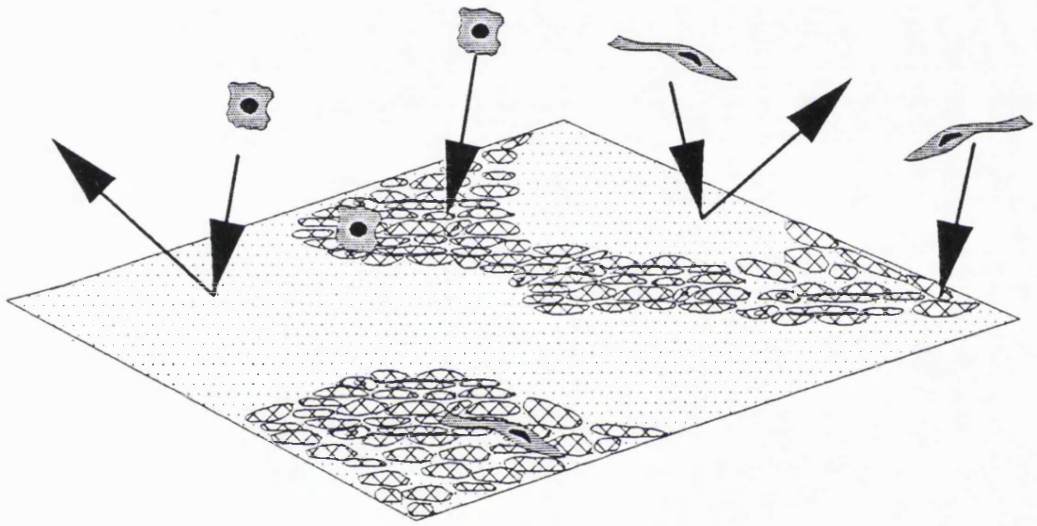


Fig. 4.4. Schematic diagram of the velcro model of complex ERM formation in which cells are wafted into the vitreal cavity and make occasional random contact with the vitreal surface of the retina. They only adhere readily when they make contact with the simple glial membranes on the surface of the retina.

help.

The findings of the attachment experiments in this thesis provide little support for a glial sheet acting as a preferential settlement and adhesion surface. Our test glia BRG, do however produce conditioned medium which enhances BRPE adhesion but not BSF adhesion. As a result RPE may be attracted to settle preferentially in the vicinity of a glial sheet but are not necessarily attracted to glial surfaces by the said having a mechanical fly paper action. BSF have no BRG produced settlement stimulant to respond to but they do release a soluble settlement enhancement agent which is as effective on BRPE as the version produced by the BRG.

The second model is one that involves active attraction. This active model demands that the RPE and fibroblasts migrate using available substrates to arrive at the glial foci on the retina. Certainly the BRG produce chemoattractants which would attract BSF to active migration, using either the vitreal collagen remnants or the ILL. On the other hand BRG release chemoattractants, which seem to be less effective on BRPE. Equally chemoattractants released from damaged retina and attractants present in PVR fluids stimulate the migration of BSF but have limited effect on BRPE. Indeed our experiments cast doubt on the previously well established presumption that RPE in pathology have a high migratory potential (Laqua and Machemer, 1975; Campochiaro and Glaser, 1986). RPE from different species have been shown by various authorities to migrate to a number of substances in the micro chemoattraction chamber. However the ours was the first study in which RPE migration was compared with that of other cell types in similar migration experiments and then subsequently after particle labelling, in identical experimental conditions.

The interactions of the three test cell types have provided some useful information about the different behavioural attributes of the cell types forming ERMs. The investigations could be expanded further to include the inflammatory components in the form of cytokines and macrophagic elements. In addition to start looking at simple ERM equivalents by coating the tissue culture flasks with basement membrane components such as type IV collagen to approximate the ILL and seeding primary or low passage glia at sufficient density to achieve colony growth. Then looking at the interactions of the BRPE and BSF with perhaps the addition of macrophages or macrophage conditioned medium.

Throughout, the BRPE have been found to be lacking. It would seem that if the findings have any meaning in the context of the in

vivo situation, then RPE are more likely to accumulate at the site of an ERM aided by adhesion stimuli whereas fibroblasts appear to respond to chemotactic and chemokinetic attraction. BRG produce soluble mitogens which have an effect on BRPE but not BSF, at least as far as the experiments are concerned. Whether or not in vivo these glial produced mitogens make their way into the subretinal fluid and contribute to the destabilisation of the RPE monolayer remains to be established. It is of particular interest that BRPE to BRG contact induced proliferation is identified by time-lapse microscopy. As this indicates that a mechanism therefore exists to amplify the glial sheet via local cell contact which, in turn, may stimulate more RPE settlement and more fibroblast chemoattraction. Although the release of soluble mitogens from proliferating glia may not have a remote effect on the RPE monolayer it could be more effective in closer proximity in the membrane itself. Our time-lapse studies showed no evidence of a tit-for-tat mitogenesis in the BRPE but an effect on BRPE might take longer to express itself than was possible to see in our time-lapse set up and so remains not proven.

The time-lapse studies show that when BSF and BRPE do come in contact with a BRG sheet the BSF are far more effective at invasion than the BRPE, the BRPE being much slower than BSF at burrowing into the sheet. However once the BRPE are there they make it even easier for BSF to become involved. The BRPE do tend to break up the sheet so some of the BSF enhancement may be mechanical due to structural alterations of the BRG sheet but the BRPE produce powerful chemoattractants for BSF which possibly promote their movement into the sheet. The release of attachment and settlement factors by BSF which promote BRPE adhesion to the surface layers of the sheet may by their very tenacity slow down BRPE invasion by helping the BRPE to adhere but not invade. This is in contrast with tumour cells and is possibly linked with selective activation of integrin receptors (Aplin et al, 1992).

Therefore we have a system of selective motogens, mitogens and attachment factors which probably serve in concert to amplify the cellular variety and bulk of the developing ERM, creating a vortex effect by which more and more cells end up at the central body of the newly forming scar. In PVR, proliferation has been emphasised in the name of the condition and in previous names such as MPP (massive periretinal proliferation) (Machemer and Laqua, 1975). Our thinking has been coloured by the emphasis on proliferation and most non-surgical therapeutic attempts at treatment have attempted therefore to inhibit division. This

thesis, while not demeaning the role of proliferation, has highlighted other important processes such as migration and settlement as behavioural targets for future therapeutic attacks.

Extrapolation from the findings in this thesis provides evidence that:-

1. RPE are slow at migration in comparison with glia and fibroblastic cells and become involved in ERMs by passive means possibly aided by attachment factors.

2. Fibroblasts actively migrate to stimuli which are likely to be present at the site of a newly forming ERM.

3. Glia are not a particularly effective velcro for the adhesion of either fibroblasts or RPE.

4. Re-enforcement mechanisms may operate in ERMs i.e. when fibroblasts are present they release stimulants for RPE migration. RPE release factors which stimulate a whole range of glial and fibroblast activity.

5. Evidence for contact stimulation of division is provided but evidence for remote cross cellular stimulation of division was poor.

6. Fibroblasts can penetrate into and become part of a glial sheet more rapidly than RPE.

Glia first, RPE second and fibroblasts third would seem to be best interpretation of the findings on the arrival of cells at the ERM.

APPENDIX I

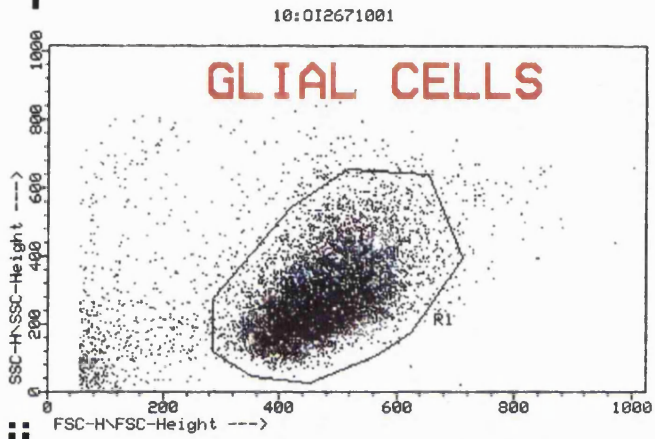
Measurement of the Relative Sizes of BRG, BRPE and BSF in Suspension Using Flow Cytometry

The relative sizes of cultured BRG, BRPE and BSF in suspension were measured by using flow cytometry in a Becton Dickinson Fluorescence Activated Cell Scanner (FAC-Scan, Becton Dickinson, UK). The cells were removed from the tissue culture flasks by trypsinsation and suspended in Isoton. The parameters of forward scatter to measure cell size and side scatter to measure cell granularity (density) were employed. The amount of scattering of the cells by a laser was detected by photomultiplier tubes and the signal converted to electronic pulses known as events which were measured in volts. Ten thousand events were recorded for each cell type and each event represented one cell. The results obtained were displayed as two dimensional dot plots of side scatter (granularity/density) on the y axis against forward scatter (diameter) on the x axis (Figs. Ia(i), Ib(i) and Ic(i)). In addition histograms of forward scatter impulse peak height (Figs. Ia(ii), Ib(ii) and Ic(ii)) were plotted and the mean cell size values displayed.

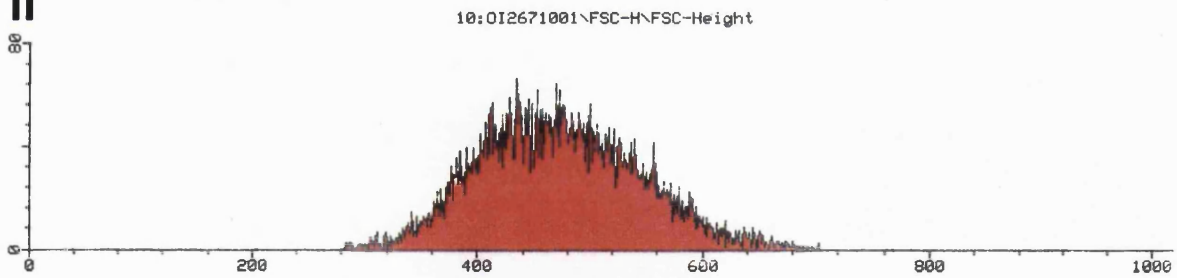
The results showed that there was no overall difference in size between the cell types (Fig. Id.), although the BRPE showed a larger amount of scattering on the two dimensional plot (Fig. Ib (i) indicating a greater range of cell sizes in comparison with the BRG and BSF. Percentage difference between the mean peak heights were calculated. The mean peak height of the BRPE was 4% larger than the BRG and 13% larger than the BSF and that of the BRG was 9% larger than that of the BSF (Figs. Ia(ii), Ib(ii) and Ic(ii)).

