MONOCLONAL ANTIBODIES TO DAMAGED AND REGENERATING ENDOTHELIAL CELLS IN VITRO.

Serena W Pringle B.Sc.

Cardiovascular Research Unit,
Hugh Robson Building,
University of Edinburgh.

Submitted for the degree of Ph.D.to
The Faculty of Medicine,
University of Edinburgh.
September 1990.

DECLARATION

The work presented in this thesis is original and unless otherwise noted in the text or references was conducted solely by the author in the Cardiovascular Research Unit, University of Edinburgh, during the period from January 1985 to January 1988.

Serena W Pringle

ACKNOWLEDGEMENTS

I would like to express my gratitude to my supervisor Professor David de Bono for his constructive critiscism, friendly advice and endless patience during the writing of this thesis. His boundless enthusiasm for the work presented has supported and encouraged me throughout.

To Dr Jeremy Pearson of the Vascular Biology Unit at Northwick Park Hospital, London, for his helpful guidance in the early stages of the project.

To all the staff and fellow Ph.D students of the Cardiovascular Research Unit, University of Edinburgh, whose friendship and program of "extracurricular activities" helped to maintain sanity.

I would also like to say thankyou to Drs Nik Hunter and Iain McGregor of the Blood Components Assay Group, Edinburgh for advice and allowing me to "beg, steal or borrow".

My thanks to Fiona of the Dept. of Cardiology, Glenfield General Hospital, Leicester for helping me master the Apple Mac. and allowing me precious time on the computers and photocopier during the writing of this thesis.

Last but not least my thanks to my parents Eon and Esther for their endless support and encouragement.

ABSTRACT

The object of this thesis was to develop and characterise monoclonal antibodies which would recognise damaged and or regenerating endothelial cells and which might therefore be useful in the diagnosis and treatment of vascular disease.

The work described was carried out with cultured vascular endothelial cells of human, bovine, or porcine origin. In vitro models of endothelial damage were devised in which cells were damaged by various methods. Mouse monoclonal antibodies were raised by inoculation with cultured endothelial cells and tested against damaged endothelium in vitro. A number of antibody producing clones were identified, isolated and grown on for characterisation of the antibody produced.

Using an immunohistochemical system for localising the sites of binding of these antibodies to cultured endothelial cells, a number of different patterns of antibody binding were identified. Attention was concentrated on antibodies which appeared to selectively bind to damaged endothelial cells.

In an attempt to identify the antigens recognised by the monoclonal antibodies the protein constituents of endothelial cell lysates were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis. The protein bands were transferred to nitrocellulose

membranes so that specific bands which reacted with individual protein bands could be identified. A number of protein bands were identified which reacted with specific monoclonals. An interaction between damaged endothelial cells and the Fc component of IgG has been previously described in the literature. The non-specific interaction of IgG with endothelial cells was confirmed in this study and the binding component identified. Reasons for believing that the monoclonal antibodies raised in this project predominantly interacted with damaged endothelial components by specific rather than non-specific binding are discussed.

Attempts were made to label some of the monoclonal antibodies with radionuclides so that they could be used in an in vivo model system. Only limited success was achieved in labelling with radioactive iodine. but it did prove feasible to produce internal labelling with tritium by growing the antibody producing cells in medium containing tritiated lysine.

The possibility of using a monoclonal antibody to target a drug to areas of endothelial damage was investigated by covalently linking urokinase to one of the monoclonal antibodies. The ability of the conjugate to produce fibrinolysis strictly limited to areas of endothelial damage was demonstrated in an in vitro

model. This agent may have possible uses as a strictly localised anti-thrombotic agent in microvascular surgery.

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CHAPTER 1

INTRODUCTION

The endothelium consists of a single layer of tightly apposed cells which lines the luminal surface of the entire cardiovascular and lymphatic systems (Altschul 1954). It constitutes a continuous and dynamic interface between the blood and the rest of the body tissues, with many complex biochemical interactions occurring within the cell and at the cell surface. (Cryer 1983, Fishman 1982).

Endothelial integrity is critical for normal vascular function, particularly in its role of blood compatibility and maintenance of blood fluidity (Jaffe 1984, Gimbrone 1986).

This chapter reviews the structural and functional properties of the endothelium and the consequences of endothelial injury and dysfunction.

1.1. GENERAL MORPHOLOGY AND STRUCTURE.

The ultrastructure of endothelial cells has been studied in some detail. Each cell has a single nucleus surrounded by scant cytoplasm. Cell thickness on cross-sectioning is approximately 0.2microns through the cytoplasm and 3microns through the nucleus (Simionescu et al 1978). This 'bulging' nucleus gives the endothelium a 'cobblestone' appearance in scanning electron micrographs of en face preparations.

In the cytoplasm, mitochondria, rough endoplasmic

reticulum, Golgi complexes, pinocytic vesicles and multivesicular bodies are present. The Golgi complexes tend to be perinuclear and numerous microtubules and microfilaments of various sizes criss-cross the cytoplasm (Haudenschild et al 1975). These microfilaments and microtubules are thought to have a crucial role in cell motility and replication (Selden & Schwartz 1979, Gotlieb et al 1983, Wong & Gotlieb 1984).

Weibel-Palade bodies (WP bodies) are electron dense, rod-shaped organelles, which consist of unit membrane bound bundles of tubules of approximately 150 Angstroms in width (Weibel & Palade 1964, Haudenschild et al 1975). These organelles are often clustered near the periphery of the cell cytoplasm. Their function is not entirely clear but they have been associated with von Willebrand factor (vWF) and may be storage sites for this substance (Jaffe et al 1973, Wayne et al 1982, Wagner et al 1982, Wagner & Marder 1984).

WP bodies appear to be exclusive to endothelial cells and are particularly abundant in human umbilical cord vein endothelium. However they have proven to be unreliable markers for endothelial cell identification since they are present in only 30 to 70% of cells otherwise identified as endothelium. They are rarely seen in capillary endothelium (Jaffe et al 1973). Large

numbers of WP bodies have been reported in injured endothelial cells but these findings are inconsistent (Trillo & Pritchard 1979, Reidy & Schwartz 1983).

Scanning electron microscopy studies of the endothelium in vivo, show a single layer of very closely apposed cells with extensive overlapping of 'cytoplasmic flaps' of neighbouring cells (Reidy & Schwartz 1981a, Reidy & Schwartz 1981b, Woolf 1983). In large vessels the endothelium consists of flattened, elongated cells (and nuclei) with the longitudinal axis aligned with the direction of blood flow. Cell shape tends to vary from vessel to vessel and within the same large vessel, with elongation being more prominent near vessel branch points. Cell shape may indicate an adaptation of the cells to blood flow and shear stress and may also reflect the elastic properties of the vessel wall (Woolf 1983). In static culture endothelial cells have a rounded polygonal shape, giving the cells a morphologically typical cobblestone appearance (Jaffe et al 1973).

In the capillaries and the post-capillary venules of the lymph nodes the endothelium has become specialised in structure. This 'high endothelium' has a columnar structure with deep troughs between individual cells, therefore effectively increasing the surface area to aid the passage of lymphocytes to and from the

circulation through the capillary walls (Cho & De Bruyn 1979, Ager 1987).

1.2. CONTACT INHIBITION AND GROWTH CONTROL.

1.2.1. Contact inhibition and the role of cell shape in growth control.

Endothelial cells differ from most other cells in culture in that they show a final growth saturation density that is independant of serum concentrations, nutrients or other growth factors (Haudenschild et al 1979, Schwartz et al 1979). This suggests that endothelial cell growth is controlled intrinsically by the cells. This has been substantiated by studies that have shown that membrane extracts from confluent cells could arrest the growth of subconfluent cultures of the same cell type (Whittenberger & Glaser 1977, Natraj & Datta 1978). Vlodavsky and co-workers (1979) demonstrated the presence of a cell surface protein of 60,000 Dalton molecular weight (CSP-60) on confluent vascular endothelial cells that was not present on the same cells at subconfluent densities. However there is no evidence that CSP-60 has an active role in growth control.

In anchorage dependant cells, cell to cell contact inhibits or reverses cell movement (Albrecht-Buehler 1977). However it seems unlikely that cell contact

alone is sufficient to inhibit growth. Work by Folkman & Moscona (1978) has shown that DNA synthesis is associated with cell shape in anchorage dependant cell growth. At sparse plating densities cells adhere and spread out along the growth substrate in a flattened and often elongated manner. These cells are active in DNA synthesis. By changing the growth substrate so that sparsely plated cells retain a more rounded spheriodal shape, DNA synthesis was shown to be inhibited. As cell density increases, as in a confluent cell monolayer, individual cells become rounded, spheroidal and 'less flattened'. In confluent monolayers DNA synthesis and hence cell replication ceases. It is therefore postulated that cell shape influences DNA synthesis and therefore has a role in controlling replication and growth.

This hypothesis may explain the pattern of cell replication in a regenerating wounded monolayer, shown by Schwartz et al (1978), where DNA synthesis was not confined to the cells at the immediate wound edge but also present in cells away from the wound edge. The cells at the wound edge, spread out to cover the denuded area, taking 'the pressure off' the cells further back, which spread out to maintain monolayer integrity. The resulting shape change initiates DNA synthesis.

1.2.2. Gap junctions and growth control.

Communication between cells in the vascular wall occurs via intercellular gap junctions, tight junctions and desmosomes. Thus maintaining cell homeostasis by transport of nutrients, growth factors / inhibitors and electrolytes, between cells and the surrounding medium (Larson & Sheridan 1985, Huttner et al 1973, Lowenstein et al 1981). Regenerating and proliferating cells do not have gap junctional structures, which only appear when regrowth is complete and the monolayer intact (Schwartz et al 1975, Lowenstein 1979, Bruzzoni & Meda 1988). For this reason gap junctions and the passage of growth controlling molecules may have a role in contact inhibition.

Cultured endothelium shows fewer gap junctions than in vivo preparations. Gap junctions also exist between endothelium and the intimal smooth muscle cells, supporting other evidence of the role of endothelium in the control of smooth muscle proliferation . In vitro, numbers of gap junctions in the endothelium decreases with increasing number of subcultures (Davies et al 1985, Joris & Majno 1977). If this is also true of aging endothelium in vivo it may support the role of gap junctional communication in the control of intimal smooth muscle cell proliferation by the endothelium and the development of atherosclerosis

as part of the ageing process.

Gap junctional communication and intercellular passage of molecules between endothelial cells and adhering leukocytes has also been reported (Guinan et al 1988).

1.2.3. Growth factors.

There is considerable evidence that endothelial cell growth is controlled by extrinsic polypeptide growth factors. These growth factors may play important roles in the body's response to injury, by promoting wound healing such as the action of epidermal growth factor (EGF) (Brown et al 1986) and also in angiogenesis and neovascularisation (Folkman & Klagsbrun 1987).

Many peptide growth factors have been identified and characterised and would appear to be multifunctional and interactive with each other (Sporn & Roberts 1988). Among the first angiogenic growth factors to be identified, and the most important to endothelial growth are a group of heparin binding polypeptides. Fibroblast growth factor (FGF) was first isolated from brain (Gospodarowizc et al 1978a). It exists in two forms, acidic and basic FGF. Endothelial cell growth factor (ECGF) was first isolated from hypothalamus (Maciag et al 1979). Two forms of ECGF, a

and b, have been isolated and are thought to be members of the acidic FGF family (Burgess et al 1985). The basic FGF family share many biochemical features with the ECGF/acidic FGF family and it is likely that the various FGF molecules are multiple forms of acidic or basic FGF. Whether this is physiologically significant or just an artifact of purification is not clear.

Heparin - binding growth factors mediate biological activity through a high affinity polypeptide receptor on the endothelial cell surface. It would appear that distinct receptors for acidic and for basic FGF forms exist (Friesal et al 1986, Huang & Huang 1986). The FGF/ECGF molecule is rapidly translocated to the nucleus where it stimulates the transcription of ribosomal genes (Bouche et al 1987).

The role of heparin in the mechanism of ECGF/FGF receptor binding is unclear, although it is known to increase the binding of ECGF to endothelial cells and it also protects FGF from inactivation (Schreiber et al 1985, Gospodarowicz & Cheng 1986).