Fig. Ia. (Opposite) Two dimensional dot plot (i) for BRG representing the size distribution of the cells measured. Side scatter impulse peak height was plotted against forward scatter impulse peak height for BRG. Both axes were measured in units scales; 1000 units represented 9 volts. The histogram (ii) was of forward scatter impulse peak height and showed the size distribution of the BRG on a linear scale, the mean peak height for BRG was 473.09.

i



ii



10:OI2671001\FSC-H\FSC-Height

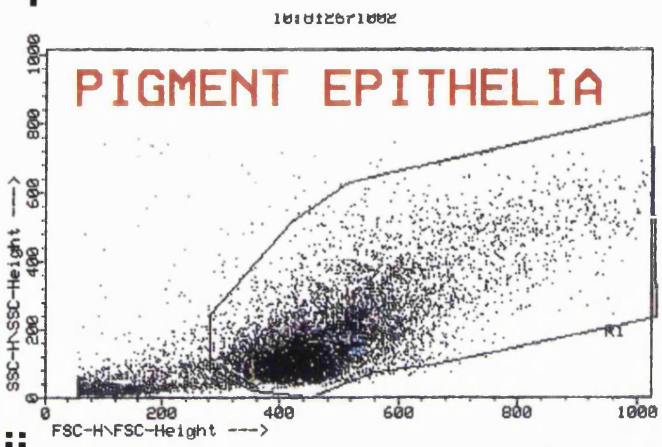
--- Arithmetic Histogram Statistics for 10:OI2671001 ---

Parameter FSC-H FSC-Height Gate G1= R1

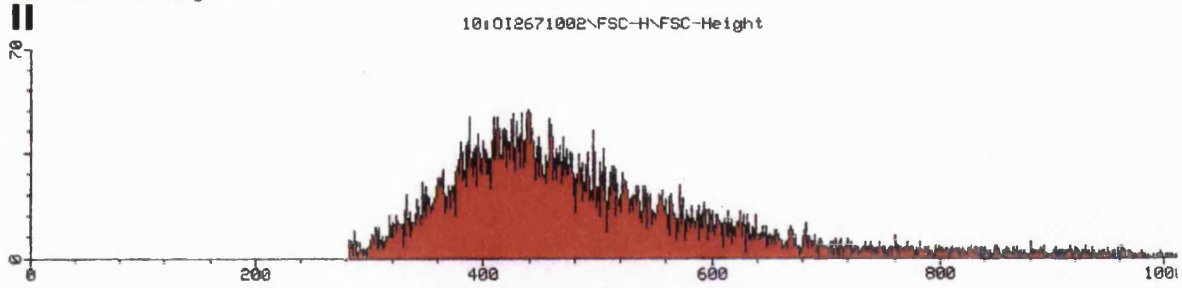
M	Left,Right	Events	%	Peak	PkChl	Mean	Median	SD	CV
0	0, 1023	9098	100.00	66	434.00	473.09	470.56	69.87	14.77

Fig. Ib. (Opposite) Two dimensional dot plot (i) for BRPE representing the size distribution of the cells measured. Side scatter impulse peak height was plotted against forward scatter impulse peak height for BSF. Both axes were measured in units scales; 1000 units represented 9 volts. The histogram (ii) was of forward scatter impulse peak height and showed the size distribution of the BRPE on a linear scale, the mean peak height for BRG was 494.03.

i



ii



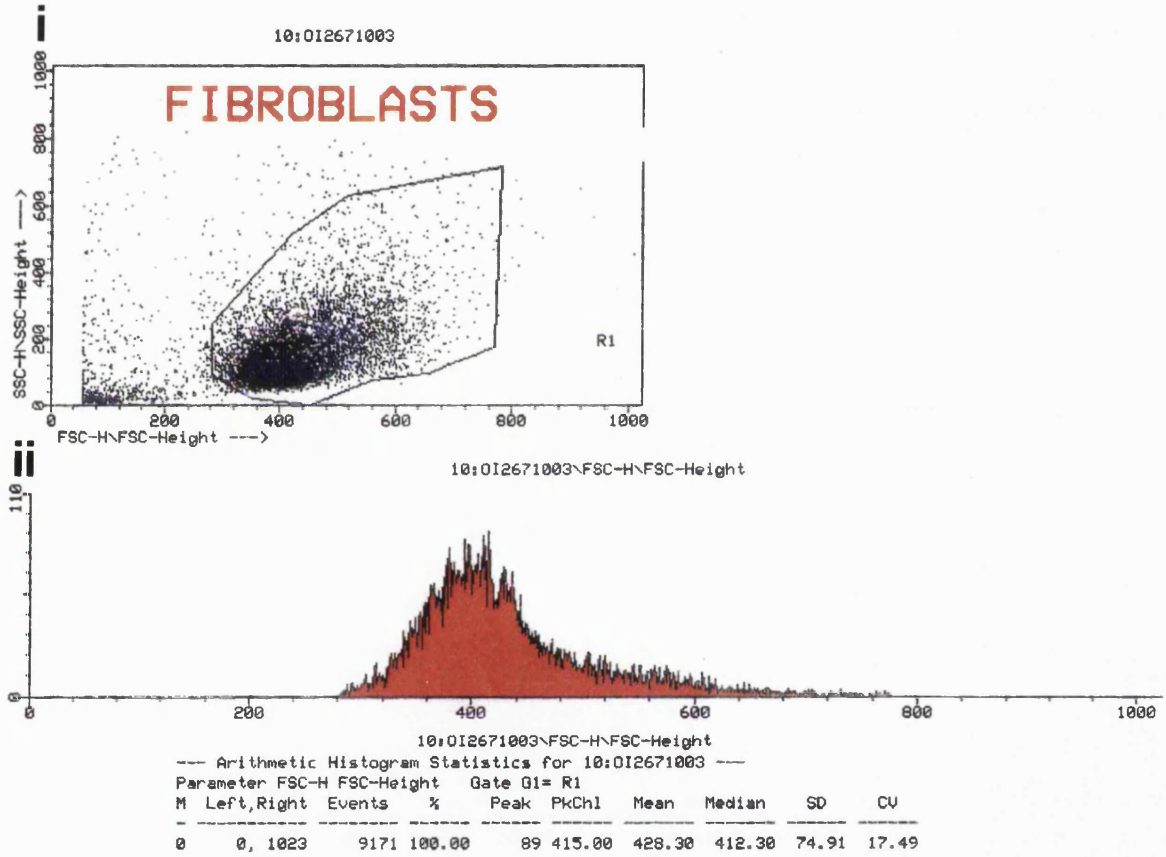
10:OI2671002\FSC-H\FSC-Height

--- Arithmetic Histogram Statistics for 10:OI2671002 ---

Parameter FSC-H FSC-Height Gate G1= R1

M	Left,Right	Events	%	Peak	PkChl	Mean	Median	SD	CV
0	0, 1023	8293	100.00	56	1023	494.03	462.19	132.43	26.80

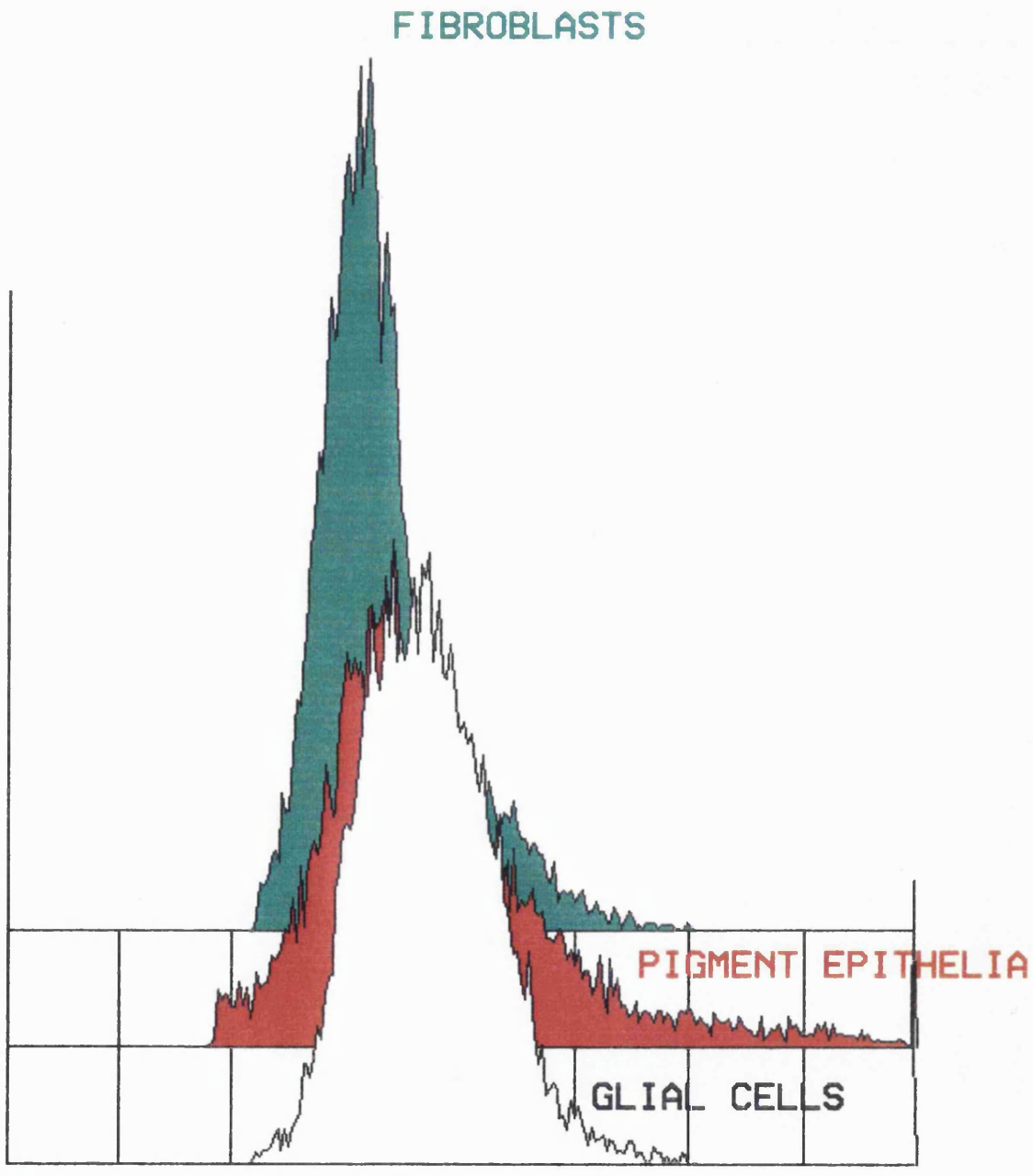
Fig. Ic. (Opposite) Two dimensional dot plot (i) for BSF representing the size distribution of the cells measured. Side scatter impulse peak height was plotted against forward scatter impulse peak height for BSF. Both axes were measured in units scales; 1000 units represented 9 volts. The histogram (ii) was of forward scatter impulse peak height and showed the size distribution of the BRPE on a linear scale, the mean peak height for BSF was 429.30.



--- Arithmetic Histogram Statistics for 10:OI2671003 ---
Parameter FSC-H FSC-Height Gate G1= R1

M	Left,Right	Events	%	Peak	PkChl	Mean	Median	SD	CV
0	0, 1023	9171	100.00	89	415.00	428.30	412.30	74.91	17.49

Fig. Id. (Opposite) Combined histograms of forward scatter, impulse peak height for BRG, BRPE and BSF, to compare the three size distributions.


FIBROBLASTS
PIGMENT EPITHELIA
GLIAL CELLS

APPENDIX II

Analysis of the Fibronectin Content of BRG, BRPE and BSF Conditioned Media by an Enzyme-Linked Immunoabsorbant Assay (ELISA)

A pilot run was performed to determine the fibronectin content of samples of conditioned medium collected from the cultures of bovine cells, employing a standard double antibody ELISA assay. A specific antibody to fibronectin was employed (Dako) and quantified using an enzyme-linked colourimetric reaction. The colour absorbency was measured on an automated photometer (ELISA plate reader, Titertek Multiscan Plus, Wellcome Diagnostics, Uk) at a wavelength of 450 nm. The results obtained from known concentrations of fibronectin were used to plot a standard curve and the concentrations of the test substances read from this. The readings obtained from the samples run (Fig.II) suggested that the BRPE and BRG conditioned media contained higher concentrations of fibronectin, than medium from the BSF.

Fibronectin		
BRG Conditioned Medium	BRPE Conditioned Medium	BSF Conditioned Medium
8.5 µg/ml	23 µg/ml	4 µg/ml
27 µg/ml	15 µg/ml	3 µg/ml
12 µg/ml	16 µg/ml	8 µg/ml
Mean 15.8 ±Sem 5.7 µg/ml	Mean 18 ±Sem 2.5 µg/ml	Mean 5 ±Sem 1.5 µg/ml

Fig. II. Fibronectin concentrations in samples of BRG, BRPE and BSF conditioned medium measured using ELISA.