Both types of FGF/ECGF stimulate endothelial cell proliferation in vitro and in vivo. In vitro, FGF is reported to be necessary for the clonal growth of endothelial cells and for prolonging the replicative life span of bovine aortic endothelial cells (Gospodarowicz et al 1976). However, bovine cells can

be grown from clonal densities and for a large number of replications without addition of FGF (Levine & Mueller 1979, Chapter 2). Human endothelial cells in vitro do seem to have a greater requirement for ECGF/FGF at sparse densities (Johnson et al 1979, Gospodarowicz et al 1978b). It has been observed in vivo, that regrowth of endothelial cells after widespread loss stops even though reendothelialisation is not complete. Cell senescence and inhibition of endothelial replication by underlying smooth muscle cells did not seem to be responsible (Reidy et al 1983, Reidy 1988). Growth of these quiescent cells could be stimulated by bFGF (Lindner et al 1990).

Almost all normal tissues produce heparin- binding growth factors, however, endothelial turnover in vitro is extremely low. This raises questions as to the role of these growth factors in vitro. Does contact inhibition and the influence of cell shape at confluence have a role in 'switching off' growth factor receptor mediated action ? Or, are growth factors sequestered within the cells of origin and do not have access to endothelial cells under normal conditions ? After damage to the cells or tissues FGF/ECGF may be released to play a major role in tissue repair. However, much is still to be learned about the exact mechanisms of growth factor action.

A number of other angiogenic growth factors have been identified in recent years which may have more relevance to the vascularisation process in tumours than in vascular repair. Angiogenin was first isolated from the conditioned medium of a human adenocarcinoma cell line and is a potent stimulator of angiogenesis (Fett et al 1975). Its cell specificity is unknown but it is not a mitogen for vascular endothelial cells.

Transforming growth factors (TGF) were originally isolated from viral - transformed rodent cells and are known to alter phenotypes of some normal cells to transformed cells (De Larco & Todaro 1980). TGF-a also stimulates microvascular endothelial cell proliferation. TGF-b on the other hand inhibits proliferation of vascular endothelial cells in vitro but seems to have some sort of stimulatory effect on neovascularisation in vivo, either directly or through mediation by another growth factor (Schreiber et al 1986).

Endothelial cells have the ability to condition their own medium, which can support the growth of other endothelial cells and fibroblasts at sparse densities in the absence of other growth factors (Greenburg et al 1980, Gajdusek et al 1980). This endothelial derived growth factor (EDGF) has not been fully characterised. It has also been reported to stimulate the growth of

monoclonal antibody producing hybridoma cells at clonal density. The existence of such a growth promoting agent supports the importance of intrinsic factors in the control of growth.

A growth factor derived from platelets (PDGF) has produced some conflicting results. PDGF levels in plasma are low, but should be increased locally at sites of endothelial injury due to platelet activation (Goldberg et al 1980). D'Amore and Shepro (1977) found that bovine aortic endothelium showed a higher rate of proliferation in the presence of PDGF and whole platelets. However, other studies have shown that both human and bovine endothelium grow readily in the absence of PDGF and other platelet factors (Schwartz et al 1979, Davies & Ross 1978, Gadjusek & Schwartz 1983, Jarrell et al 1984). This is also true for cells at the wound edge.

The role of growth factors in general cell growth control is unclear. Studies on the role of these agents have all been done in vitro, which may reflect the behaviour of cells under culture conditions rather than true responses in vivo.

1.3. LIFE SPAN AND CELL SENESCENCE.

In culture, endothelial cells have been shown to have a finite life span with cellular senescence

occurring after repeated subculture (Jaffe et al 1973, Schwartz 1978, Gajdusek & Schwartz 1983, Jarrell et al 1984, Gimbrone et al 1974). This finite life span appears to be a pre-programmed event that is dependant on the number of cell divisions rather than chronologic time. Studies by Levine et al (1984) have shown that the exact point in terms of population doublings , at which senescence occurred, in cloned bovine endothelial cells, varied from clone to clone but was reproducible for each clone.

The loss of proliferative capacity of serially cultivated endothelium is accompanied by other cellular changes. Giant multinuclear cells are a common feature of senescing cultures. These cells have a protein content three to four times greater than that of normal cells, with an increased number of vacuoles, multivesicular bodies, lipid droplets and Golgi complexes (Rosen et al 1981). Senescent cells show no Weibel-Palade bodies (Haudenschild et al 1975). Chromosomal abnormalities are also observed with the loss of proliferative capacity in models of cellular senescence (Miller et al 1975). Synthetic and metabolic capabilities are also affected. The decrease and eventual loss of production of angiotensin converting enzyme in cells of increasing number of subcultures is well documented (Levine et al 1983, Auerbach 1984). The

loss of Weibel-Palade bodies in giant senescing cells may reflect a change in distribution and storage of vWF rather than loss of synthetic capabilities by these aging cells.

The finite life span of cultured endothelial cells is due to their low frequency of spontaneous transformation . Some bovine endothelial cell cultures with indefinite or greatly expanded life spans in the absence of growth factors have been reported but are thought to be the result of cell transformation (Schwartz et al 1980, Gaynor 1971, Caplan & Schwartz 1973, Schwartz & Benditt 1976). These studies have also shown focal areas of increased cell turnover which are frequently found near vessel branch points and may be due to hypertension (Schwartz & Benditt 1977), the effects of flow and shear stress (Caplan & Schwartz 1973) or hyperlipidemia (Bondjers et al 1977). The daily cell turnover rate of these areas has been reported to be as high as 10%. Such constant high cell turnover may exhaust the cells' life span, leaving areas of senescent cells and eventual denudation. Altered subendothelium affecting the replacement of such areas by adjacent 'normal' cells must be considered. The implications of this phenomenon in age-related disease process is discussed below.

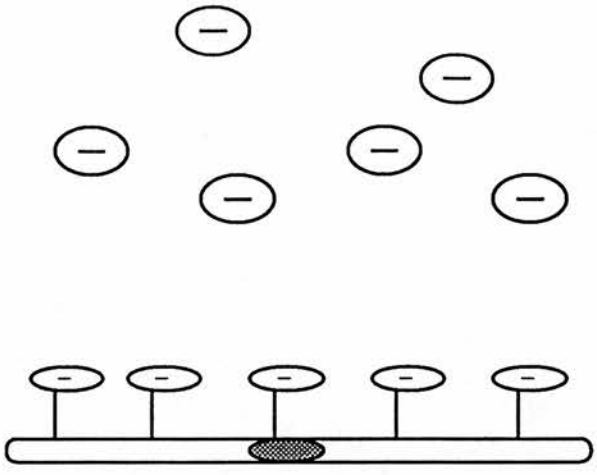
1.4. ENDOTHELIAL REGENERATION.

The initial phase of re-endothelialisation after a denuding injury is by movement of the adjacent cells into the wound, followed by cell replication. This movement is unidirectional and highly coordinated, requiring the presence of nucleotides (Haudenschild & Harris-Hooker 1984, Gotlieb et al 1981, Gotlieb & Spector 1981). Static non-migrating cells have microtubules radiating from a perinuclear organising centre, in a random fashion. Migrating cells show alignment of microtubules parallel to the direction of movement with the microtubule organising centre in front of the nucleus facing the direction of movement (Gotlieb et al 1979, Gotlieb et al 1983). Small single cell wounds are closed very rapidly (within 30 to 40 minutes) by movement of cytoplasmic lamellopodia from surrounding cells into the denuded area (Wong & Gotlieb 1984). This does not seem to be followed by cell replication.

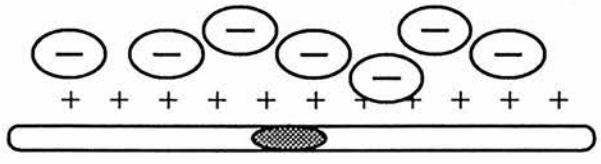
In larger denuding injuries, cell movement is followed by cell replication but the latter is not initiated for up to 20 hours after the initial wounding event (Schwartz et al 1978). Thymidine index values remain elevated for approximately 96 hours after wounding. In extensive denuding injuries, cell replication may stop before reendothelialisation is

complete. This is not due to contact inhibition, cell senescence or inhibition by smooth muscle cells (Reidy et al 1983, Reidy 1988). Regrowth could be stimulated by the addition of bFGF (Lindner et al 1990).

Wound regeneration in vivo has two major differences from that in vitro. First, cells in vivo regenerate faster in a direction parallel to blood flow. Second, unlike wounding in vitro where response is confined to cells proximal to the wound edge, a zone of regeneration extending approximately 100 cells back from the line of injury occurs in vivo. In this zone cells show an increased proliferation, in excess of that required for simple cell replacement. Such areas of wounding in vivo can be recognised by endothelium of increased cell density which seems to persist for some time after injury (Schwartz et al 1978, Reidy & Schwartz 1981b). In non-denuding cell injuries such as that caused by bacterial endotoxin where cell death occurs slowly over a period of time, cell regeneration occurs before cell loss. Healthy cells migrate underneath the injured cells, possibly stimulated by breakdown in intercellular communication, pushing dead cells off the vessel wall once the re-endothelialisation process is complete (Reidy & Schwartz 1983).



NORMAL ENDOTHELIAL SURFACE WITH A NET NEGATIVE CHARGE
ELECTROSTATICALLY REPELS PLATELETS, ALSO WITH
A NET NEGATIVE CHARGE.



"ALTERED" ENDOTHELIAL SURFACE LOSES NEGATIVE CHARGE
CAUSING IT TO BECOME "STICKY" TO PLATELETS.

DIAG. 1.1 Non-thrombogenicity of endothelium in terms of electrostatic repulsion.

1.5. NON-THROMBOGENIC AND FIBRINOLYTIC ACTIVITIES OF ENDOTHELIUM.

1.5.1. Physical properties of intact endothelium.

The non-thrombogenic properties of endothelium was first recognised by Virchow (1856) and until fairly recently this function was attributed to physico-chemical properties of the luminal cell surface and the glycocalyx (Pelikan et al 1979, Skutelsky et al 1975). Both the endothelial and platelet surface membranes have a high sialoglycoprotein content, which gives the cells a net negative charge. It has been suggested that the forces of electrical repulsion prevent platelet aggregation on the endothelial surface (Born & Palinski 1984)(Diag.1.1). However, this process alone seems highly unlikely in preventing the initiating stages of haemostasis which is a highly complex and coordinated process.

1.5.2. Prostacyclin production by endothelial cells.

A more active role of the endothelium in controlling haemostatic / thrombotic balance was considered with the discovery that endothelial cells synthesize prostacyclin (PGI_2)(Weksler et al 1977, Moncada et al 1977, MacIntyre et al 1978). PGI_2 is a potent inhibitor of platelet aggregation as well as being a powerful vasodilator .

PGI_2 works by stimulation of adenylate cyclase which raises intracellular levels of cAMP which in turn limits the active transport of calcium ions into the the cell cytoplasm. This inhibits calcium - dependant phospholipase activity (Gorman et al 1977, Moncada 1983). Physiologically it inactivates platelet aggregation by inhibiting shape change, platelet factor 3 activity and blocking the formation of membrane receptors for fibrinogen and vWF (Moncada et al 1976, Ehrman & Jaffe 1980, Fujimoto et al 1982).

High concentrations of PGI_2 also inhibit platelet adherence to subendothelial matrix (fibrinogen and vWF being major components of this matrix)(Weiss & Turitto 1979).