APPENDIX III

Control Experiments to Measure Absorbency of ^{14}C Adenine by the BRG Target Monolayer

Control experiments were run to assess whether uptake of label by the BRG target monolayer due to cell to medium leakage of label in the scintillation counting cell settlement experiments would prove a problem. The target cell layer was incubated for 16 hours with the filtered media which had contained ^{14}C adenine labelled test cells. Then the cultures were washed with PBS as and incubated as described in section 2.5.1. in the presence of excess adenine for 24 hours. The cells were disrupted with a solution of 0.5 ml 0.25% trypsin/0.5 ml 0.2% EDTA and 0.1 ml Triton X. Scintillant Safe (10 ml) was added. The radioactivity was measured in the scintillation counter (CA 1900) and the DPMs converted to cell numbers using the calibration graphs in Appendix IV. The absorbency of the label by the target cell layer was shown to be negligible (Fig. III.).

Cell Supernatant	BRG
Mean DPM ± SEM (n=6)	136.0 ± 9.3
Mean number of cells/well containing ^{14}C Adenine ± SEM	281.8 ± 9.1
Mean BRG cell number per confluent well of a 24 well plate (n=6)	6×10^5
Percentage absorbency per well	0.05%

Fig. III. Table to show the negligible absorbency of 14C adenine by the target BRG cell layer.

APPENDIX IV

Scintillation Counting: Conversion of Disintegrations Per Minute to Cell Numbers

The relationship between DPMs and cell numbers for each cell type was established by constructing calibration curves as follows. The cells were labelled with the ^{14}C adenine isotope as described in section 2.5.1. and serial dilutions were performed to give 20, 200, 2000, 20,000 and 200,000 cells/ml. The radioactivity in log mean DPM in each of these samples was plotted against log calculated cell number for each cell type (Fig. IVa). The slope "a" of the graph was calculated as follows:-

$$a = (\text{mean})y - b(\text{mean})x$$

Where:-

$$b = \frac{[N\Sigma xy - \Sigma x\Sigma y]}{[N\Sigma x^2 - (\Sigma x)^2]}$$

The line of best fit was drawn and the correlation coefficient "r" was calculated for each cell type from the following equation:-

$$r = \frac{N\Sigma xy - \Sigma x\Sigma y}{\sqrt{(N\Sigma x^2 - (\Sigma x)^2)(N\Sigma y^2 - (\Sigma y)^2)}}$$

The correlation coefficient r was close to 1 for all three cell types (Fig. IVa). As the regression values were linear it was assumed that the amount of radioactivity estimated by the scintillation counter was directly proportional to the cell numbers present (Fig. IVb.). The cell numbers in the subsequent experiments were calculated by converting the mean DPM values for each run to log values, reading the curve and taking the anti-log of the cell number.

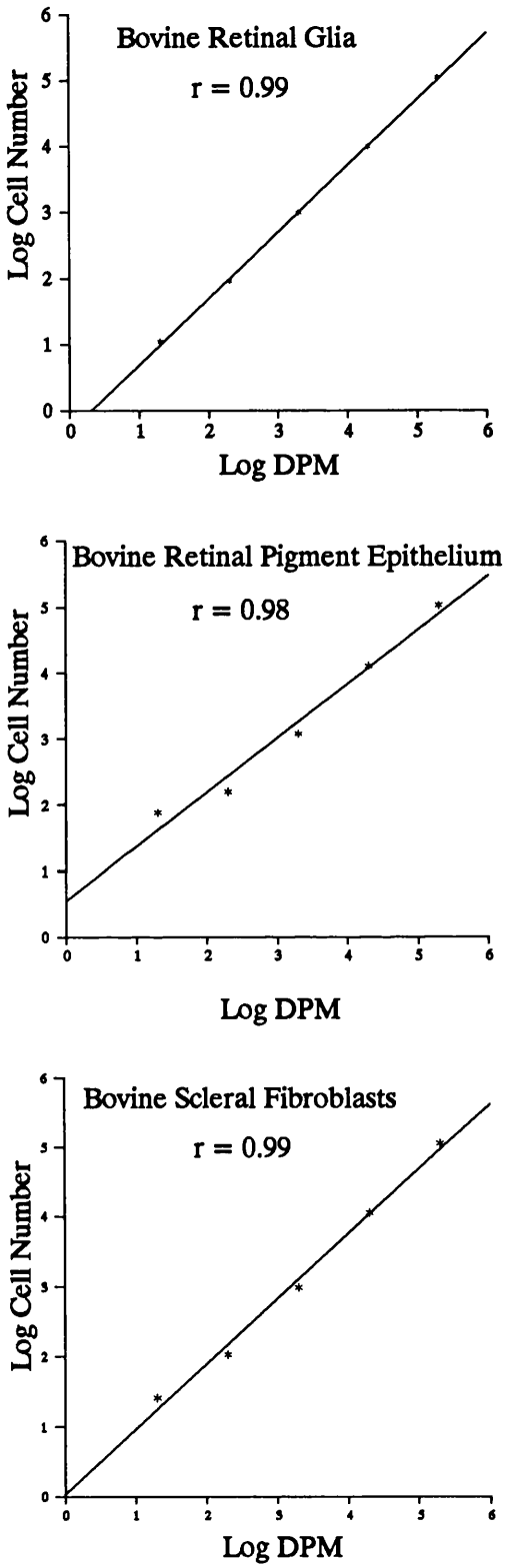


Fig. IVa. Regression curves and r values for the conversion of DPMs to cell numbers.

(i) Bovine retinal glia			
x axis		y axis	
Cell Number	Log Cell Number	Mean DPM	Log Mean DPM
20	1.30	11	1.04
200	2.30	91	1.96
2,000	3.30	982	2.99
20,000	4.30	9,736	3.99
200,000	5.30	110,184	5.04

(ii) Bovine retinal pigment epithelium			
x axis		y axis	
Cell Number	Log cell Number	Mean DPM	Log Mean DPM
20	1.30	75	1.88
200	2.30	159	2.20
2,000	3.30	1,213	3.08
20,000	4.30	12,856	4.11
200,000	5.30	106,037	5.03

(iii) Bovine scleral fibroblasts			
x axis		y axis	
Cell Number	Log Cell Number	Mean DPM	Log Mean DPM
20	1.30	26	1.41
200	2.30	107	2.03
2,000	3.30	987	2.99
20,000	4.30	11,274	4.05
200,000	5.30	112,083	5.05

Fig. IVb. Tables of values for the conversion of DPMs to cell numbers for the three test cell types.

BIBLIOGRAPHY

Abercrombie, M., Contact inhibition in tissue culture. In Vitro, 1970, 6, 128-142.

Abercrombie, M., The basis of the locomotory behaviour of fibroblasts. Exp. Cell. Res., 1961, Suppl. 8, 188-198.

Abercrombie, M., The cells: fibroblasts. J. Clin. Path., 1978, 31, Suppl., 1-6.

Abercrombie, M., The Croonian lecture: the crawling movement of metazoan cells. Proc R, Soc. Lond., 1980, B207, 129-147.

Adler, R., Magistretti, P.J., Hyndman, A.g. and Shoemaker, W.J., Purification and cytochemical identification of neuronal and non-neuronal cells in chick embryo retina cultures. Dev. Neurosci., 1982, 5, 27-39.

Aguayo, A.J., David, S. and Bray, G.M., Influence of the glial environment on the elongation of axons after injury: transplantation studies in adult rats. J.Exp. Biol., 1981, 95, 231-240.

Akiyama, S.K. and Yamada, K.M., Fibronectin. Adv. Enzymol., 1987, 59, 1-57.

Akhmeteli, L. M., Kasavina, B. S. and Petropavlovoskaja, G.A., Biochemical investigation of the subretinal fluid. Brit. J. Ophth., 1975, 59, 70-77.

Albini, A., Adelmann-Grill, B.C., Mueller, P.K., Fibroblast chemotaxis, Coll. Rel. Res., 1985, 5, 283-296.

Albini, A., Allavena, G., Melchiori, A., Giancotti, F., Richter, H., Comoglio, P.M., Martin, G.R., Tarone, G., Chemotaxis of 3T3 and SV3T3 cells to fibronectin is mediated through the cell-attachment site in fibronectin and a fibronectin cell surface receptor. J. Cell Biol., 1987, 105, 1867-1872.

Albrecht-Buehler, G., The phagokinetic tracks of 3T3 cells. Cell, 1977, 11, 395-404.

Alexander, R. A., Hiscott, P., McGalliard, J. and Grierson, I., Oxytalan fibres are component of proliferative vitreoretinopathy membranes. Ger. J. Ophth., 1992, 1, 382-387.

Ali, J.U. and Hynes, R.O., Effects of LETS glycoprotein on cell motility. Cell, 1978, 14, 439-446.

Altman, D.G., Comparing groups - continuous data: In: Practical Statistics for Medical Research. Chapman and Hall, London, 1991, 179-228.

Anderson, D.H., Guérin, C.J., Matsumoto, B. and Pfeffer, B.A., Identification and localization of a Beta-1 receptor from the integrin family in mammalian retinal pigment epithelial cells. Invest. Ophth. Vis. Sci., 1990, 31, 81-93.

Anderson, D.H., Stern, W.H., Fisher, S.K., Erickson, R.A. and Borgula, G.A., The onset of pigment epithelial proliferation after retinal detachment. Invest. Ophth. Vis. Sci., 1981, 21, 10-16.

Andujar, M.B., Melin, M., Guerret, S. and Grimaud, J.A., Cell migration influences gel contraction. J. Submicrosc. Cytol. Path., 1992, 24, 145-154.

Antoniades, H.N. and Scher, C.D., Radioimmunoassay of a human serum growth factor for Balb/c-3T3 cells: derivation from platelets. Proc. Natl. Acad. Sci. USA., 1977, 74, 1973-1977.

Aplin, J.D., Expression of integrin alpha 6 beta 4 in human trophoblast and its loss from extravillous cells. Placenta, 1993, 14, 203-215.

Aplin, J.D., Sattar, A. and Mould, A.P., Variant choriocarcinoma (BeWo) cells that differ in adhesion and migration on fibronectin display conserved patterns of integrin expression. J.Cell Sci., 1992, 103, 435-444.

Apple, D.J., Naumann, G.O.H. and Manthy, R.M., Microscopic anatomy of the eye; Retina. In: Pathology of the Eye. Eds. Naumann, G.H. and Apple, D.J., Springer-Verlag, 1986, 38-50.

Armstrong, P.B. and Lackie, J.M., Studies on intercellular invasion in vitro using rabbit peritoneal neutrophil granulocytes

(PMNs). J. Cell Biol., 1975, 65, 439-462.

Arruti, C. and Courtois, Y., Morphological changes and growth stimulation of bovine epithelial cells by a retinal extract in vitro. Exp. Cell Res., 1978, 117, 283-292.

Avery, R.L. and Glaser, B.M., Inhibition of retinal pigment epithelial cell attachment by a synthetic peptide derived from the cell-binding domain of fibronectin. Arch. Ophth., 1986, 104, 1220-1225.

Balazs, E.A., Toth, L.Z.J., Eckl, E.A. and Mitchell, A.P., Studies on the structure of the vitreous body. Exp. Eye Res., 1964, 3, 57-71.

Balazs, E.A., Toth, L.Z.J. and Ozanics, V., Cytological studies on the developing vitreous as related to the hyaloid vessel system. Graefe's Arch. Clin. Exp. Ophth., 1980, 213, 71-85.

Bailey, N.T.J., The correlation of measurements: the calculation of an estimated correlation coefficient. In: Statistical Methods in Biology. Unibooks, English Universities Press Ltd., London, 1959, 81-84.

Baird, A., Esch, F., Gospodarowicz, D. and Guiellmin, R., Retina- and eye-derived retinal endothelial growth factors; partial molecular characterisation and identity with acidic and basic fibroblast growth factors. Biochem., 1985, 24, 7855-7860.

Baron-Van Evercooren, A., Kleinmann, H.K., Seppa, H.E., Rentier, B. and Dubois-Dalcq, M., Fibronectin promotes rat Schwann cell growth and motility. J. Cell Biol., 1982, 93, 211-216.