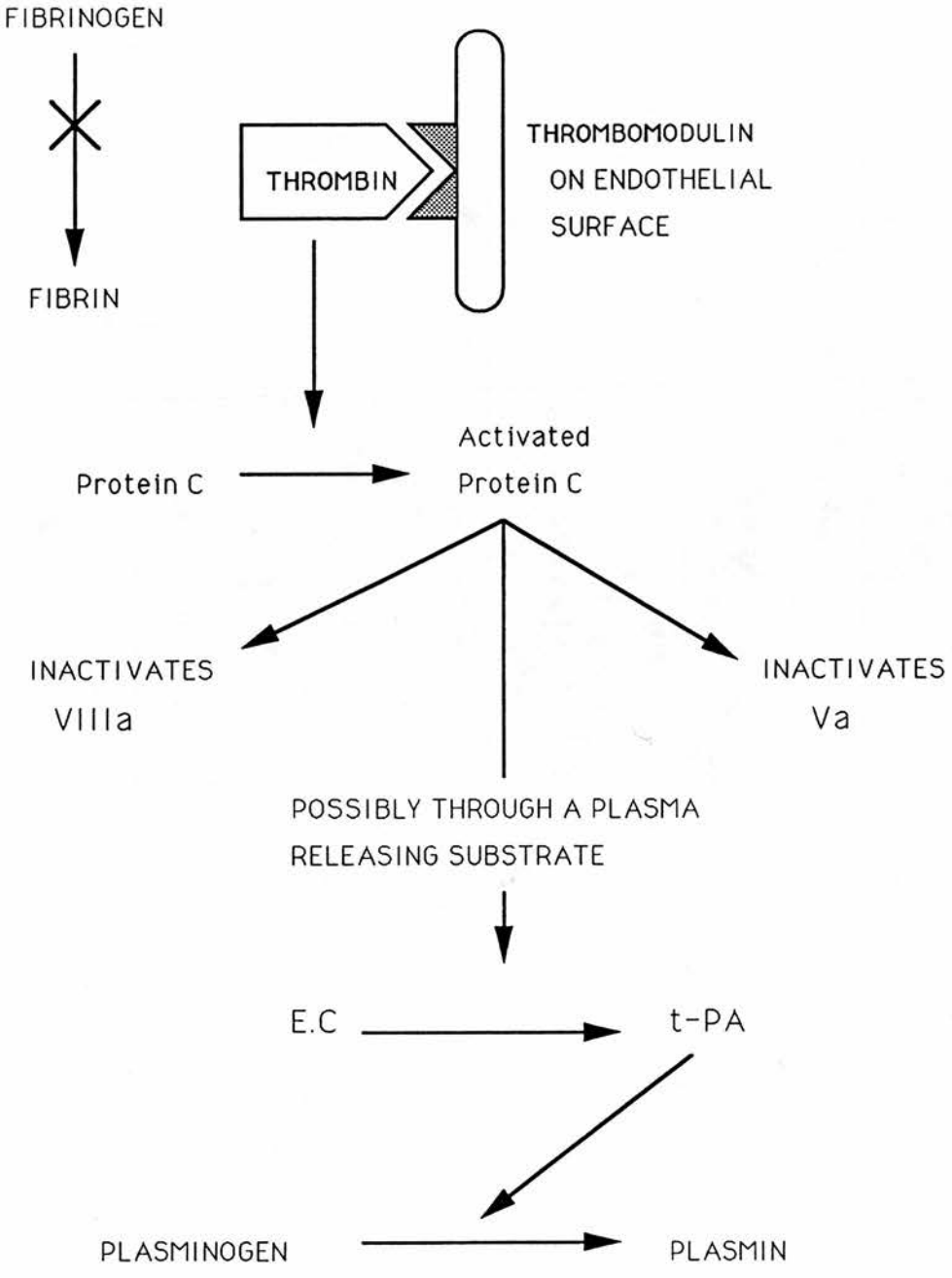
PGI_2 does not inhibit its own synthesis since endothelial cells possess a very active cAMP phosphodiesterase which maintains a low cAMP level during PGI_2 synthesis and in the presence of exogenous PGI_2. It has also been found that high cAMP does not depress endothelial synthesis of PGI_2 (Hopkins & Gorman 1981, Brotherton & Hoak 1982).

Age and sex hormones both affect the production of PGI_2 by the endothelium. Male rats produce more PGI_2 than females (Pomerantz et al 1980). When female rabbits were given ethynyl estradiol, PGI_2 production dropped and conversely cultured female porcine

endothelial cells increased PGI_2 synthesis when given testosterone (Elam et al 1980, Seillan et al 1983).

The state of growth of endothelial cells in culture also affects synthesis. Human umbilical vein endothelium produce more when pre-confluent than when fully confluent. Bovine cells produce more the longer they are maintained at confluence and can be subcultured numerous times before PGI_2 synthesis is diminished. In contrast, adult human and porcine endothelial cells in culture lose their capacity to produce PGI_2 during routine subculture (Eldor et al 1983, Weksler et al 1977). These effects may reflect the consequences of tissue culture conditions rather than the intrinsic differences in PGI_2 synthesis. Extrapolation of these results from in vitro studies to in vivo behaviour in terms of PGI_2 production must be dealt with carefully.

Numerous substances such as ATP, bradykinin and histamine stimulate the production of PGI_2 (Pearson et al 1983, Hong 1980, Baezinger et al 1981). Thrombin causes a rapid but transient increase in human and porcine cells but not in bovine cells (Czervionke et al 1979). Cell injury such as that caused by hypoxia and hypotonicity induces a rapid release of PGI_2 followed by a marked decrease in capacity to synthesize it (Needleman et al 1978).



DIAG.1.2. Activation of protein C and initiation of fibrinolysis by binding of thrombin to thrombomodulin.

The physiological role of PGI_2 remains uncertain since there is very little in the normal 'resting' circulation and inhibition does not cause catastrophic thrombosis. It seems more likely that PGI_2 functions as a part of the defence mechanisms of the endothelium against local injury.

1.5.3. Thrombomodulin and activated protein C.

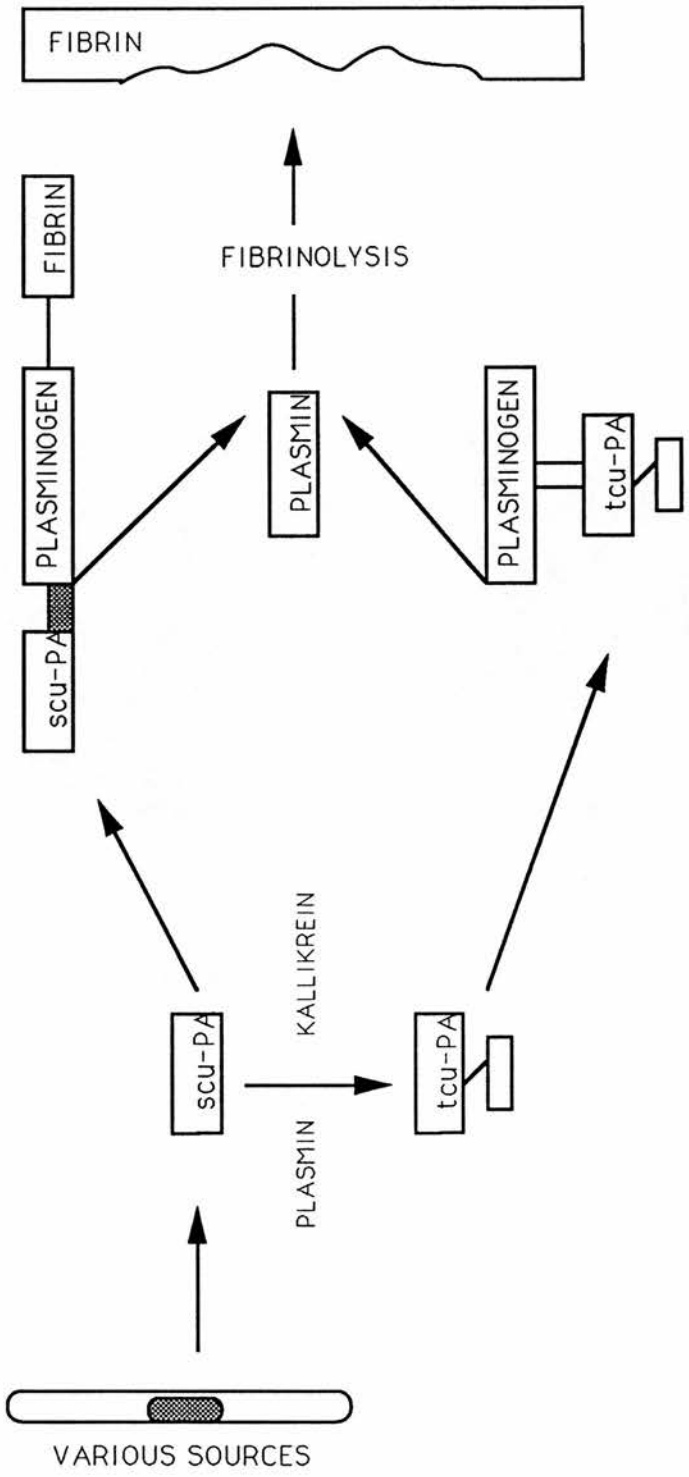
Studies with cells sub-lethally damaged by irradiation, which causes a decrease in PGI_2 production, show that although no PGI_2 is produced the cells still maintain non-thrombogenic properties (Eldor et al 1983b). This is due to a number of other properties of the endothelium in maintaining the balance between anti-coagulation and pro-coagulation.

Endothelial cells have a unique membrane protein, thrombomodulin, which binds thrombin reversibly and with high affinity. This inactivates the enzyme's pro-coagulant activity and at the same time thrombin acquires the capacity to activate protein C (Esmon et al 1982a, Bauer et al 1983, Esmon & Owen 1981, Esmon et al 1982b). Activated protein C in turn inactivates factor VIIIa and factor Va and may also mediate the release of tissue plasminogen activator (Comp et al 1982)(Diag.1.2).

1.5.4. Fibrinolysis.

Fibrin formation is a central feature of haemostasis and tissue repair with the endothelium playing a major role in the control of fibrin formation and dissolution. Injury to the vessel wall and disruption of the endothelial continuity results in the formation of a fibrin / platelet plug. This process is the result of a series of complex cascade events initiated by exposure of the basement membrane. Fibrin deposition on intact endothelial monolayers results in cell contraction, disorganisation of the monolayer and exposure of basement membrane thereby promoting further fibrin formation (Schleef & Birdwell 1984, Weimar & Delvos 1986). The mechanisms of fibrin induced monolayer disruption are however poorly understood. The fibrinolytic activity of normal endothelium may therefore represent a self protecting mechanism by preventing fibrin formation beyond the area of injury.

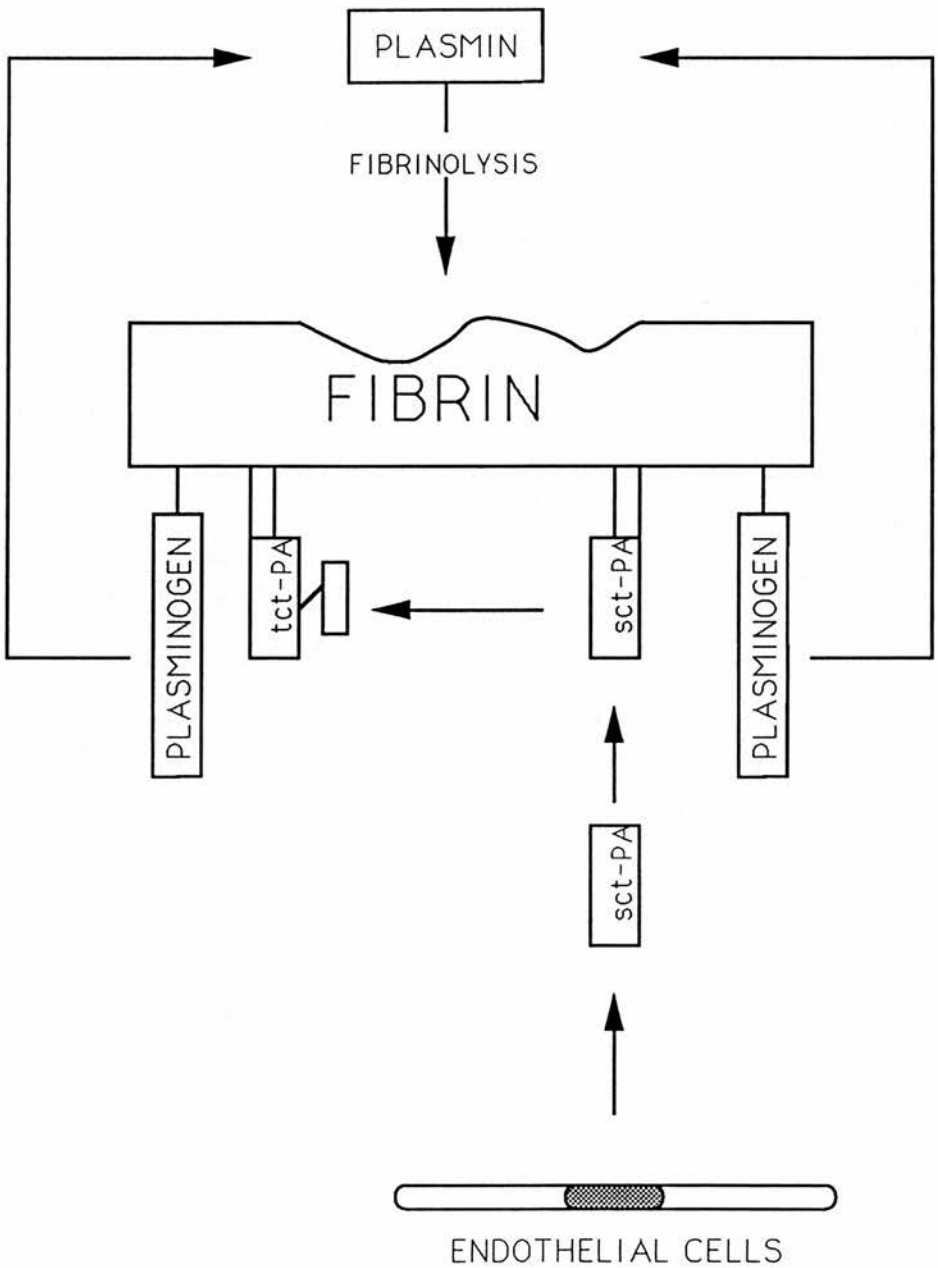
The primary fibrinolytic enzyme is plasmin, which is formed by cleavage of its inactive circulating precursor plasminogen by the action of plasminogen activators (PA's). Two immunologically distinct molecules with similar modes of action have been characterised. These activators cleave the plasminogen molecule at the same arginine-valine bond to produce plasmin.



DIAG.1.3 Fibrinolytic pathway of u-PA

Urokinase or urinary type plasminogen activator (u-PA) was originally found in the urine (Stump et al 1986a, Husain et al 1983). It exists in two enzymatically active forms, single chain u-PA (scu-PA) or pro-urokinase (PRO-UK) and two chain u-PA (tcu-PA) or high molecular weight urokinase (HMW-UK). Scu-PA is produced by various cells in culture including endothelium (Stump et al 1986b, Loskutoff 1986). Human plasma concentrations have not been clearly determined. Scu-PA has fibrin specific plasminogen activator activity but does not actually bind to the fibrin molecule (Gurewich & Pannell 1987). Without fibrin,scu-PA has no plasminogen activator activity. This would suggest a conformational change in plasminogen when bound to fibrin that makes it receptive to scu-PA activation.

In the presence of plasmin and kallikrein scu-PA is converted to tcu-PA . Cleavage of the scu-PA molecule occurs at the Phe-Lys bond at position 158, resulting in a two chain molecule held together by disulphide bonds (Ichinose et al 1986). Tcu-PA activity does not require the presence of fibrin which suggests a role in the activation of free circulating plasminogen (Diag.1.3). This is substantiated by studies which have shown scu-PA to have 3 to 4 times greater thrombolytic activity than tcu-PA, and that



DIAG.1.4. Fibrinolytic pathway of t-PA

tcu-PA causes systemic fibrinogen depletion (Zamarron et al 1984, Stump et al 1987).

Plasminogen activator inhibitors, also produced by the endothelium, bind to circulating u-PA. PAI-1 and PAI-2 react with tcu-PA but not with scu-PA (Verheijen et al 1984, Kruithof et al 1986). Thrombin can cleave the scu-PA molecule resulting in inactivation (Ichinose 1986).

Tissue type plasminogen activator (t-PA) is predominantly produced by endothelial cells (Shepro et al 1980, Levin & Loskutoff 1979, Laug 1981, Levin & Loskutoff 1982b). Like u-PA, t-PA cleaves plasminogen at the same arginine - valine bond. However, t-PA activity requires binding of the molecule to fibrin (Hoylaerts et al 1982), whereas u-PA acts on fibrin bound plasminogen without binding to fibrin itself. (Diag.1.4).

T-PA exists in two variants, single chain t-PA (sct-PA) and a proteolytically degraded two-chain form (tct-PA). These molecules differ in molecular weight and carbohydrate content (Rijken et al 1985). In the presence of small amounts of plasmin and tissue kallikrein, sct-PA is converted to tct-PA. Some functional differences exist between sct-PA and tct-PA but the physiological significance of these two variants is unclear (Tate et al 1987, Rijken et al

1985, Ranby & Wallen 1985). In the absence of fibrin both forms of t-PA are poor activators of plasminogen, sct-PA more so than tct-PA. Turnover rates of plasminogen activation at physiological concentration increase 200 to 400 times in the presence of fibrin (Nieuwenhuizen et al 1983). Sct-PA has a greater affinity for fibrin, but when bound to fibrin both forms activate plasminogen equally well. The conversion of the sct-PA form to the two chain form occurs on the fibrin surface which would suggest that physiological fibrinolysis induced by sct-PA occurs mainly via the two chain derivative but this conversion does not appear to play a significant role in the regulation of fibrinolysis (Rijken et al 1982). Although local concentrations of t-PA can be high, the circulating levels in human plasma are very low with most of it being complexed with PAI-1. This process appears to occurs at the endothelial cell surface, with t-PA covalently binding to membrane associated PAI-1. In vitro, the resulting complex dissociates from the cell surface (Barnathan et al 1988, Russell et al 1990). Both sct-PA and tct-PA are inactivated equally well by PAI-1, but PAI-2 inactivates tct-PA 100 times more efficiently than sct-PA (Astedt et al 1985, Verheijen et al 1984).

In cultured endothelial cells the synthesis and

secretion of plasminogen activators is influenced by the cell source, growth rate, state of nutrition and a variety of external stimuli (Levin & Loskutoff 1982a, Levin & Loskutoff 1980). Bovine endothelial cells produce both u-PA and t-PA in culture (Levin & Loskutoff 1982b). In log phase growth these cells show a low rate of synthesis and release of t-PAs. On reaching confluence and being maintained at confluence, u-PA production is increased 20 to 30 times. Cultured human endothelial cells synthesise more t-PA than bovine cells (Levin & Loskutoff 1979).

Recent work has shown that cultured human endothelial cells exhibit discrete binding sites for both t-PA and u-PA (Hajjar & Hamel 1990, Barnathan et al 1990). These binding sites are surface membrane associated proteins of 40,000 and 48,000 KDa molecular weights respectively (Hajjar & Hamel 1990). The presence of these membrane binding sites may play a role in the localisation of plasmin formation at the vessel wall surface and maintain the non-thrombogenic properties of the intact vessel.

1.5.5. Interaction of the endothelium with leukocytes.

Endothelial cell-leukocyte interactions play a major role in the inflammatory and immune responses. Vascular cell adhesion molecules are known to be

important modulators of endothelial cell-leukocyte interactions. A number of endothelial cell adhesion molecules have been identified and characterised. Cytokines such as interleukin-1 (IL-1) and tumour necrosis factor-alpha (TNF-a) rapidly induce adhesion molecule expression (Cotran et al 1988). In situ adhesion molecule expression can be demonstrated, immunohistochemically at sites of endothelial activation (Cotran et al 1986). ELAM-1 (endothelial-leukocyte adhesion molecule-1) appears to be absent in normal endothelium, but can be detected in post-capillary venule endothelial cells (high endothelium) in delayed hypersensitivity reactions and after immunotherapy with IL-1. ELAM-1 may have a role in the initial phase of transendothelial neutrophil and lymphocyte migration (Bevilacqua et al 1989).

ICAM-1 (intercellular adhesion molecule-1) is found on a number of cells including lymphocytes and endothelial cells (Simmons et al 1988). ICAM-1 is present on normal endothelium and expression is upregulated following cytokine treatment (Munro et al 1988).

PECAM-1 (platelet -endothelial cell adhesion molecule) is an integral membrane protein that is widely distributed in human endothelial cells and is also cytokine inducible. This protein is predominant at

intercellular junctions (Muller et al 1989).

GMP-140 is a member of the same family of adhesion molecules as ELAM-1. It mediates the adhesion of neutrophils and monocytes, and is stored in the alpha-granules of platelets and the Weibel-Palade bodies of endothelial cells. It is mobilised to the cell surface after stimulation by thrombin and other products of the coagulation cascade (Johnston et al 1989, Larsen et al 1989).

1.6. ENDOTHELIAL INJURY AND THE RESPONSE OF THE BLOOD / VESSEL WALL.

1.6.1. Injury.

The endothelium, because of its unique position at the interface between the blood and the tissues, is subjected to a variety of foreign and endogenous substances that may prove injurious. Ultrastructurally, cell injury appears to have two phases, the early and possibly reversible phase is characterised by breakdown of intercellular junctions and loss of cell to cell communication, and dilatation of cytoplasmic vacuoles and vesicles. The late and probably irreversible phase, shows breakdown of mitochondria, destruction of plasma and intracellular membranes, accumulation of calcium (Trump & Arstila 1975, Farber et al 1981). Dead cellular material is removed from the system by

macrophages (Wyllie et al 1980). Whether Ca^{2+} influx is a result of loss of mitochondrial function and plasma membrane channels and has an active part in cell death, or whether it accumulates, coincidentally as a result of increased permeability of the plasma membrane, is debatable (Jennings et al 1975, Farber et al 1981, Smith et al 1981, Hansson & Schwartz 1988).

1.6.2. Assays of cell injury.

A number of methods for assaying cell death both in vitro and in vivo have been developed. The most commonly used methods involve the principle of anionic dye exclusion by intact plasma membrane. On cell death or damage to the plasma membrane dyes such as trypan blue, Evans blue and lissamine green gain access to intracellular proteins to which they bind (Holmberg 1961). In effect these dyes are indicators of plasma membrane injury.

Deposition of intracellular calcium is a characteristic of cell death. The antibiotic chlorotetracycline binds to intracellular calcium deposits and can be detected by fluorescence (Hansson & Schwartz 1983). Similarly the accumulation of IgG has been shown to occur in damaged cells. Damaged plasma membranes allow the entry of IgG molecules into the cell where they bind to the vimentin component of

intermediate filaments. This occurs via the Fc component of the Ig molecule (Hansson et al 1979, Hansson et al 1984).

An alternative approach to detecting cell damage is to look for cytoplasmic materials which leak into the extracellular surroundings. A commonly used cell damage marker is the cytoplasmic enzyme lactate dehydrogenase (Morgan et al 1978). Such a method may be useful in looking at large scale endothelial damage, but cannot be used for localisation of small focal areas of injury.

Various metabolic labels can be used to localise damage with varying degrees of sensitivity. Fluoroscein diacetate and acridine orange / ethidium bromide are compounds that are metabolised by viable cells to give a green fluorescence (Rotman & Papermeister 1966). Adenine uptake gives an indication of nucleotide production and hence the state of metabolism and viability (de Bono et al 1977). Similarly ^{86}Rb efflux can be employed as a fairly sensitive measure of potassium efflux, an indicator of cell viability (Ager et al 1984).

1.6.3. Types of endothelial injury.

Injury can be classified at two levels. That which results in the loss of endothelial integrity, exposing

the underlying subendothelium, and that which causes endothelial dysfunction but without cell loss. Most studies of denuding endothelial injury come from in vivo and in vitro models of deliberate physical damage (Schwartz et al 1975, Clowes & Karnovsky 1977). This type of damage may have some relevance to the sort of pathological damage that occurs to the endothelium after accidental trauma, during routine surgery and also during some routine invasive diagnostic and therapeutic techniques such as cardiac catheterisation and balloon angioplasty.

Increased endothelial cell turnover has been postulated as having a role in the initiation of denuding injuries. Continuous high endothelial cell turnover depletes the cells replicative life-span leading to senescence and cell death . The reason for observed focal areas of increased endothelial cell turnover is unclear, but disturbances in flow, hypertension, high shear stress and homocystinuria have all been implicated (Davies 1988, Caplan & Schwartz 1973, Starkebaum & Harlan 1986). There is no evidence of spontaneous denudation although it is known that endothelial cells undergo spontaneous loss and replacement (Coutard et al 1986). Cell injury, death and increased endothelial cell turnover does not necessarily imply denudation. Studies

by Reidy and Schwartz (1983) have shown that endothelial regeneration can be rapid and in cases where cell death occurs over a prolonged period of injury, as with endotoxin damage, dead cells are replaced before denudation occurs.