Barritault, D., Arruti, C. and Courtois, Y., Is there a ubiquitous growth factor in the eye? Proliferation induced in different cell types by eye derived growth factors. Differentiation, 1981, 29-42.

Barritault, D., Plonet, J., Courty, J. and Courtois, Y., Purification, characterisation and biological properties of the eye-derived growth factor: analogies with brain-derived growth factor. J. Neurosci. Res., 1980, 8, 477-490.

Basu, P.K., Sarkar, P., Menon, I., Carre, F. and Persad, S.,

Bovine retinal pigment epithelial cells cultured in vitro: growth characteristics, morphology, chromosomes, phagocytosis ability, tyrosinase activity and effect of freezing. Exp. Eye Res. 1983, 36, 671-683.

Baudouin, C., Fredji-Grobellet, D. and Gordon, W.W., Immunohistologic study of epiretinal membranes in proliferative vitreoretinopathy. Am. J. Ophth., 1990, 110, 593-598.

Ben Ze'ev, A., Cell-cell interaction and cell configuration related control of cytokeratins and vimentin in epithelial cells and fibroblasts. Ann. N.Y. Acad. Sci., 1986, 455, 597-613.

Bignami, A. and Dahl, D., The radial glia of Müller in the rat retina and their response to injury. An immunofluorescence study with antibodies to the glial fibrillary acidic (GFA) protein. Exp. Eye Res., 1979, 28, 63-69.

Birk, D.E., Zycband, E.I., Winkleman D.A. and Trelstad, R.L., Collagen fibrillogenesis in situ: fibril segments are intermediates in matrix assembly. Proc. Natl. Acad. Sci. USA, 1989, 86, 4549-4553.

Bishop, P., Crossman, M., McLeod, D. and Ayad, S., The glycosaminoglycan composition of bovine vitreous type IX collagen proteoglycan. Invest. Ophth. Vis. Sci., 1993, Suppl., 34, 949.

Bleckmann, H., Glycosaminoaminoglycan metabolism of cultured fibroblasts from bovine vitreous. Graefe's Arch. Clin. Exp. Ophth., 1984, 222, 90-94.

Bloom, W. and Fawcett, D.W., Fibroblasts. In: A Textbook of Histology, 10th Ed., W.B. Saunders Co., London, 1975, 171-172.

Bok, D. and Hall, M.O., The role of pigment epithelium in the etiology of inherited retinal dystrophy in the rat. J. Cell Biol., 1971, 49, 664-682.

de Bono, D. and Green, C., Interaction between vascular endothelial cells and vascular intimal spindle-shaped cells in vitro. J. Cell Sci., 1983, 60, 89-102.

de Bono, D. and Green, C., The adhesion of different cell types to cultured vascular endothelium: effects of culture density and

age. Br. J. Exp. Path., 1984, 65, 145-54.

Boulton, M. and Marshall, J., Repigmentation of retinal pigment epithelial cells in vitro. Exp. Eye Res., 1985, 41, 209-218.

Boulton, M., Moriarty, P. and Gregor, Z.J., Biological activities of vitreous gel, retrohyaloid fluid and subretinal fluid from diabetic and non-diabetic eyes. Brit. J. Ophth., 1992, 76, 79-83.

Borel, J-P. et Maquart, F-X., La cicatrisation. La Recherche, 1991, 22, 1174-1181.

Bowersox, J.C. and Sorgente, N., Chemotaxis of aortic endothelial cells in response to fibronectin. Cancer Res., 1982, 42, 2547-2551.

Boyden, S., The chemotactic effects of mixtures of antibodies and antigen on polymorphonuclear leucocytes.
J. Exp. Med., 1962, 115, 453-456.

Bresgen, M., Botz, N., Wiedemann, P. and Heimann, K., In vitro screening of polypeptide growth factors: an adaptation of the MTT colorimetric assay to RPE cells. Invest. Ophth. Vis. Sci., 1991, Suppl., 32, 769.

Brody, A. R., Bonner, J. C., Overby, L.H., Badgett, A., Kalter, V., Kumar, R.K. and Bennett, R.A., Interstitial pulmonary macrophages produce platelet-derived growth factor that stimulates rat lung fibroblast proliferation. J. Leuco. Biol., 1992, 51, 640-648.

Bronner-Fraser, M.E., Latex beads as probes of a neural crest pathway: the effects of laminin, collagen and surface changes on bead translocation. J. Cell Biol., 1984, 98, 1947-80.

Brown, P.J. and Juliano, R.L., Association between fibronectin receptor and the substratum: spare receptors for cell adhesion. Exp. Cell Res., 1987, 171, 376-388.

Bryan, J.A. and Campochiaro, P.A., A retinal pigment epithelial derived growth factor. Arch. Ophth., 1986, 104, 422-425.

Bryan, J.A., Hackett, S., Campochiaro, P.A. and de Juan, E.,

Migration and proliferation of rat retinal glial cells. Invest. Ophth. Vis. Sci., 1985, Suppl., 26, 1987.

Buckley-Sturrock, A. Woodward, S.C., Senior, R.M., Griffin, G.L., Klagsbrun, M. and Davidson, J.M., Differential stimulation of collagenase and chemotactic activity in fibroblasts derived from rat wound repair and human growth by growth factors. J. Cell Physiol., 1989, 138, 70-78.

Bunge, R., Tissue culture observations relevant to the study of axon-Schwann cell interactions during peripheral nerve development and repair. J. Exp. Biol., 1987, 132, 21-34.

Bunge, R. and Bunge, M., Interrelationship between Schwann cell function and extracellular matrix production. Trends. Neurosci., 1983, 6, 499-505.

Bunge, R., Glaser, L., Lieberman, M., Raben, D., Salzer, J., Whittenburger, B. and Woolsey, T., Growth control by cell to cell contact. J. Supramolec. Struct., 1979, 11, 175-187.

Burke, J.M., Cell-cell contact promotes DNA synthesis in retinal glia but not in fibroblasts. Exp. Cell Res., 1983, 146, 204-206.

Burke, J.M., Cell interactions in proliferative vitreoretinopathy: Do growth factors play a role? In: International Symposium on Proliferative Vitreoretinopathy Köln, Ed. Heinmann, K. and Wiedemann, P., Kade Verlag, 1989, 80-87.

Burke, J.M., Cultured retinal glial cells are insensitive to platelet derived growth factor. Exp. Eye Res., 1982, 35, 663-669.

Burke, J.M., Growth in retinal glial cells in vitro is affected differentially by two types of cell contact mediated interactions. Exp. cell Res., 1989, 180, 13-19.

Burke, J.M., Stimulation of DNA synthesis in human and bovine RPE by peptide growth factors: the response to TNF-alpha and EGF is dependent upon culture density. Curr. Eye Res., 1989, 8, 1279-1286.

Burke, J.M., Abrams, G., Aaberg, T. and Williams, G., Chemotaxis-

and proliferation-stimulating activities in human vitrectomy specimens: correlation with PVR intensity. Invest. Ophth. Vis. Sci., 1985, Suppl., 26, 283.

Burke, J.M. and Foster, S.J., Injured vitreous stimulates DNA synthesis in retinal pigment epithelial cells in culture and within the vitreous. Graefe's Arch. Clin. Exp. Ophth., 1982, 218, 153-155.

Burke, J.M. and Foster, S.J., Culture of adult rabbit retinal glial cells: methods and cellular origin of explant outgrowth. Curr. Eye Res., 1984, 3, 1169-1178.

Burke, J.M. and Foster, S.J., Induction of DNA synthesis by co-culture of retinal glia and pigment epithelium. Invest. Ophth. Vis. Sci., 1985, 26, 636-642.

Burke, J.M. and Twinning, S.S., Vitreous macrophage elicitation: generation of stimulants for pigment epithelium in vitro. Invest. Ophth. Vis. Sci., 1987, 28, 1100-1107.

Calthorpe, C.M. and Grierson, I., Fibronectin induces migration of bovine trabecular meshwork cells in vitro. Exp. Eye Res., 1990, 51, 39-48.

Calthorpe, C.M., Grierson, I. and Hitchings, R.A., Chemoattractants produced by ocular cells induce trabecular meshwork cell migration. Int. Ophth., 1991, 15, 185-192.

Campochiaro, P.A. and Glaser, B.M., Platelet-derived growth factor is chemotactic for human retinal pigment epithelial cells. Arch. Ophth., 1985, 103, 576-579.

Campochiaro, P.A., Jerdan, J.A., Glaser, B.M., The extracellular matrix of human retinal pigment epithelial cells in vivo and its synthesis in vitro. Invest. Ophth. Vis. Sci., 1986, 27, 1615-1621.

Campochiaro, P.A., Jerdan, J.A., Glaser, B.M., Cardin, A. and Michels, R.G., Vitreous aspirates from patients with vitreoretinopathy stimulate retinal pigment epithelial migration. Arch. Ophth., 1985, 103, 1402-1405.

Campochiaro, P.A. and Glaser, B.M., A retina-derived

stimulator(s) of retinal pigment epithelial cell and astrocyte proliferation. Exp. Eye. Res., 1986, 43, 449-457.

Campochiaro, P.A. and Glaser, B.M., Serum contains chemoattractants for human retinal pigment epithelial cells. Arch. Ophth., 1984, 102, 1830-1833.

Campochiaro, P.A., Sugg, R., Grotendorst, G. and Hjelmeland, L., Retinal pigment epithelial cells produce PDGF-like proteins and secrete them into their media. Invest. Ophth. Vis. Sci., 1988, Suppl., 29, 305.

Capentandes, A. and Gerritsen, M.E., Simplified method for consistent and selective culture of bovine retinal endothelial cells and pericytes. Invest. Ophth. Vis. Sci., 1990, 31, 1738-1744.

Carpenter, G. and Cohen, S., Human epidermal growth factor stimulates the proliferation of human fibroblasts. J. Cell Physiol., 1975, 88, 227-238.

Chakravarthy, U., McCormick, D., Maguire, C.J.F. and Archer, D.B., An in vitro study of irradiated vitreoretinal membranes. Eye, 1987, 1, 126-135.

Charteris, D.G., Hiscott, P.S., Grierson, I. and Lightman, S., Proliferative vitreoretinopathy: lymphocytes in epiretinal membranes. Ophth., 1992, 99. 1364-1367.

Chignell, A.H., Pathogenesis, predisposing factors, presentation and methods of examination. In: Retinal detachment surgery, Springer-Verlag, London, 1988, 1-3.

Chignell, A.H., Preoperative management and operative planning. In: Retinal detachment surgery, Springer-Verlag, London, 1988, 91-92.

Chignell, A.H., Carruthers, M. and Rahi, A.H.S., Clinical, biochemical, and immunoelectrophoretic study of subretinal fluid. Brit. J. Ophth., 1971, 55, 525-532.

Chu, P. and Grunwald, G.B., Functional inhibition of retinal pigment epithelial cell-substrate adhesion with a monoclonal antibody against the $\beta 1$ subunit of integrin. Invest. Ophth. Vis.

Sci., 1991, 32, 1763-1769.

Chu, P. and Grunwald, G.B., Identification of an adhesion associated protein of the retinal pigment epithelium. Invest. Ophth. Vis. Sci., 1990, 31, 847-855.

Cibis, P.A., Recent methods in the surgical treatment of retinal detachment procedures. Trans. Ophth. Soc. UK, 1965, 85, 111-126.

Clark, V.M., The cell biology of the retinal pigment epithelium. In: The Retina a Model for Cell Biology Studies, Part II. Ed. Adler, R. and Farber, D., Academic Press, 1986, 129-159.

Cleary, P.E. and Ryan, S.J., Histology of wound healing, vitreous retina in experimental posterior penetrating eye injury in the Rhesus monkey. Am. J. Ophth., 1979, 88, 221-231.

Cleary, P.E. and Ryan, S.J., Mechanisms in traction retinal detachment. Dev. Ophth., 1981, 2, 328-333.