Agents injurious to the endothelium are numerous. Some such as bacterial endotoxins, viruses and cytotoxic sera are foreign to the body, while others are endogenous components which are 'activated' or produced as a result of pathological or disease processes.

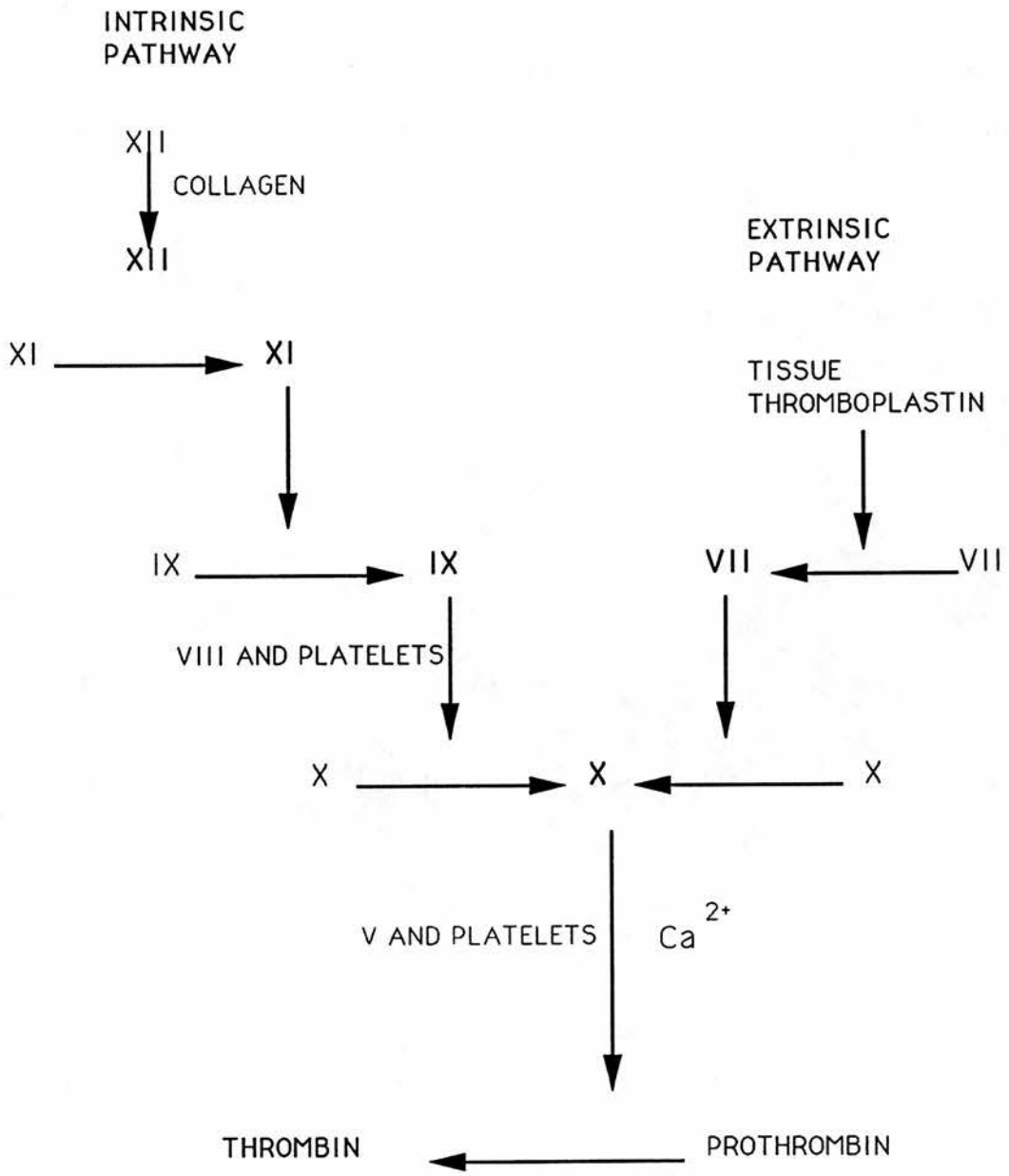
The effects of endotoxin on endothelium is well documented. At sublethal doses it is known to induce the production of tissue factor, a cofactor for the initiation of coagulation by endothelial cells (Galdal 1984, Schorer et al 1985). It has been implicated in the pathogenesis of disseminated intravascular coagulation in man and animals (Bradley 1979), and it promotes neutrophil adhesion (Schleiner & Rutledge 1986).

Viruses have been found in vascular cells associated with atherosclerotic plaques in both humans and animals (Minick et al 1979, Fabricant et al 1978, Cunningham 1988). Whether they have an active role in the initiation of atherosclerosis or are simply opportunistic pathogens is not yet clear. Viral

infections of endothelial cells have been reported to elevate the biosynthetic levels of interferon (Einhorn et al 1985). Interferon in turn enhances the susceptibility of infected cells to cytotoxic T cells (Bukowski & Welsh 1985, Yu et al 1985). Interferon and tumour necrosis factor (TNF) increases endothelial permeability by structural changes to the monolayer (Stolpen et al 1986). TNF and interleukin-1 promote pro-coagulation activity of endothelial cells (Pober et al 1986, Bevilacqua et al 1986).

Cytotoxic antibodies which are induced after transplantation are also reported to increase pro-coagulant activity of endothelium and similarly in immune - mediated thrombocytopenia (de Bono 1977, Cines et al 1987). A similar effect of cigarette smoke through the inhibition of PGI_2 production has also been observed (Reinders et al 1986). Nicotine has been reported to increase endothelial cell turnover (Zimmerman & McGeachie 1985).

Neutrophil activation during the inflammatory response produces superoxide anions, hydroxyl radicals and hydrogen peroxide which have all been implicated in endothelial injury (Varami et al 1985, Stroncek et al 1986). The reaction of xanthine with xanthine oxidase to produce hydrogen peroxide has been used as a model for granulocyte activation and endothelial injury (Ager



DIAG.1.5. Intrinsic and Extrinsic pathways of thrombin formation. Bold characters indicate active components.

et al 1984, Ager & Gordon 1984). Hydrogen peroxide is also thought to be the injurious agent in homocystinuria (Starkebaum & Harlan 1986). Oxygen free radicals are thought to cause vascular damage by causing increased cell permeability, cellular contraction which leads to disrupted monolayer morphology and eventual cell loss.

1.6.4. Thrombosis as a response to endothelial injury.

Injury to vascular endothelium or loss of endothelial integrity initiates the haemostatic process. The exposure of the subendothelial collagen, vWF and fibrinectin initiates platelet adhesion and activation which in turn initiates the production of thrombin which activates fibrin polymerisation. The formation of thrombin from its inactive precursor is initiated by a complex cascade system. (Diag.1.5). Many of the reactions of the intrinsic pathway occur at the endothelial cell surface (factors XII, IX, X are activated at the cell surface, and factor V is synthesised by endothelial cells)(Anjanayate et al 1986). Tissue thromboplastin, the initiator in the extrinsic pathway is an endothelial cell surface protein (Maynard et al 1977).

The pro-coagulant and anti-coagulant capacities of the endothelium are normally kept in haemeostatic

balance by a number of other endothelial cell derived modulators. Thrombin is rapidly inactivated by antithrombin III which is catalysed by cell surface glycosaminoglycans (Busch & Owen 1982). It can also be inactivated by binding to thrombomodulin, an endothelial membrane protein. This process activates protein C which promotes fibrinolysis by plasmin formation through factors Va and VIIIa (Diag.1.2). Thrombin itself is reported to have a damaging effect on endothelial cells by causing an increase in permeability and cell contractility (Lough & Moore 1975, Killacky 1986, Galdal et al 1982).

Disturbance of the haemostatic balance between these two processes under pathological conditions may lead to thrombosis.

1.6.5. Atherosclerosis.

The normal artery wall consists of three distinct layers, intima , media and adventitia. The intima which is the lining of endothelial cells bound by a sheet of elastic fibres (internal elastic lamina), with the occasional smooth muscle cell (SMC) between the two. The media consists of SMC surrounded by collagen, proteoglycans and some small elastic fibres. The adventitia is separated from the media by the external

elastic lamina and is made up of a loose arrangement of fibroblasts, SMC, collagen and proteoglycan .

Two types of lesion are characteristic of atherosclerotic disease, the fatty streak and the atherosclerotic plaque. The fatty streak is a focal accumulation of lipid bearing cells (foam cells) in the intima of the vessel wall. Microscopically and immunologically these foam cells are thought to be of monocyte / macrophage origin (Schaffner et al 1980, Gerrity 1981). These lesions cause little or no obstruction and appear to have no associated clinical symptoms.

Atherosclerotic plaques are structurally more complex lesions. Their principal characteristic is the proliferation and accumulation of lipid laden SMC in the subendothelial intimal zone. Advanced lesions have a fibrous cap of SMC and dense connective tissue consisting of elastin, collagen, proteoglycans and basement membrane. The core is often a necrotic mixture of cellular debris, extracellular matrix, foam cells, cholesterol crystals and calcium deposits (Cotran & Munro 1988). Atherosclerotic plaques protrude into the vessel lumen causing decreased and disturbed blood flow and sometimes total occlusion. They are complicated by thrombosis, calcification and rupture. SMC accumulation and proliferation is the central feature of

atherosclerotic lesions and it is now generally accepted that the initiating factor in this event is endothelial cell injury (Ross & Glomset 1976) Endothelial cells are in direct communication with SMC and exert some control over their growth. It is thought that endothelium produces SMC growth inhibitors (Castellot 1980). In the event of endothelial cell loss this inhibitory function is also lost and adhering platelets release growth factors which stimulate SMC migration and proliferation . Endothelial denudation stimulated SMC proliferation is a self limiting process, with cessation of SMC proliferation with endothelial regeneration. It seems unlikely that this sort of injury alone is responsible for the formation of atherosclerotic lesions. Evidence exists to indicate that endothelial denudation is actually a late event in atherosclerosis (Davies et al 1988).

Non-denuding endothelial injury, dysfunction or altered behaviour is initiated in a number of ways. Hypertension, hypercholesterolemia / lipidemia, lymphokine activation, viral transformation, disturbed blood flow and inflammatory reaction products, all promote increased endothelial adhesiveness to both platelets and circulating macrophage / monocytes. This probably leads to the migration of lipid filled macrophages into the subendothelium leading to fatty

streak formation. The relationship between fatty streaks and the development of fibrous plaques is unclear. Fatty streak development seems to precede atherosclerotic plaque formation in the vessels and their distribution in the vasculature is similar. Therefore it has been suggested that they are precursors of the advanced atherosclerotic lesion (Ross & Glomset 1976). More recent evidence has supported this theory by showing platelet adherence to endothelium at sites of fatty streaks and the subsequent SMC proliferation (Faggiotto et al 1984, Schwartz et al 1986). THe intrusion of foam cells underneath the endothelium does not disrupt endothelial integrity but raises the area of cells above the level of the surrounding normal endothelium. These raised cells are prone to the effects of disturbed blood flow and shear stress (Davies 1988). Therefore the initial reaction to focal cell damage (fatty streak formation) may in turn promote further cell damage leading to progression of atherosclerotic lesions.

1.7. AIMS OF THE PROJECT.

Thrombosis and atherosclerosis are the pathological manifestations of the breakdown in homeostatic interaction between the vessel wall and blood elements.

Elucidation of the cellular and molecular events of

vascular disease have provided a greater understanding of the development of atheroma and have shown it to be a complex multifactorial process. The role of endothelial injury in acute thrombotic events is well established and there is no lack of evidence in experimental models to show that changes in the endothelial cell are associated with atherogenesis. It is however not entirely clear if atherosclerotic lesions develop at sites of endothelial injury or if endothelial changes occur as a later event in well established atheroma.