Coca-Prados, M. and Wax, M.B., Transformation of human ciliary epithelial cells by simian virus 40: induction of cell proliferation of β_2-adrenergic receptors. Proc. Natl. Acad. Sci. USA, 1986, 83, 8754-8758.

Connor, T., Roberts, A., Sporn, M., Davis, J. and Glaser, B., RPE cells synthesise and release transforming growth factor-beta, a modulator of endothelial cell growth and wound healing. Invest. Ophth. Vis. Sci., 1988, Suppl., 29, 307.

Constable, I.J., Horne, R., Slatter, D.H., Chester, G.H. and Cooper, R.L, Regeneration of retinal limiting membrans after chorioretinal biopsy in dogs. Invest. Ophth. Vis. Sci., 1981, 20, 246-251.

Constable, I.J., Oguri, M., Chesney, C.M., Swann, D.A. and Colman, R.A., Platelet induced vitreous membrane formation, Invest. Ophth. Vis. Sci., 1973, 12, 680-685.

Constable, I.J., Tolentino, F.I., Donovan, R.H., Schepens, C.L., Clinico-pathologic correlation of vitreous membranes. In: Retinal

Congress. New York, Appleton Century Crofts, 1974, 254-257.

D'Amore, P.A., Glaser, B.M., Brunson, S.K. and Feneslau, A.H., Angiogenic activity from bovine retina: partial purification and characterisation. Proc. Natl. Acad. Sci., 1981, 78, 3068-3072.

D'Ardenne, J.D. and McGee, J. O'D., Fibronectin in disease. J. Path., 1984, 142, 123-151.

Davidson, J.M. and Broadley, K.N., The response of experimental wounds to endogenous and exogenous growth factors, In: Wound Healing, Eds. Janssen, R. and Robertson, J.I.S., Wrightson Biomedical Publishing Ltd., 1991, 81-90.

Day, J., Grierson, I., Unger, E.G. and Robbins, E., Some effects of phagocytosis on bovine meshwork cells in culture. Exp. Eye Res., 1986, 43, 1077-1087.

Daya-Grosjean, L., Azzarone, B., Maunoury, R., Zaech, P., Elia, G., Zaniratti, S. and Benedetto, A., SV40 immortalization of adult human mesenchymal cells from neuroretina. Biological, functional and molecular characterization. Int. J. Cancer, 1984, 33, 319-329.

Diegelmann, R.F., Schuller-Levis, G., Cohen, I.K. and Kaplan, A.M., Identification of a low molecular weight, macrophage-derived chemotactic factor for fibroblasts. Clin. Immun. Immunopath., 1986, 41, 331-341.

Del Monte, M.A. and Maumenee, I.H., In vitro culture of human retinal pigment epithelium for biochemical and metabolic study. Vis. Res., 1981, 21, 137-142.

Dipasquale, A., Locomotory activity of epithelial cells in culture. Exp. Cell Res., 1975, 94, 191-215.

Dixon, R.G. and Eng, L. F., Glial fibrillary acidic protein in the retina of the developing albino rat: an immunoperoxidase study of paraffin imbedded tissue. J. Comp. Neurol., 1981, 195, 305-321.

Dowrick, P.G. and Warn, R.M., The cellular response to factors which induce motility in mammalian cells. In: Cell Motility Factors, Ed. Goldberg, I.D., Birkhausen Verlag, Basel,

Switzerland, 1991, 89-108.

Dunphy, J.L., The fibroblast - a ubiquitous ally for the surgeon. New Eng.J. Med., 1963, 268, 1367-1377.

Dutt, K., Waldrep, J.C., Kaplan, H.J., Del Monte, M., Semple, E., Verly, G., In vitro phenotypic and functional characterisation of human pigment epithelial cell lines. Curr. Eye Res., 1989, 8, 435-440.

Eagle, H., Amino acid metabolism in mammalian cell cultures. Science, 1959, 130, 432-437.

Eagle, H. and Levine, E.M., Growth regulatory effects of cellular interaction. Nature, 1967, March 18, 1102-1106.

Erickson, P.A., Fisher, S.K., Guérin, C.J, Anderson, D. H. and Kaska, D.D., Glial cell fibrillary acidic protein increases in Müller cells after retinal detachment. Exp. Eye. Res., 1987, 44, 37-48.

Falk, W., Goodwin, R.H. and Leonard, E.J., A 48-well micro chemotaxis assembly for rapid and accurate measurement of leucocyte migration. J. Immunol. Meth., 1980, 33, 239-47.

Feeney, L., Burns, R.P. and Mixon, R.M., Human subretinal fluid. Its cellular and subcellular components. Arch. Ophth., 1975, 93, 62-69.

Feeney, L. and Mixon, R.M., An in vitro model of phagocytosis in bovine and human retinal pigment epithelium. Exp. Eye Res., 1976, 22, 533-548.

Finkelstein, S.P., Benowitz, L.I., Olson, A.J., Perrone-Bizzozero, N.I., Majocha, R.E. and Apostolides, P.J., Conditioned media from injured lower vertebrate CNS promote neurite outgrowth from mammalian brain neurons in vitro. Brain Res., 1987, 413, 267-274.

Fisher, S.K. and Linberg, K.A., Intercellular junctions in the early human embryonic retina. J. Ultrastruct. Res., 1975, 51, 69-78.

Foos, R.Y., Non-vascularised proliferative extraretinal retinopathies. Am. J. Ophth., 1978, 86, 723-5.

Foos, R.Y., Vitreoretinal juncture epiretinal membranes and vitreous. Invest. Ophth. Vis. Sci., 1977, 16, 416-422.

Foos, R.Y. and Gloor, B.P., Vitreoretinal juncture; healing of experimental wounds. Graefe's Arch. Clin. Exp. Ophth., 1975, 218, 213-230.

Freshney, R.J. Quantitation and experimental design: Cytotoxicity and viability: short term tests - viability. In: Culture of Animal Cells. Alan R. Liss Inc., 1983, 207-209.

Friedenwald, J. S. and Chan, E., Pathogenesis of retinitis pigmentosa with a note on the phagocytic activity of Müller's fibres. Arch. Ophth., 1932, 8, 173-181.

Gabbiani, G., The myofibroblast: a key cell for wound healing and fibrocontractive diseases. In: Connective tissue research. Alan.R. Liss. Inc., New York, 1981, 183-194.

Gartry, D.S., Chignell, A.H., Franks, W.A. and Wong, D., Pars plana vitrectomy for the treatment of rhegmatogenous retinal detachment uncomplicated by advanced proliferative vitreoretinopathy. Brit. J. Ophth., 1993, 77, 199-203.

Gaudric, A., Falquhero, L., Clement, G., Caruelle, D., Moses, H., Lyons, R., Caruelle, J.P., Coscas, G. and Barritault, D., Fibroblast growth factors, transforming growth factor beta in patients with proliferative retinopathy. Invest. Ophth. Vis. Sci., 1988, Suppl., 29, 221.

Gauss-Müller, V., Kleinman, H.K., Martin, G.R. and Schiffmann, E., Role of attachment factors and attractants in fibroblast chemotaxis. J. Lab. Clin. Med., 1980, 96, 1071-1079.

Gherardi, E., Growth factors and cell movement. Eur. J. Cancer, 1991, 27, 403-405.

Gilbert, C., Hiscott, P. S., Unger, W., Grierson, I. and McLeod, D., Inflammation and the formation of epiretinal membranes. Eye, 1988, Suppl., 2, 140-156.

Glaser, B.M., D'Amore, P.A., Michels, R.G., Patz, A. and Feneselau, A., Demonstration of vasoproliferative activity from mammalian retina. J. Cell Biol., 1980, 84, 298-304.

Glaser, B.M., Cardin, A. and Biscoe, B., Proliferative vitreoretinopathy. The mechanism of vitreoretinal traction. Ophth., 1987, 94, 327-332.

Gloor, B.P. and Daicker, B.C., Pathology of the vitreoretinal border structures. Trans. Ophth. Soc. UK, 1975, 95, 387-390.

Gospodarowicz, D., Growth factors and their action in vivo and in vitro. J.Path., 1983, 141, 201-233.

Gospodarowicz, D. and Moran, J.S., Mitogenic effect of fibroblast growth factor on early passage cultures of human and murine fibroblasts. J. Cell Biol., 1975, 66, 451-556.

Gotleib, A.I. and Spector, W., Migration into an in vitro wound. Am. J. Path., 1981, 103, 271-282.

Grabner, G., Boltz, G. and Förster, O., Macrophage-like properties of human hyalocytes. Invest. Ophth. Vis. Sci., 1980, 19, 333-340.

Grant, M.B., Guay, C. and Marsh, R., Insulin-like growth factor stimulates proliferation, migration and plasminogen activator release by human retinal pigment epithelial cells. Curr. Eye Res., 1990, 9, 323-335.

Grierson, I. and Forrester, J.V., Vitreous haemorrhage and vitreal membranes. Trans. Ophth. Soc., 1980, 100, 140-150.

Grierson, I., Hiscott, P., Hitchins, C.A., McKechnie, N., White, V. and McCleod, D., Which cells are involved in the formation of epiretinal membranes? Sem. Ophth., 1987, 2, 99-109.

Grierson, I., Joseph, J., Miller. M. and Day J.E., Wound repair: the fibroblast and the inhibition of scar formation. Eye, 1988, 2, 135-148.

Grierson, I., Hiscott, P., Hogg, P., Robey, H., Mazure, A., Larkin, G., Development, repair and regeneration of the retinal pigment epithelium. Eye, 1994, 8, in press.

Grillo, H.C. and Potsaid, M.S., Studies in wound healing: IV Retardation of contraction by local X-irradiation and observations leading to the origin of fibroblasts in repair. Ann. Surg., 1961, 154, 741-750.

Grinnell, F., Cellular adhesiveness and extracellular substrata. Int. Rev. Cytol., 1978, 58, 65-144.

Grinnell, F., Fibronectin and wound healing. J. Cell Biochem., 1984, 26, 107-116.

Grisanti, S., Heimann, K., Wiedemann, P., Origin of fibronectin in epiretinal membranes of proliferative vitreoretinopathy and proliferative diabetic retinopathy. Brit. J. Ophth., 1993, 77, 238-242.

Grisanti, S., Wiedemann, P.,Weller, M., Heimann, K. and Zilles, K., The significance of complement in proliferative vitreoretinopathy. Invest. Ophth. Vis. Sci., 1991, 32, 2711-2717.

Grünwald, J., Bloom, R. and Wülfroth, P., Migration von monozyten und granulozyten in co-kultur mit endothelzellen. Zeitschrift für Kardiol., 1989, 78, 113-116.

Hackett, S.F., Conway, B.P., Campochiaro, P.A., Subretinal fluid stimulation of RPE migration and proliferation is dependent upon certain features of the detachment or its treatment. Invest. Ophth. Vis. Sci., 1988, Suppl., 29, 306.

Ham, R.G., An improved solution for diploid chinese hamster and human cell lines. Exp. Cell Res., 1963, 29, 515-526.

Hamilton, A.M. and Taylor, W., Significance of pigment granules in the vitreous. Brit. J. Ophth., 1972, 56, 700-702.

Harada, T., Chavaud, D., Pouliquen, Y., An electron microscopic study of the epiretinal membranes of human eyes. Graefe's Arch. Clin. Exp. Ophth., 1981, 215, 327-339.

Harris, A. K., Cell motility and the problem of anatomical homeostasis. J. Cell Sci., 1987, Suppl., 8., 121-140.

Harris, A. K., Stopak, D. and Wild, P., Fibroblast traction as

a mechanism for collagen morphogenesis. Nature, 1981, 290, 249-251.

Harris, A. K., Wild, P. and Stopak, D., Silicone rubber substrata: A new wrinkle in the study of cell locomotion. Science, 1980, 208, 177-179.

Harvey, A., Roberge, F. and Hjelmeland, L., Chemotaxis of rat retinal glia to growth factors found in repairing wounds. Invest. Ophth. Vis. Sci., 1987, 28, 1029-1099.