Clinically the effects of thrombosis and atherosclerosis are most notable in coronary artery disease. Diagnostic and therapeutic procedures carried out in the management of coronary artery disease are not entirely free of drawbacks . Balloon angioplasty is frequently used to re-establish blood flow in occluded atheromatous coronary arteries. It is also used to induce endothelial damage in experimental models. In using the procedure therapeutically , the risk of local thrombosis due to endothelial injury is increased. It is common clinical practise to use systemic anticoagulants and thrombolytic agents to control local thrombosis. However the inability of these agents to distinguish between thrombi and haemostatic plugs increases the risk of systemic bleeding.

Monoclonal antibodies have been used extensively in endothelial cell research for identification (Schlingeman 1985, Drenk 1987) and in the elucidation of structure and function (Darnule et al 1983, Kaplan et al 1983, Arvieux et al 1986, Ketis et al 1986, Sage et al 1986, Favaloro et al 1990).

Monoclonal antibodies to structurally and functionally "altered" or damaged endothelial cells and to regenerating endothelium may prove useful in detection and elucidation of the early phases of vascular disease, to monitor endothelial regeneration after invasive diagnostic or therapeutic techniques and may provide a way of targetting coagulant or anti-coagulant agents to specific sites.

It was the aim of this project to 1. develop monoclonal antibodies specific to damaged and/or regenerating endothelial cells. 2. To characterise the antigens recognised by these antibodies. 3. To assess the suitability of these antibodies for diagnostic purposes. 4. To conjugate these antibodies with thrombolytic agents and target such activity specifically to areas of damage.

CHAPTER 2

GENERAL METHODS

2.1 ENDOTHELIAL CELL CULTURE

2.1.1. Introduction

In 1963 Maruyama described a method of isolating and culturing human endothelial cells from umbilical cord vein. This was an easily obtainable and abundant source of human material which could yield large numbers of cells. However, Maruyama and other workers at that time (Pollak & Kasai, 1964 ; Pomerat & Slick, 1963 ; Fryer et al, 1966.) were limited by a difficulty in growing and maintaining a heterogenous culture for more than a few weeks. The cells they were culturing were never positively identified as being endothelial.

Jaffe et al (1973) and Gimbrone (1974) extended the work of Maruyama and devised the method for isolating and culturing human umbilical endothelial cells which is widely used at present. They also established the morphologic and immunological criteria on which endothelial identification is based. However, it was found that human umbilical vein endothelial cells (HUVEC) could not be grown reproducibly for more than 2 to 3 passages (Gimbrone et al 1974 and Gimbrone 1976). Work by Gospodarowicz et al (1978) showed that vascular endothelial cells could be grown and maintained in culture long term, if the culture medium was

supplemented with high concentrations of bovine brain or pituitary fibroblast growth factor (FGF). Later studies showed that HUVEC do not respond mitogenically at low seeding densities to high concentrations of FGF but to crude preparations of bovine pituitary, brain and hypothalamus (Maciag et al 1979). This neural-derived endothelial cell growth factor (ECGF) is an acid- and heat-labile protein which is physically and chemically distinct from FGF (Maciag et al 1981). Addition of ECGF to low density cultures of HUVEC results in a significant increase in cell growth and is now routinely used in the growth mediums for long term serial propagation of human vascular endothelial cells.

This work set the standard for the development of methods of isolating and culturing of endothelium from a variety of animal sources. Schwartz (1978) provided the method of growing bovine aortic endothelium, which is very easy to maintain for long periods in culture and can be grown very quickly in large quantities without special growth factor requirements.

A very simple method for cloning bovine aortic endothelial cells developed by Gadjusek & Schwartz (1983) , allows growth of cell lines for much longer periods of time by eliminating contaminating and senescent cells. The method can also be adapted to human and porcine cells.

More recent research has shown an increase in techniques of isolating and growing microvessel endothelium from a variety of sources both human and animal (Goetz et al, 1983 ; Kern et al, 1983 ; Wagner & Matthews, 1975).

The techniques used in the following section were based on the methods of Jaffe (1973) , Schwartz (1978) and Gadjusek & Schwartz (1983).

2.1.2. Bovine Aortic Endothelial Cells.

Bovine vessels were obtained from the local abattoir at the time of slaughter. Segments of thoracic aorta approximately 30 cm in length were collected no more than 15 minutes after the death of the animal and processed immediately. Both ends of the vessel segments were tied off with sterile " Kwill" filling tubes inserted. Enzyme and washing mediums were added to the vessel lumen by way of these tubes. The aorta was then filled with cold Earle's balanced salts (EBS) containing 2 ug/ml gentamicin and placed in a cold box for transport back to the lab.

Fat and connective tissue were dissected from the aorta and the intercostal vessels were ligated. The lumen was then rinsed well with sterile saline and filled with 10 ml of 0.1% collagenase in EBS. The

vessel was wrapped in cling-film (to aid the transfer of heat around the vessel and prevent evaporative cooling) and incubated at 37^{o}C for 15 to 20 minutes. Gentle massaging of the vessel aided the release of loosened cells before the collagenase/cell solution was removed. The lumen was rinsed twice with Dulbecco's modification of Eagles medium (DMEM) supplemented with 2 ug/ml gentamicin and 20% newborn calf serum (NBCS) (DMEM/NBCS). The collagenase/cell suspension was centrifuged at 1000xg for 10 minutes and the cell pellet was rinsed once with DMEM/NBCS. The cells were finally resuspended in 20 ml of DMEM/NBCS and placed in a 75 cm^{2} glass "medical flat" culture bottle.

Collagenase treatment removed the cells from the vessel wall in large sheets which made an accurate cell count impossible, therefore the cells obtained from one such length of aorta were initially cultured in 20 ml of medium in a 75 cm^{2} bottle. If the cells were free from contamination and in general good condition they usually reached confluence after 48 hours.

2.1.3. Pig Aortic Endothelial Cells.

Pig cells were obtained by exactly the same method as described for the bovine aortic endothelium. The growth medium for pig cells was DMEM supplemented with 2 ug/ml of gentamicin,15% NBCS and 5% pig serum (PS).

The cells generally reached confluence after 24 to 48 hours. However the pig cells were more susceptible to infection than the bovine cells and many of the primary cultures would not adhere or grow and had to be discarded.

2.1.4. Human Umbilical Vein Endothelial Cells.

Umbilical cords were obtained from the labour wards of the Simpson Memorial and the Elsie Inglis Maternity Hospitals, Edinburgh, with the kind help of the nursing staff.

The procedure used for endothelial cell isolation was an adaptation of the method of Jaffe et al (1973).

Pieces of cord between 20 and 30 cm long were drained of as much blood as possible and placed in cold sterile EBS solution containing 2 ug/ml gentamicin and kept refrigerated until processed. Cords which had been heavily clamped were discarded. The vein at each end of the cord was cannulated using the blunt plastic sheath of a 1.7 mm gauge intravenous cannula and held in place with surgical ties. Blood was flushed from the vein with sterile saline and then with 0.1% collagenase solution. One end was then clamped and 2 to 5 ml of collagenase/EBS was infused into the vessel. Over-filling of the vessel was avoided since this tended to lead to rupturing and leakage and

probably to contamination with non-endothelial cell types such as fibroblasts and smooth muscle cells. The cord was wrapped in cling-film and incubated at 37^{o}C for 15 to 20 minutes.

The collagenase/cell suspension was flushed out with 10 ml of DMEM supplemented with 150 ug/ml of endothelial cell growth factor (ECGF ; from bovine hypothalamus), 15% foetal calf serum (FCS), 5% adult human serum (AHS) and 2ug/ml gentamicin. The cells were pelleted by centrifuging at 1000 x g for 10 minutes and rinsed once before being resuspended in 5ml of the above medium. The cells from one piece of cord this length were initially cultured in a 25 cm^{2} plastic tissue culture bottle which had been previously coated with fibronectin or with 0.2% gelatin. Cells reached confluence after 3 to 5 days with medium changes every other day.

Cords were usually processed within 2 to 3 hours of delivery. However some sections of cord were kept overnight under refrigeration without any apparent effects on cell recovery or viability.

The ECGF and fibronectin were very kindly donated by Dr N Hunter of the Scottish National Blood Transfusion Headquarters, Forrest Road, Edinburgh.

2.1.5. Adult Human Saphenous Vein Endothelial Cells.

Adult saphenous vein was obtained from coronary artery bypass patients. Pieces of vein surplus to the graft requirement, which ranged from 10 to 15 cm long, were placed immediately in ice cold EBS/ 2 ug/ml gentamicin solution and kept in the fridge until they could be processed . Some sections of vein were kept overnight under refrigeration without any apparent effects on cell recovery or viability .

The vein was cannulated and tied with the same procedure as for the umbilical cords and processed in exactly the same way. Adult human cells were also cultured on fibronectin or gelatin coated plastic and medium was changed every other day. Cells from one 10 to 15 cm segment of vein were grown in 25 cm^2 flasks. Cells from shorter segments were cultured initially in one 16mm diameter well of a 24 well plate, and subcultured to the other wells of the plate once confluence was reached. Confluence was generally attained after 5 to 10 days, depending on cell condition and initial seeding density.

2.1.6. Subculturing.

All uncloned cell lines were subcultured as soon as they had reached confluence. The same basic procedure was used for all types of endothelial cells.

The old growth medium was decanted and the cell monolayer was rinsed once with a small volume of 10mM sucrose / 1mM EDTA in 20mM HEPES buffered PBS (Kaplan 1983). A small volume of this buffer (5ml/25cm^2 flask and 10ml/75cm^2 flask) at 37^{o}C, was incubated with the cells at 37^{o}C for 5 minutes or until they had detached from the surface of the culture vessel. Monolayers which did not resuspend within 10 to 15 minutes in this buffer were transferred to a fresh aliquot of the same buffer containing 0.02% trypsin and incubated for a further 2 to 3 minutes at 37^{o}C. Human cells which were grown on fibronectin coated vessels usually required trypsin treatment.

Cells were recovered from the sucrose/EDTA buffer by centrifugation at 300 x g for 5 minutes. The cell pellet was resuspended in the appropriate amount of growth medium and dispensed into tissue culture flasks. Bovine cells in good condition and growing fast were usually split at a 1:3 or a 1:4 ratio. Human cells were always split at a 1:2 ratio.

2.1.7. Cloning.

Primary isolates of endothelium are quite often contaminated with non-endothelial cell types such as smooth muscle cells and fibroblasts. Mixed cultures are quickly overgrown by fibroblasts. Several

techniques for isolating pure endothelial cell cultures have been reported. Semi-selective mediums, such as those containing d-valine which slows the growth of smooth muscle cells and fibroblasts (Picciano et al 1984) and some with agents cytocidal to the non-endothelial cell types (Schwartz 1978) have been used. More specific methods have utilised endothelial cell capacity for low density lipoprotein (LDL) uptake. By fluorescent labelling of acetylated LDL the endothelial cells can be separated by fluorescent activated cell sorting (FACS) (Voyta et al 1984).

The method of obtaining pure endothelial cell lines by cloning is time consuming but it was chosen because of its simplicity and because it does not require any special materials or equipment. The procedure used was based on that of Gajdusek and Schwartz (1983) and was used for all endothelial cell types.

Confluent cultures in their first or second passage were resuspended using 10mM sucrose / 1mM EDTA / 0.02% trypsin in 20mM HEPES buffered PBS. The cell suspension was aspirated through a 25 gauge needle 2 to 3 times to break up cell clumps, and diluted 50:50 with serum - containing growth medium to inactivate the trypsin. The cells were recovered by centrifugation at 1000 x g for 5 minutes and washed once in growth medium before being

finally resuspended in a small amount of medium. Cells were counted with an improved Neubauer haemocytometer and the appropriate dilutions were made in complete growth medium.

Bovine and porcine cells were diluted so that on average there was one cell per well in 100 ul aliquots in a 96 well plate. Human cells seemed to be a bit more reluctant to grow at low densities and were therefore cloned by limiting dilution. Cells were diluted to 230 cells in 4.6 ml of complete growth medium and plated into 36 wells of a 96 well pre-coated plate, in 100 ul aliquots. To the remaining 1 ml of cell suspension a further 4 ml of growth medium was added and another 36 wells were plated with 100 ul. To the remaining cell suspension, 1.4 ml of medium was added and the remaining 24 wells were plated with 100 ul. The first 36 wells should on average have 5 cells/well, the second 36 wells, 1 cell/well and the remaining 24 wells, 0.5 cells/well. Therefore if the cells did not grow at single cell density, colonies from the higher densities were grown up and recloned.