Herbst, T.J., McCarthy, J.B., Tsilibary, E.C. and Furcht, L., Differential effects of laminin, intact type IV collagen, and specific domains of type IV collagen on endothelial cell adhesion and migration. J. Cell Biol., 1988, 106, 1365-1373.

Hergott, G.J., Nagai, H. and Kalnins, V.I., Inhibition of retinal pigment epithelial cell migration and proliferation with monoclonal antibodies against the $\beta 1$ integrin subunit during wound healing in organ culture. Invest. Ophth. Vis. Sci., 1993, 34, 2761-2768.

Herman, I.M and D'Amore, P.A., Microvascular pericytes contain muscle and nonmuscle actins. J. Cell Biol., 1985, 101, 43-52.

Hertzberg, E.L., Lawrence, T.S. and Gilula, N.B., Gap junctional communication. Ann. Rev. Physiol., 1981, 43, 479-491.

Hicks, D. Bugra, K., Faucheux, B., Jeanny, J-C.,Laurent, M., Malecaze, F., Mascarelli, F., Raulais, D., Cohen, S.Y. and Courtois. Y., Fibroblast growth factors in the retina. Prog. Ret. Res., 1991, 11, 339-374.

Hicks, D. and Courtois, Y., Fibroblast growth factor stimulates photoreceptor differentiation. Invest. Ophth. Vis. Sci., 1991, Suppl., 32, 1298.

Hilton, G., Machemer, R., Michels, R., Okun, E., Schepens, C. and Schwartz, A., The classification of retinal detachment with proliferative vitreoretinopathy. Ophth., 1983, 90, 121-125.

Hiscott, P.S., Morino, I., Alexander, R., Grierson, Z. and Grierson, I., Cellular components of subretinal membranes in proliferative vitreoretinopathy. Eye, 1989, 3, 606-610.

Hiscott, P.S., The morphology of epiretinal membranes, Ph.D. Thesis, London, 1986, 45-58.

Hiscott, P.S., Grierson, I., Hitchins, C.A., Rahi, A.H.S. and McLeod, D., Epiretinal membranes in vitro. Trans. Ophth. Soc., 1983, 103, 89-102.

Hiscott, P.S., Grierson, I. and McLeod, D., Natural history of fibrocellular epiretinal membranes: a quantitative, autoradiographic and immunohistochemical study. Brit. J. Ophth., 1985, 69, 810-823.

Hiscott, P.S., Grierson, I., Trombetta, C.J., Rahi, A.H.S., Marshall, J. and McLeod, D., Retinal and epiretinal glia: an immunohistochemical study. Brit. J. Ophth., 1984, 68, 698-707.

Hiscott, P.S., Unger, W., Grierson, I. and McLeod, D., The role of inflammation in the development of epiretinal membranes. Curr. Eye Res., 1988, 7, 877-892.

Hiscott, P.S., Waller, A., Grierson, I. Butler, M.G., Scott, D.L., Gregor, Z. and Morino, I., Fibronectin synthesis in subretinal membranes. Brit. J. Ophth., 1992, 76, 486-490.

Hitchins, C. and Grierson, I., Experimental scar membranes in the rabbit's vitreous. An autoradiographic and quantitative morphological study. Act. Ophth., 1985, 63, 557-566.

Hitchins, C. and Grierson, I., Intravitreal injection of fibroblasts: the pathological effects on the ocular tissues of the rabbit following an intravitreal injection of autologous skin fibroblasts. Brit. J. Ophth., 1988, 72, 498-510.

Hitchins, C. and Grierson, I. and Hiscott, P.S., The effects of injections of cultured fibroblasts into the rabbit vitreous. Graefe's Arch. Clin. Exp. Ophth., 1985, 223, 237-249.

Hjelmeland, L.M. and Harvey, A.K., Gliosis of the mammalian retina: Migration and proliferation of retinal glia. Prog. Ret. Res., 1988, 7, 259-282.

Hjelmeland, L.M. and Harvey, A.K., Hohman, T.C. and de Juan Jr.E., Primary culture and chemotactic responses of human retinal glia. Invest. Ophth. Vis. Sci., 1987, Suppl., 28, 208.

Hogan, .J.M., Alvarado, I.A., and Weddell, J.E., Retina. In: Histology of the human eye an atlas and textbook. W.B.Saunders Company, Philadelphia, 1971, 393-522.

Hogan, .J.M., Alvarado, I.A., and Weddell, J.E., Vitreous. In: Histology of the human eye an atlas and textbook. W.B.Saunders Company, Philadelphia, 1971, 607-637.

Hogg, N., An integrin overview. Chem. Immunol., 1991, 50, 1-12.

Hollyfield, J.G., Phagocytic capabilities of the pigment epithelium. Exp. Eye Res., 1976, 22, 457-468.

Hook, M., Molecular mechanisms of cell-substrate adhesion. In: Self Assembling Architecture, Alan R. Liss, Inc., 1988, 119-127.

Horan, P.K. and Slezak, S.E., Stable cell membrane labelling. Nature, 1989, 340, 167-168.

Hudspeth, A.J. and Yee, A. G., The intercellular junctional complexes of retinal pigment epithelia. Invest. Ophth., 1973, 12, 354-365.

Hughes, R. C., Pena, S.D.J. and Dourmashkin, R.R., Molecular requirements for the adhesion and spreading of hamster fibroblasts. Exp. Cell Res., 1979, 121, 307-314.

Hunt, R.C., Bernstein, P.S., Box, D., Turner, J. and Davis, A.A., A human retinal pigment epithelial that retains epithelial cell characteristics. Invest. Ophth. Vis. Sci., 1994, Suppl., 35, 1761.

Hunt, T.K., Goodson III, W.H. and Scheuenstuhl, H., A Strategy for human studies: thoughts on models. In; Wound Healing, Eds., Janssen, H. and Robertson, J.I.S., Wrightson Biomedical Publishing Ltd., 1991, 177-187.

Hynes, R.O., Integrins: A family of cell surface receptors. Cell, 1987, 48, 549-554.

Hynes, R.O. and Yamada, K. M., Fibronectins : Multifunctional modular glycoproteins. J. Cell Biol., 1982, 95, 369-377.

Immonen, I., Salonen, E.-M., Laatikinen, L. and Vaheri, A., Fibronectin in subretinal fluid. In: International Symposium on Proliferative vitreoretinopathy. Ed. Heimann, K. and Wiedemann, P., Kaden-Verlag, 1989, 109-117.

Isaacs, W.B., Cook, R.K., Van-Atta, J.C., Redmond, C.M. and Fulton, A.B., Assembly of vimentin in cultured cells varies with cell type. J. Biol. Chem., 1989, 264, 17953-17960.

Izzard, C.S. and Lochner, L.R., Cell-to-substrate contacts in living fibroblasts: an interference reflexion study with an evaluation of the technique. J.Cell Sci., 1976, 21, 129-159.

Johnson, N.F. and Foulds, W., Observations on the retinal pigment epithelium and retinal macrophages in experimental retinal detachment. Brit. J. Ophth., 1977, 61, 564-572.

Joseph, J.P., Grierson,I. and Hitchins, R.A., Normal rabbit aqueous humour, fibronectin and fibroblast conditioned medium are chemoattractant to Tenon's capsule fibroblasts. Eye, 1987, 1, 585-592.

de Juan, E., Dickson, M.S. and Hjelmeland, L., Serum is chemotactic for retinal-derived glial cells. Arch. Ophth., 1988, 106, 986-990.

Kampik, A., Keynon, K.R., Michels, R.G., Green, W.R. and de la Cruz, Z.C., Epiretinal and vitreous membranes. Comparative study of 56 cases. Arch. Ophth., 1981, 99, 1445-1454.

Kang, A.H., fibroblast activation. J. Lab. Clin. Med., 1987, 92, 1-4.

Kelley, C., D'Amore, P., Hechtmann, H.B. and Shepro, D., Microvascular pericyte contractility in vitro: comparison with other cells of the vascular wall. J. Cell Biol., 1987, 483-490.

Kirchof, B., Kirchof, E., Ryan, S. J. and Sorgente, N., Human retinal pigment epithelial cell cultures: Phenotypic modulation by vitreous and macrophages. Exp. Eye Res., 1988, 47, 457-463.

Kirchof, B., Kirchof, E., Sorgente, N. and Ryan, S. J., Interleukin 1 stimulates migration but not proliferation of

pigment epithelial cells in vitro. Invest. Ophth. Vis. Sci., 1987, Suppl., 28, 208.

Kirchof, B. and Sorgente, N., Pathogenesis of proliferative vitreoretinopathy. Dev. Ophth., Straub, W., (Ed.), Basel, Karger, 1989, 16, 1-53.

Kovacs, E.J., Fibrogenic cytokines: the role of immune mediators in the development of scar tissue. Immunol. Today, 1991, 12, 17-23.

Kroll, A.J. and Machemer, R., Experimental retinal detachment in the owl monkey III. Electron microscopy of retina and retinal pigment epithelium. Am. J. Ophth., 1968, 64, 410-427.

Lackie, L.M., Anisotropic environments, In: Cell movement and cell behaviour, Allen and Unwin, 1986, 198-217.

Laplante, C. and Lemaire, I., Interactions between alveolar macrophage subpopulations modulate their migratory function. Am. J. Path., 1990, 16, 199-206.

Laqua, H. and Machemer, R., Glial cell proliferation in retinal detachment (massive periretinal proliferation). Am. J. Ophth., 1975, 80, 602-618.

Laqua, H. and Machemer, R., Clinico-pathological correlation in massive preretinal proliferation. Am. J. Ophth., 1975, 80, 396-410.

Lean, J.S., Origin of simple glial epiretinal membranes in and animal model. Graefe's Arch. Clin. Exp. Ophth., 1987, 225, 421-425.

Lean, J.S., Stern, W.A., Irvine, A.R. and Azen, S.P., and the Silicone Study Group, Classification of proliferative vitreoretinopathy used in the Silicone Study. Ophth., 1989, 96, 765-771.

Lemor, M., Hyer, K., Davis, J., Connor, T., Glaser, B., Subretinal fluid from experimental detachments stimulates RPE cell and astrocyte proliferation. Invest. Ophth. Vis. Sci., 1987, Suppl., 28, 206.

Leschey, K. H., Hackett, S.F., Singer, J.H. and Campochiaro, P.A., Growth factor responsiveness of human retinal pigment epithelial cells. Invest. Ophth. Vis. Sci., 1990, 31, 839-846.

Lessel, S. and Kuwbara, T., Retinal neuroglia. Arch. Ophth., 1963, 70, 671-678.

Lévesque, J.P., Hatzfeld, A. and Hatzfeld, J., Mitogenic properties of major extracellular proteins. Immunol. Today, 1991, 12, 258-262.

Lewis, G., Guerin, C. and Erickson, P., Anderson, D.H. and Fisher, S.K., Basic fibroblast growth factor (BFGF) stimulates proliferation of non neuronal retinal cells in vivo. Invest. Ophth. Vis. Sci., 1991, Suppl., 32, 754.

Lewis, J., Morphogenesis by fibroblast traction. Nature, 1984, 307, 413-414.

Lillie, R.D., Cytoplasmic granules and organelles.
In: Histopathological Techniques and Practical Histochemistry, 3rd edition, McGraw Hill Book Co., USA, 1965, 286.

Liotta, L. A., Rao, C.N. and Barsky, S.H., Tumour invasion and the extracellular matrix. Lab. Invest., 1983, 49, 636-649.

Machemer, R., Pathogenesis and classification of massive periretinal proliferation. Brit. J. Ophth., 1978, 62, 737-747.

Machemer, R. and Laqua, H., Pigment epithelium proliferation in retinal detachment (massive periretinal proliferation). Am. J. Ophth., 1975, 80, 1-23.

Machemer, R., Aaberg, T.M., Freeman, H.M., Irvine, A.R., Lean, J.S. and Michels, R.M., An updated classification
of retinal detachment with proliferative vitreoretinopathy. Am. J. Ophth., 1991, 112, 159-163.