Immediately after plating a further 100 ul of conditioned medium (see section 2.1.8.) was added to each well. The wells were checked after 24 hours for cell growth. Those wells containing single cells or small single colonies were noted and considered to be a

clone. After 5 days the wells were examined again and only those with single colonies of 8 to 10 cells were marked and received further attention. The medium was changed every 4 to 5 days using a 50 : 50, fresh : conditioned medium mixture, until the cells had reached confluence. The cells were then transferred, on a well to well basis, to a 24 x 16mm well plate in normal growth medium, using standard subculturing techniques. If the cells of each well retained the cobblestone morphology of endothelium on reaching confluence and were not obviously contaminated with smooth muscle cells or fibroblasts, they were expanded to 25 cm^2 flasks and grown on in the usual way.

2.1.8. Preparation of Conditioned Medium.

Confluent cultures of endothelial cells were maintained in the appropriate complete growth medium for 5 days. The medium was removed from the cells, centrifuged for 15 minutes at 1000 x g to remove dead cells and debris and passed through a 0.22 um Millipore filter to ensure sterility. It was stored at $-20^{o}C$.

2.1.8. Fibroblast and Smooth Muscle Cell Culture.

Primary endothelial cell cultures contaminated with smooth muscle cells were preferentially kept and cloned

accordingly. Growth medium was exactly the same as that used for endothelium.

2.1.10. Liquid Nitrogen Storage.

When good primary cell and cloned cell lines were obtained, a quantity was stored in liquid nitrogen for future use.

Cells in their first or second passage at confluence were resuspended as in section 2.1.6 . Cells were counted before being centrifuged and rinsed once in EBS. Final resuspension was in 90% FCS and 10% DMSO at $5x10^5$ cells/ml for human cells and $2.5x10^5$ cells/ml for bovine and pig endothelium. Aliquots of 0.5 ml in sterile cryotubes were cooled slowly to $-70^{o}C$ and kept at this temperature for 12 to 24 hours before being transferred to vapour phase liquid nitrogen (Oi & Herzenberg 1980).

2.1.11. Recovery of Cells from Liquid Nitrogen Storage.

Vials were removed from the liquid nitrogen immediately before use and thawed quickly,but not completely in a $37^{o}C$ waterbath. 5 ml of cold growth medium was added dropwise. Slow addition of cold medium at this stage prevents rupture of the cells through heat or osmotic shock.

Cells were recovered by gentle centrifugation (200

x g for 10 minutes) and resuspended slowly as before. Final resuspension was in 5 ml of growth medium at 37^{o}C. Cells were then plated in 25 cm^{2} flasks. Confluence was reached between 5 and 10 days depending on the number remaining viable after storage and recovery.

2.2. ENDOTHELIAL CELL IDENTIFICATION.

2.2.1. Morphological Criteria.

In the majority of the bovine and porcine cultures, identification was based on morphological criteria.

Endothelial cells tend to be polygonal in shape and form a tight cobblestone appearance when confluent. Pure endothelia grow in a single uniform monolayer with cells showing contact inhibition. At confluence cell growth becomes static and cultures of pure endothelium can be maintained for long periods of time without subculturing. With frequent medium changes cell turnover is relatively slow.

By contrast fibroblasts and smooth muscle cells, the two common contaminants of endothelial cultures from large vessels, are elongated and less well defined in shape. Fibroblasts grow in multiple layers and very quickly overgrow any endothelial cells present in a culture. The striking difference in morphology between these cells helps to eliminate contaminated cultures

early on.

Human umbilical vein and adult saphenous vein endothelium are less easily discriminated from contaminating cells in early primary cultures. Human cells in non - confluent cultures have a tendency to grow in an elongated manner until the cells are confluent and then they assume a more typical polygonal - cobblestone appearance. They do exhibit contact inhibition and monolayer growth.

2.2.2 Immunological Criteria.

The most widely used immunological marker for endothelial cells is von Willebrand factor. Cells incubated with a fluorescently labelled anti-vWF antibody are identified by fluorescent microscopy.

More recent work with monoclonal antibodies and lectins have produced two more reliable markers, PAL-E which is a pan endothelial cell antibody (Schlingeman 1985) and the lectin Ulex europaeus which is highly specific for endothelial cells (Holthofer et al 1982). Biochemical markers such as angiotensin converting enzyme (ACE) have been used but would appear to unreliable (Del Vecchio & Smith 1982). In one cloned cell line of bovine aortic origin isolated and grown in this laboratory, ACE production was not detectable at passages 15. In terms of morphology and growth

characteristics, these cells appeared normal. It was not known whether these cells had lost this function through an ageing process, or a non-ACE producing cell line had been unwittingly selected at cloning.

Endothelial cells were identified by morphology and by the presence of vWF antigen.

2.3 MONOCLONAL ANTIBODY PRODUCTION

2.3.1. Introduction.

The technique of hybridising mouse antibody producing spleen cells with "immortal" mouse myeloma cells to produce a cell line secreting monospecific tailor - made antibodies was developed by Kohler & Milstein in 1975. The technique was first used for the derivation of anti - sheep red cell antibodies but since then has been used widely.

There are a few variations on the original hybridisation method and the following procedure is based on that of Oi & Herzenberg (1980). The few alterations to the method were on the kind advice of Dr L. Micklem of the Department of Surgery, University of Edinburgh.

2.3.2. Immunisation of Mice.

Three to five week old BALB/c mice were used to

provide the immune spleen cells needed for fusion. Two types of immunogen were used in separate groups of mice. Mouse immunisation protocol was the same for both types of immunogen.

Immunogen 1.

Endothelial cells were isolated and grown as described in section 2.1. When the monolayers were confluent the cells were resuspended by scraping them off the vessel surface with a glass rod in the sucrose/EDTA buffer (without trypsin) described in section 2.1.6. The suspension was then aspirated through a 25 gauge needle to break up cell clumps.

Cells were recovered by centrifugation and the pellet was rinsed 3 times in serum - free EBS with a 15 second mix (vortex mixer) at each rinse. This step was to free the cell surface of any serum albumin from the growth medium which may have adhered. Cells were finally suspended in a volume of EBS such that each mouse received between 10^5 and 10^7 cells in a 0.2 - 0.3 ml volume.

Each mouse in the group received 2 intraperitoneal (i.p.) injections of the cell suspension at 21 day intervals. No Freund's adjuvant was used. After the second injection, tail bleed plasma was tested for antibody production, as described in section 2.3.2.

Pre-immunisation tail bleeds (pooled sample) were used as an immunological base line for this particular group of mice. Those mice which were immuno-positive for endothelial cell proteins were given a booster injection 3 days before being killed. Mice which were immuno-negative were dropped from the programme. These mice were used in the production of mixed thymocyte medium. (section 2.2.8.)

Immunogen 2.

In the mouse the spleen is a good source of immunologically competant cells. Studies on endothelial - lymphocyte interactions show that viable lymphocytes actively bind to the endothelial cell surface. After 4 to 6 hours incubation the lymphocytes detach (de Bono 1977) . It was thought that during this detachment the lymphocytes may "remove" a significant part of the endothelial cell surface and in turn present it to the immune systems of the injected mice.

Monolayers of BAEC at their second or third subculture were used 2 days after becoming confluent. A suspension of cells in complete growth medium was prepared from the spleens of non - immunised BALB/c mice. The old medium on the endothelial monolayers was removed and replaced with the spleen cell preparation and incubated overnight at 37^{o}C. After gentle agitation

the spleen cells were removed leaving an apparently intact endothelial monolayer. The spleen cells were recovered by spinning at 1500 x g for 10 minutes and were very gently rinsed twice in EBS before being finally resuspended in a volume of EBS such that each mouse received 0.3 ml.

To the cells in one 75 cm^2 flask was added the cells of one 4 to 5 week old BALB/c spleen, which was then used to immunise 5 mice. Again immunisation was by 2 i.p. injections 21 days apart. Immunity was checked before the mice were given a final booster of whole endothelial cells, prepared in the same way as Immunogen 1.

2.3.3. Myeloma Parent Cell Line.

P3-NSI-1 abbreviated to NS-1 is a cell line derived from the BALB/c myeloma cell line MOPC-21. NS-1 cells synthesise the MOPC-21 k light chain but are incapable of producing the MOPC-21 y_1 heavy chain. Hybrid cells lines from this fusion partner will therefore produce mixed molecules with only the MOPC-21 k light chain (McMichael & Fabre 1982.).

NS-1 cells were very kindly provided by Dr Micklem. These cells were grown in RPMI 1640 medium supplemented with 0.85 g/l sodium bicarbonate, 20 mM HEPES, 10 mM sodium pyruvate, 2 ug/ml gentamicin and 5% FCS. Cells

which were taken from liquid nitrogen storage were initially grown in the above medium supplemented with 10% FCS and in a 5% CO_2, 95% air atmosphere. Once growth was well established the FCS content of the medium was reduced to 5%. Cell density was maintained at $5 \times 10^4 - 1 \times 10^6$ per ml. With the cells in logarythmic growth phase, cultures were split every 2 to 3 days.

For fusion, NS-1 cells in log phase growth (a density of 10^5 cells per ml) were collected by centrifugation at 500 x g and rinsed once in serum free RPMI 1640 medium.

NS-1 cells are 8-azaguanine resistant and are therefore HAT sensitive. The mechanism of HAT selection is explained in section 2.3.6. Due to spontaneous genetic drift, NS-1 cells which had been kept in culture for long periods tended to lose HAT sensitivity. Those cell lines which had done so were grown in growth medium to which 8-azaguanine was added to a final concentration of 10^{-4}M. The cells were then re-cloned and those cultures strongly HAT sensitive were kept as stock.

2.3.4. Mouse Spleen Parent Cell Preparation.

The mice primed three days prior to fusion were killed by cervical dislocation. The spleens were

removed under sterile conditions and a suspension of cells was made in serum free RPMI 1640 medium by forcing the whole spleen through aa 19 gauge needle with a 1ml syringe. After allowing the large clumps of fatty connective tissue to settle, the suspension was transferred to a fresh sterile tube and centrifuged at 1000 x g for 10 minutes. The cells were then rinsed three times in serum free RPMI medium.

2.3.5. Fusion.

The following procedure is a variation on the method of Oi & Herzenberg (1980).

The parent cell lines were prepared as described and counted . They were then mixed together in a sterile centrifuge tube at a ratio of 1:4 (NS-1:spleen). The cells were then centrifuged at 400 x g for 10 minutes at room temperature to form a tight pellet. The supernatant was removed completely and all consequent procedures were carried out at 37^{o}C.

A 50% solution of polyethylene glycol 1500 (PEG) was prepared by autoclaving 20 - 50g of the material for 20 minutes at 121 - 132^{o}C and adding an equivalent volume of sterile RPMI medium to it before it solidified. The PEG solution was stored at room temperature. During storage the solution turns alkaline which did not seem to affect it as a fusion agent,

therefore the pH was not altered.

The PEG solution , serum free and 5% FCS RPMI mediums were all pre-warmed to 37^{o}C . Using a sterile pasteur pipette, 1ml of 50% PEG was slowly and gently stirred into the cell pellet over 1 minute. Stirring was continued for a further 1 minute. Using the same pipette, 1ml of serum free medium was added in the same way over 1 minute. This was repeated once. A further 7ml of serum free medium was added by gentle stirring over a 4 minute period. Pipetting of the cell suspension was avoided during this procedure to prevent breaking up cell clumps and therefore reducing the chances of cell fusion.

The suspension was centrifuged at 200 x g for 10 minutes at room temperature. The supernatant was carefully decanted and a volume of 5% FCS RPMI medium was added to the cell pellet to give 10^{6} cells per 0.1ml. At this stage the cells may be pipetted once before aliquoting into 96 well flat-bottomed plates at 0.1ml per well and incubated in a 5% CO_2 in air mix at 37^{o}C.

2.3.6. HAT Selection.

The fusion procedure is a random process and results in a mixture of cell pairs e.g NS-1:NS-1 , NS-

1:spleen , spleen:spleen as well as unfused parent cells. Only the NS-1:spleen cell hybrid is of importance in antibody production. The addition of hypoxanthine, aminopterin and thymidine (HAT) to the growth medium positively selects the growth of NS-1:spleen hybrids by the following mechanism.