Machemer, R., Van Horn, D. and Aaberg, T.M., Pigment epithelial proliferation in human retinal detachment with massive periretinal proliferation. Am. J. Ophth., 1978, 85, 181-191.

Magalhaes, M.M., Functional architecture of the retina Müller cell. In: The structure of the eye III, Eds. Yamada and

Mishima, 1976, 33-340.

Majno, G., The story of the myofibroblasts. Am. J. Surg. Path., 1979, 3, 535-542.

Mano, T. and Puro, D.G., Phagocytosis by human retinal glial cells in culture. Invest. Ophth. Vis. Sci., 1990, 31, 1047-1055.

Marzona, L., Olivo, O.M., Volpi, G. and Toni, G., Effects of carmine and carminic acid on embryonic tissue cell cultures. Experimentia, 1977, 33, 755-756.

Mascarelli, F., Raulais, D., Counis, M.F. and Courtois, Y., Characterization of acidic and basic fibroblast growth factors in brain, retina and vitreous chick embryo. Biochem. Biophys. Res. Comm., 1987, 146, 478-486.

Mayne, R., Brewton, R.G., Mayne, P.M. and Baker, J.R., Isolation and characterisation of the chains of type V/type XI collagen present in bovine vitreous. J. Biol. Chem., 1993, 268, 9381-9386.

Mazure, A. and Grierson, I., In vitro studies of the contractility of cell types involved in proliferative vitreoretinopathy. Invest. Ophth. Vis. Sci., 1992, 33, 3407-3416.

Mc Carthy, J. B. and Furcht, L.T., Laminin and fibronectin promote the haptotactic migration of B16 mouse melanoma cells in vitro. J. Cell Biol., 1984, 98, 1474-1480.

Mc Carthy, J. B., Sas, D.F. and Furcht, L.T., Mechanisms of parenchymal cell migration into wounds.
In: The molecular and cell biology of wound repair. Eds. Clark, R.A.F. and Hensen, P.M., Plenum Press, New York and London, 1988, 281-319.

McGaw, W.T and Ten-Cate, A.R., A role for collagen phagocytosis by fibroblasts in scar remodelling: an ultrastructural stereologic study. J.Invest. Derm., 1983, 81, 375-378.

McKechnie, N., Boulton, M., Robey, H., Savage, F.J. and Grierson, I., The cytoskeletal elements of human retinal pigment epithelium

in vitro and in vivo. J. Cell Sci., 1988, 91, 303-312.

McKeown, M. Knowles, G. and McCulloch, C.A.G., Role of the cellular attachment domain of fibronectin in the phagocytosis of beads by human gingival fibroblasts in vitro. Cell Tiss. Res., 1990, 262, 523-530.

McLeod, D., Hiscott, P.S. and Grierson, I., Age-related cellular proliferation at the vitreoretinal juncture.
Eye, 1987, 1, 263-281.

Melchiori, A., Allavena, G., Negrone, R., Parodi, S. and Albini, A., Effect of different cell detachment techniques on fibroblast chemotaxis to fibronectin and conditioned medium. Boll. Soc. It. Biol. Sper., 1986, 62, 637-643.

Mensing, H., Pontz, B.F., Müller, P.K. and Gauss-Müller, V., A study on fibroblast using fibronectin and conditioned medium as chemoattractants. Eur. J. Cell Biol., 1983, 29, 268-273.

Michels, R.G., A clinical and histopathological study of epiretinal membranes affecting the macula and removed by vitreous surgery. Trans. Am. Ophth. Soc., 1982, 80, 580-656.

Middleton, C.A., The control of epithelial cell locomotion in tissue culture. Ciba Foundation Symp., 1973, 14, 251-262.

Middleton, C.A. and Sharp, J.A., Cell locomotion in culture. In: Cell locomotion in vitro techniques and observations. 1984, 101-136.

Miller, B., Millar, H., Patterson, R., Ryan, S.,J., Retinal wound healing - cellular activity at the vitreoretinal interface. Arch. Ophth., 1986, 104, 281-285.

Moll, R., Franke, W.W. and Schiller, D.L., The catalog of the human cytokeratins: patterns of expression in normal epithelia, tumours and cultured cells. Cell, 1982, 31, 11-24.

Morino, I., Hiscott, P.S., McKechnie, N. and Grierson, I., Variation in epiretinal membrane components with clinical duration of the proliferative tissue. Brit. J. Ophth., 1990, 74, 393-399.

Morris, J.E., Steric exclusion of cells. A mechanism of glycosaminoglycan-induced cell aggregation. Exp. Cell Res., 1979, 120, 141-153.

Morse, L.S., Terrell, J. and Sidikaro, Y., Bovine retinal pigment epithelium promotes proliferation of choroidal endothelium in vitro. Arch. Ophth., 1989, 107, 1659-1663.

Müller-Jensen, L., Machemer, R. and Azarina, R., Autotransplantation of retinal pigment epithelium in intravitreal diffusion chamber. Am. J. Ophth., 1975, 80, 530-537.

Nakamura, T., Yoshimoto, K., Nakayama, Y., Tomita, Y. and Ichihara, A., Reciprocal modulation of growth and differentiated functions of mature rat hepatocytes in primary culture by cell-cell contact and cell membranes. Proc. Natl. Acad. Sci. USA., 1983, 80, 7229-7233.

Nathan, C.F., Secretory products of macrophages. J. Clin. Invest., 1987, 79, 319-326.

Newsome, D.A., Rodrigues, M.M. and Machemer, M.D., Human massive periretinal proliferation: in vitro characteristics of cellular components. Arch. Ophth., 1981, 99, 873-880.

Norris, D.A., Clark, A.F., Swigart, L.M., Huff, J.C., Weston, W.L. and Howell, S.E., Fibronectin fragments are chemotactic for human peripheral blood monocytes. J. Immunol., 1982, 129, 1612-1618.

Ogden, T.E., Nerve fibre layer astrocytes of the primate retina: Morphology, distribution and density. Invest. Ophth. Vis. Sci., 1978, 17, 499-510.

Oppenheimer-Marks, N., Davis, l.S., Bogue, D.T., Ramberg, J. and Lipsky, P.E., Differential utilisation of ICAM-1 and VCAM-1 during the adhesion and transendothelial migration of human T lymphocytes. J. Immun., 1991, 147, 2913-2921.

Oster, G., The biophysics of the leading lamella. Cell Motil. Cytoskel., 1988, 10, 164-167.

Parish, R.W., Schmidhauser, C., Schmidt, T. and Dudler, R.K., Mechanisms of tumour cell metastasis. J. Cell Sci., 1987,

Suppl., 8., 181-197.

Parsons, J.H., The retina, In: The pathology of the eye. Hodder and Stoughton, London, 1905, vol.2, 452-600.

Peacock, E.E., Inflammation and the cellular response to wound injury. In: Wound repair. W. B. Saunders Company, Philadelphia, 1984, 1-14.

Pepper, M.S., Spray, D.C., Chanson, M., Montesano, R. and Moda, P., Junctional communication in migrating capillary endothelial cells. J.Cell Biol., 1989, 109, 3027-3038.

Pessa, M. E., Bland, K. I. and Copeland, E.M., Growth factors and determinants of wound repair. J. Surg. Res., 1987, 42, 207-217.

Petrakis, N.L., Davis.M. and Lucia, S.P., The in vivo differentiation of leucocytes into histiocytes, fibroblasts and fat cells in subcutaneous diffusion chambers. Blood, 1961, 17, 109-118.

Pollack, S.V., The wound healing process. Clinics in Derm., 1984, 2, 8-16.

Pontén, J., Westermark, B. and Hugosson, R., Regulation of proliferation and movement of human glia like cells in culture. Exp. Cell Res., 1969, 58, 393-400.

Postlethwaite, A.E., Keski-Oja, J., Balian, G. and Kang, A.H., Induction of fibroblast chemotaxis by fibronectin. Localization of the chemotactic region to a 140, 000 -molecular weight non-gelatin-binding fragment. J. Exp. Med., 1981, 153, 494-499.

Postlethwaite, A.E., Keski-Oja, J., Moses, H., and Kang, A.H., Stimulation of the chemotactic migration of human fibroblasts by transforming growth factor β. J. Exp. Med., 1987, 165, 251-256.

Postlethwaite, A.E., Snyderman,R. and Kang,A.H., The chemoattraction of human fibroblasts to a lymphocyte derived factor. J. Exp. Med., 1976, 144, 1188-1203.

Puro, G., Roberge, F. and Chan, C., Retinal glial cell

proliferation and ion channels: A possible link. Invest. Ophth. Vis. Sci., 1989, 30, 521-529.

Rachal, W.F. and Burton, T.C., Changing concepts of failures after retinal detachment surgery. Arch. Ophth., 1979, 97, 480-483.

Raymond, L. and Jacobson, B., Isolation and identification of stimulatory and inhibitory cell growth factors in bovine vitreous. Exp. Eye Res., 1982, 34, 267-286.

Rhodes, R.H., An ultrastructural study of the complex carbohydrates of the mouse posterior vitreoretinal juncture. Invest. Ophth. Vis. Sci., 1982, 22, 460-477.

Robey, H.L., Hiscott, P. and Grierson, I., Cytokeratins and epithelial cell behaviour. J. Cell Sci., 1992, 102, 329-340.

Ross, R., Platelet-derived growth factor. Lancet, 1989, 8648, 1179-1181.

Ross, R., The fibroblast and wound repair. Biol. Rev., 1968, 43, 51-96.

Ross, R., Glomset, J., Kariya, B. and Harker, L., A platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscles in vitro. Proc. Nat. Acad. Sci. USA., 1974, 71, 1207-1210.

Ross, R. and Vogel, A., The platelet derived growth factor. Cell, 1978, 14, 203-209.

Rowen, S., Chandler, C., Lutty, G., Human vitreous is mitogenic for astrocytes. Invest. Ophth. Vis. Sci., 1986, Suppl., 27, 304.

Rowen, S., Glaser, B.M., Retinal pigment epithelial cells release a chemoattractant for astrocytes. Arch. Ophth., 1985, 103, 704-707.

Ruoslathi, E., Integrins. J. Clin. Invest., 1991, 87, 1-5.

Ruoslathi, E. and Pierschbacher, M.D., New perspectives in cell adhesion: RGD and integrins. Science, 1987, 238,

491-497.

Salzmann, M., The pigment epithelium of the choroidea. In: The anatomy and histology of the human eyeball in the normal state its development and senescence. University of Chicago press, Chicago, 1912, 60-63.

Samlowski, W.E., Robertson, B.A., Draper, B.k., Prystas, E. and McGregor, J.R., Effects of supravital fluorochromes used to analyse the in vivo homing of murine lymphocytes on cellular function. J. Immun. Meth., 1991, 144, 101-115.

Savage, F.J., Day, J.E., Hogg, P. and Grierson, I., Tissue culture of retinal glial cells. Eye, 1989, Suppl, .2 , 164-179.

Schepens, C.L., Clinical aspects of pathological changes in the vitreous body. Am. J. Ophth., 1954, 38, 8-21.

Schnitzer, J., Astrocytes in mammalian retina. Prog. Ret. Res., 1988, 7, 209-232.

Schnitzer, J., Distribution and immunoreactivity of glia in the retina of the rabbit. J. Comp. Neurol., 1985, 240, 128-142.

Schroeder, F. and Kinden, D.A., Measurement of phagocytosis using fluorescent latex beads. J. Biochem. Biophys. Meth., 1983, 8, 15-27.

Schwartz, M., Cohen, A., Stein-Izsak, C. and Belkin, M., Dichotomy of the glial response to axonal injury and regeneration. FASEB., 1989, 3, 2371-2378.

Schweigerer, L., Malerstein, B., Neufeld, G. and Gospodarowicz, D., Basic fibroblast growth factor is synthesised in cultured retinal pigment epithelial cells. Biochem. Biophys. Res. Commun., 1987, 143, 934-940.

Sebag, J., Anatomy and pathology of the vitreoretinal interface. Eye, 1992, 6, 541-552.

Seery, C.M and Davison, P.F., Collagens of the bovine vitreous. Invest. Ophth. Vis. Sci., 1991, 32, 1540-1550.

Senior, R. M., Huang, S.S., Griffin, G. and Huang, J.S., Brain-derived growth factor is a chemoattractant for fibroblasts and astroglial cells. Biochem. Biophys. Res. Commun., 1986, 141, 67-72.

Seppä, H.E.J, Yamada, K.M., Seppä, S.T., Silver, M.H., Kleinmann, H.K. and Schiffmann, E., The cell binding fragment of fibronectin is chemotactic for fibroblasts. Cell Biol. Int. Rep., 1981,5, 813-819.

Seppä, H., Grotendorst, G., Seppä, S., Schiffman, E. and Martin, G., Platelet derived growth factor is chemotactic for fibroblasts. J. Cell Biol., 1982, 92, 584-588.

Shipley, G. D., Rosenbaum, J.T., Tsai, J., Hendrickson, J., Keeble, W. and Robertson, J., Retinal pigment epithelial cells bind and probably secrete type-2 heparin binding growth factor. Invest. Ophth. Vis. Sci., 1987, Suppl., 28, 207.

Shirakawa, R., Yoshimura, N., Yamakawa, R., Matsumara, M., Okada, M. and Ogino, N., Cell components in proliferative retinopathy. Immunofluorescent double staining of cultured cells from proliferative tissues. Ophthalmologic., 1987, 194, 56-62.

Siegal, S., The case of two independent samples: the Mann Whitney U test. In: Nonparametric Statistics for the Behavioural Sciences McGraw Hill International Book Company, 1956, 116-127.

Singer, I. I., Kazizis, D.M. and Scott, S., Scanning electron microscopy of focal contacts on the substratum attachment surface of fibroblasts adherent to fibronectin. J. Cell Sci., 1989, 93, 147-54.

Smiddy, W.E., Maguire, A.M., Green, W.R., Michels, R.G., De La Cruz, Z., Enger, C., Jaeger, M. and Rice, T., Idiopathic epiretinal membranes: ultrastructural characteristics and clinicopathological correlation. Ophth., 1989, 96, 811-821.

Smith, P.J.S., Howes, E.A. and Treherne, J.E., Mechanisms of glial regeneration in an insect central nervous system. J. Exp. Biol., 1987, 132, 59-78.

Sonnenberg, A., Calafat, J., Janssen, H., Daams, H., van der Raaij-Helmer, L.M., Falcioni, R., Kennel, S.J., Aplin, J.D.,

Baker, J. and Loizidou, M., Integrin alpha 6/beta 4 complex is located in hemidesmosomes, suggesting a major role in epidermal cell-basement membrane adhesion. J. Cell Biol., 1991, 113, 907-917.

Springer, T. A., Adhesion receptors of the immune system. Nature, 1990, 346, 425-434.

Sternfeld, M.D., Robertson, J.E., Shipley, G.D., Tsai, J. and Rosenbaum, J.T., Cultured human retinal pigment epithelial cells express basic fibroblast growth factor and its receptor. curr. Eye. Res., 1989, 8, 1029-1037.

Tahery, M.M. and Lee, D.A., Review: Pharmacologic control of wound healing in glaucoma filtration surgery. J. Ocular Pharm., 1989, 5, 155-178.

Teng, C.C., An electron microscopic study of cells in the vitreous of the rabbit eye. Part I: The macrophage. Eye Ear Nose Throat Month., 1969, 48, 91-104.

Teng, C.C., An electron microscopic study of cells in the vitreous of the rabbit eye. Part II: Fibrocytes, plasma cells and an unidentified cell. Eye Ear Nose Throat Month., 1969, 48, 80-87.

Tooney, P.A., Agrez, M.V. and Burns, G.F., A re-examination of the molecular basis of cell movement. Immun. Cell Biol., 1993, 71, 131-139.

Tolentino, F.I., Schepens, C.L. and Freeman, H.M., Massive preretinal retraction - a biomicroscopic study. Arch. Ophth., 1967, 78, 16-22.

Toyohara, A. and Inaba, K., Transport of phagosomes in mouse peritoneal macrophages. J. Cell Sci., 1989, 94, 143-153.

Tsukamoto, Y., Helsel, W.E. and Wahl, S.M., Macrophage production of fibronectin, a chemoattractant for fibroblasts. J. Immunol., 1981, 127, 673-681.

Tucker, R.W., Butterfield, C.E. and Folkmán, J., Interaction of serum and cell spreading affects the growth of neoplastic and non-neoplastic fibroblasts. J. Supramol. Struct. Cell Biochem.,

1981, 15, 29-40.

Twl Hewitt, T.A., Extracellular matrix molecules: Their importance in the structure and function of the retina. In: The Retina II A Model for Cell Biology Studies, Ed. Adler, R. and Farber, D., Academic Press, 1986, 170-214.

Uchihori, Y. and Puro, D.J., Mitogenic and chemotactic effects of platelet derived growth factor on human retinal glial cells. Invest. Ophth. Vis. Sci., 1991, 32, 2689-2695.

Vallés, A.M., Boyen, B. and Thiery, J.P., Adhesion systems in embryonic epithelial-to-mesenchyme transformations and in cancer invasion and metastasis. In: Cell Motility Factors, Birkhausen Verlag, Basel, Switzerland, 1991, 17-34.

Vaheri, A., Ruoslathi, E., Westermark, B. and Pontén, J., A common cell type specific surface antigen in culture human glial cells and fibroblasts: loss in malignant cells. J. Exp. Med., 1976, 143, 64-72.

Vasiliev, J.M., Spreading and locomotion of tissue cells: Factors controlling the distribution of pseudopodia. Phil. Trans. R. Soc. Lond., 1982, B299, 159-167.

Vasiliev, J.M., Actin cortex and microtubular system in morphogenesis: Cooperation and competition. J. Cell Sci., 1987, Suppl., 8, 1-18.

Verdoorn, C., Renardel de Lavalette, V.W., Dalma-Weizhausz, J., Orr, G.M., Sorgente, N. and Ryan, S.J., Cellular migration, proliferation and contraction - an in vitro approach to a clinical problem - proliferative vitreoretinopathy. Arch. Ophth., 1986, 104, 1216-1219.

Vidaurri-Leal, J.S. and Glaser, B.M., Effects of fibrin on morphologic characteristics of retinal pigment epithelial cells. Arch. Ophth., 1984, 102, 1376-79.

Vinores, S.A., Campochiaro, P.A., McGhee, R., Orman, W., Hackett, S.F. and Hjelmeland, L.M., Ultrastructural and immunocytochemical changes in retinal pigment epithelium, retinal glia and fibroblasts in vitreous culture. Invest. Ophth. Vis. Sci., 1990, 31, 2529-2549.

Warn, R.M. and Dowrick, P., Motility factors on the march. Nature, 1989, 340, 186-187.

Warner, A., The gap junction. J. Cell Sci., 1988, 89, 1-7.

Warwick, R. and Williams, P.L., The peripheral visual apparatus. In: Gray's Anatomy 35th edition, Longman Group Ltd., UK, 1973, 1095-1133.

Watanabe, K., Nakagawa, S. and Nishida, T., Chemotactic and Haptotactic activities of fibronectin for cultured rabbit corneal epithelial cells. Invest. Ophth. Vis. Sci., 1988, 29, 572-577.

Weiss, P., Guiding principles in cell locomotion and cell aggregation. Exp. cell Res., 1961, Suppl. 8, 260-281.

Weissmann, G., The role of neutrophils in vascular injury: a summary of signal transduction mechanisms in cell/cell interactions. Springer Sem. Immunopath., 1989, 11, 235-258.

Weller, M., Heimann, K. and Wiedemann, P., Immunochemical analysis of periretinal membranes. Dev. Ophth., 1989, 16, 55-74.

Weller, M., Wiedemann, P., Bresgen, M. and Heimann, K., Vitronectin and proliferative disorders. II. Expression of cell surface receptors for fibronectin and vitronectin in periretinal membranes. Int. Ophth., 1991, 15, 103-108.

Weller, M., Wiedemann, P., Heimann, K., Proliferative vitreoretinopathy: Is it anything more than wound healing at the wrong place? Int. Ophth., 1990, 14, 105-117.

Weller, M., Wiedemann, P., Heimann, K. and Zilles, K., Fibronectin quantification in plasma and vitreous by a noncompetitive ELISA technique. Doc. Ophth., 1988, 69, 31-351.

Weller, M., Wiedemann, P., Heimann, K. and Zilles, K., The significance of fibronectin in vitreoretinal pathology. Graefe's Arch. Clin. Exp. Ophth., 1988, 226, 294-298.

Wetzel, M.G. and Korn, E.D., Phagocytosis of latex beads by

Acanthamoeba castellanii: III isolation of the phagocytic vesicles and their membranes. J. Cell Biol., 1969, 43, 90-104.

Wiedemann, P., Growth factors in retinal diseases: Proliferative vitreoretinopathy, proliferative diabetic retinopathy and retinal degeneration. Surv. Ophth., 1992, 36, 373-384.

Wiedemann, P., Ryan, S.J., Novak, P. and Sorgente, N., Vitreous stimulates proliferation of fibroblasts and retinal pigment epithelial cells. Exp. Eye. Res., 1985, 41, 619-628.

Wiedemann, P. and Weller.M., The pathology of proliferative vitreoretinopathy. Acta Ophth. (Copenh.), 1988, Suppl., 189, 7-15.

Wilson-Holt, N., Khaw, P., Savage, F. and Grierson, I., The chemoattractant activity of the vitreous to human scleral fibroblasts following retinal detachment surgery and proliferative vitreoretinopathy. Brit. J. Ophth., 1992, 76, 159-162.

Winthrop, S.R., Cleary, P.E., Minckler, D.S. and Ryan, S.J., Penetrating eye injuries: a histopathological review. Brit. J. Ophth., 1980, 64, 809-817.

Wolter, J.R., The astroglia of the human retina and other glial elements of the retina under normal and pathologic conditions. Am. J. Ophth., 1955, 40, 88-99.

Wolter, J.R. and Lichter, P.R., Fibroblast-like cells on intraocular lens implants: phagocytosing erythrocytes. Brit. J. Ophth., 1983, 67, 641-645.

Wong, D., Chignell, A.H., Inglesby, D.V., Little, B.C. and Franks, W., The treatment of bullous retinal detachment. Graefe's Arch. Clin. Exp. Ophth., 1992, 230, 218-220.

Wong, H.C., Boulton, M., McLeod, D., Bayly, M., Clark, P. and Marshall, J., Retinal pigment epithelial cells in culture produce retinal vascular mitogens. Arch. Ophth., 1988, 1439-1443.

Wong, H.C., Boulton, Marshall, J. and Clark, P., Growth of retinal capillary endothelia using pericyte conditioned medium.

Invest. Ophth. Vis. Sci., 1987, 28, 1767-1775.

Xu, X., Ho, P.C.P., Ip, S.M., Basic fibroblast growth factor levels in subretinal fluid of retinal detachment with proliferative vitreoretinopathy. Invest. Ophth. Vis. Sci., 1994, Suppl., 35, 2042.

Yamada, K.M., Cell surface interactions with extracellular materials. Ann. Rev. Biochem., 1983, 52, 761-..

Yamakawa, R., Shirakawa, H., Okada, M., Yoshimura, N., Matsuma, M. and Ogino, N., Retinal pigment epithelial cells produce fibronectin. Ophth. Res., 1987, 19, 338-343.

Zigmond, S.J., and Hirsch, J.G., Leucocyte locomotion and chemotaxis: new methods for evaluation and demonstration of a cell derived chemotactic factor. J. Exp. Med., 1973, 137, 387-410.

Zinn, K.M. and Benjamin-Henkind, J.V., Anatomy of the retinal pigment epithelium. In: The retinal pigment epithelium. Harvard University Press, Cambridge, Massachusetts, 1979, 3-31.