Aminopterin is an analogue of folic acid and blocks the biosynthesis of purines and pyrimidines. Therefore to survive the cells must utilise the hypoxanthine and thymidine provided in the HAT medium to synthesise the nucleotides via a salvage pathway. NS-1 cells are however lacking the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRTase) which is required in the salvage pathway of nucleotide biosynthesis. Therefore in HAT medium NS-1 and NS-1:NS-1 hybrids cannot survive. Normal spleen cells do however have this enzyme and so a NS-1:spleen hybrid will be able to utilise the hypoxanthine and thymidine in the HAT. There is no need for positive selection against the growth of the spleen cells since they have limited growth in culture and most will have died within 2 - 3 days.

The 50X HAT solution was diluted 1:50 in 5% FCS RPMI medium. The day the perfusion was done was denoted day 0. On day 1, 0.1ml of HAT medium was added to each well. On days 2, 3, 5, 8 and 11, half the medium was

aspirated from the wells and replaced with 0.1ml of fresh HAT medium, taking care not to disturb the cells. After day 11 half the medium was replaced every 3 - 4 days.

After 2 - 3 days the HAT treatment drastically reduced the number of cells and the cultures appeared dead. However, after 14 - 20 days small but distinct colonies of hybrid cells were seen at low magnification (their appearance being very similar to the parent NS-1 cells). When 50 - 90% of the well surface was covered by cell colonies, the first antibody screening assay (section 2.3.2) was done and positive colonies were selected for expansion and cloning.

Colony expansion was done in conjunction with the change to HT medium, part of the process of weaning the cells off HAT medium. The contents of each small well was transferred to 0.5ml of 1X HT 5% FCS RPMI medium in a 16mm diameter well of a 24 well plate. A further 0.5ml of this medium was added to each well after 24 hours if the cells looked settled. If cell numbers were low or if the cells did not start to increase in number after 48 hours then mouse thymocytes were added as feeder cells.

Cells were kept in these plates until growth was well established. At this stage a small aliquot of cells was taken for storage in liquid nitrogen, some

for cloning and the rest were expanded to 25 cm^2 flasks in 5% FCS RPMI medium (5 - 10ml volume per flask).

2.3.7. Cloning.

At this stage the cultures contain a mixture of a variety of antibody producing hybrids and non-producing hybrids. To produce cultures of a single cell type secreting the desired antibody, the cultures were cloned by limiting dilution (Oi & Herzenberg 1980).

The number of viable cells in the sample to be cloned was determined by counting acridine orange / ethidium bromide (AO/EB) stained cells. This stain shows viable cells as bright green and dead cells as brown under fluorescent light.

The sample was diluted so that 230 live hybrids were suspended in 4.6ml of thymocyte containing growth medium or mixed thymocyte medium (2.2.8.). The first 36 wells of a 96 well plate were then aliquoted with 0.1ml each well with this suspension. To the remaining 1.0ml , 4.0ml of the thymocyte medium was added and another 36 wells were plated. Finally 1.4ml of thymocyte medium was added to the remaining cell suspension and the last 24 wells were plated.

The clones were then fed at days 5 and 12 by adding 0.1ml of fresh RPMI 1640 with 15% FCS to each well with as little disturbance as possible to the cells. By day 14 the colonies of cloned cells were large enough to

test for antibody production. The cells producing the desired antibody were then expanded to larger numbers as described in section 2.2.5.

By this method of cloning, the first 36 wells should, on average, contain 5 cells per well, the second 36 wells, 1 cell per well and the 24 wells, 0.5 cells per well. At least one of these plating concentrations should give monoclonal growth. However, depending on which concentration the antibody producing cells were found in, the cells were frequently re-cloned until monoclonal growth was achieved.

2.3.8. Cloning Medium.

Thymocyte Cloning Medium

The purpose of the thymocytes in the cloning medium was to act as feeder cells in culture and also as carrier cells in diluting the hybrid cell sample.

The thymuses from 4 - 5 week old BALB/c mice were removed aseptically and a cell suspension prepared in RPMI 1640 medium containing 15% FCS. The cells were suspended by aspirating the whole thymus through a 19 gauge needle. The suspension was used at a concentration of 10^7 - 10^8 thymocytes per ml.

If cloning was done directly from the master plate, the above medium contained 1X HT.

Mixed Thymocyte Medium

This medium was used as a substitute for thymocyte containing medium on some occasions. It did not appear to make any difference to the rate of growth of the clones and it was advantageous in some respects. It could be made in large quantities some time in advance and stored at -20^{o}C for short periods or at -80^{o}C for long term storage. It also left the clones free of cell debris making them easier to see under low power light microscopy and therefore easier to keep track of their growth progress.

The thymuses from equal numbers of 4 - 6 week old BALB/c mice and CBA mice were removed aseptically. A cell suspension was made in serum free RPMI 1640 medium as previously described. Large clumps of connective tissue were allowed to settle and the fine cell suspension was removed and centrifuged at 450 x g for 8 minutes. The cells were washed 3 times with serum free RPMI 1640. The thymocytes were resuspended in RPMI 1640 with 15% FCS at a concentration of 5 x 10^{6} cells/ml and cultured at 37^{o}C for 48 hours only. After 48 hours the medium becomes suppressive to hybridomas.

The cells and debris were then removed from the medium by centrifugation at 1000 x g for 10 minutes and the supernatant was filtered (0.22u) to ensure

sterility. Aliquots were stored at either $-20^{o}C$ or $-80^{o}C$. This medium was diluted 1:2 with fresh sterile RPMI 1640/15% FCS before being used.

2.3.9. Bulk Growth of Monoclonal Hybridomas.

Large amounts of monoclonal antibody can be produced simply by increasing the quantity of hybridoma cells in culture. The cultures of interest were expanded gradually to large tissue culture vessels which had a capacity of 200 - 300ml. Depending on the rate of growth of the cells, fresh medium was added every day or every other day until the volume capacity had been reached and the cell concentration at its maximum. The antibody containing growth medium was harvested by spinning down the cells and the supernatant was stored at $4^{o}C$, sterile or with 0.02% sodium azide as a preservative. A quantity of cells were replaced in the vessel for further expansion.

2.3.10. Ascites.

Large amounts of monoclonal antibody with high titre can be obtained by growing the hybribomas as ascites tumours in mice.

The mineral oil, pristane when injected i.p. induces ascites in BALB/c mice, which provide a favourable environment for the growth of hybridomas.

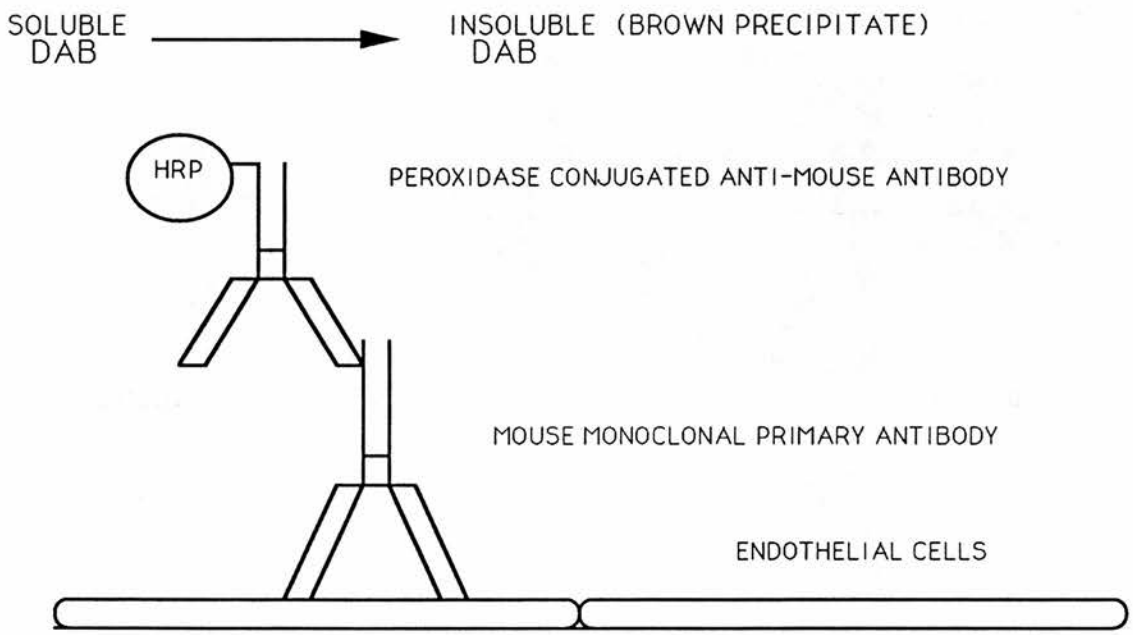
Mice 4 - 5 weeks old were given 2 intraperitoneal injections of 0.3ml of pristane, 1 week apart. The cloned hybridoma cells were then injected 7 to 10 days later. Injections of 1 - 5 x 10^6 cells were given to each mouse i.p. in a 0.3ml volume.

The mice were checked daily for abdominal swelling and when this appeared the fluid was drained using a 19 gauge needle. The fluid was pooled and centrifuged and stored with 0.02% sodium azide at $4^{o}C$. Ascitic fluids containing IgM monoclonal antibody stored at $4^{o}C$ since the molecule is unstable at temperatures below 0, but those fluids containing IgG antibody were stored at $-20^{o}C$.

2.3.11. Liquid Nitrogen Storage of Hybridomas.

At each stage in the expansion and cloning procedures, a sample of cells were taken for storage as a back up to the cells in culture. It is particularly important to have stocks of the uncloned cell lines since cloned cells which have been in culture for long periods can lose their antibody producing capacity.

Cells in log phase growth were harvested by centrifugation at 400 x g for 5 minutes and rinsed once in 5% FCS RPMI 1640 medium and counted on an improved Neubauer haemocytometer. The cells were pelleted again



DIAG.2.1. Horseradish peroxidase (HRP) labelled second antibody detects monoclonal primary antibody bound to endothelial cells. The HRP catalyses the precipitation of diaminobenzidine (DAB) to form a brown insoluble complex.

and finally resuspended at 1 - 5 x 10^6 cells per ml in cold freezing medium. The freezing medium was prepared by adding DMSO dropwise to ice cold FCS to a final concentration of 10% and gently shaking.

The cell suspension was distributed in 1ml aliquots in sterile cryotubes and cooled slowly to - 70^{o}C . After 12 to 24 hours at this temperature the samples were transferred to the gas phase of a liquid nitrogen refrigerator.

Cells were recovered from storage by thawing quickly but incompletely at 37^{o}C. The contents of the cryotube was transferred to a sterile 30ml centrifuge tube and 5 ml of 10% FCS RPMI 1640 medium was added dropwise to the cells while gently stirring. The cells were washed once in this medium by centrifuging as above and finally suspended at 2 x 10^5 cells per ml for culture.

2.4. MONOCLONAL ANTIBODY SCREENING

2.4.1. Introduction.

The screening procedure is an indirect ELISA method using a horseradish peroxidase (HRP) conjugated antibody. The principle of the reaction is summarised in Diag.2.1.

In step 1 the primary antibody binds to the antigen

(endothelial component). The HRP conjugate is added and binds to the Fc region of the primary antibody, in step 2. The addition of o - phenylenediamine (oPD) or 3,3-diaminobenzidine tetra-hydrochloride (DAB) which are substrates for peroxidase, results in oxidation of the substrate to a brown insoluble complex. (Ritchie et al 1983).

2.4.2. Primary Screening - Fixed Cell Elisa.

The hybridomas from the initial fusion plate were tested for antibody production as soon as cell growth covered 70 - 90% of the well surface.

Endothelial cells from the appropriate source were plated into 96 well plates and cultured until confluent. The growth medium was removed and replaced with 0.01% glutaraldehyde in PBS and the fixation process was allowed to proceed for 15 minutes at room temperature. The fixed cells were rinsed 3 times with PBS and once with distilled water, drained and stored at $-20^{o}C$ until used.

The plates were thawed by adding PBS to each well and letting the plate stand at room temperature for 15 minutes. The PBS was tipped out and 0.1ml of growth medium was carefully removed from each well of the fusion plate to be tested and added to the equivalent

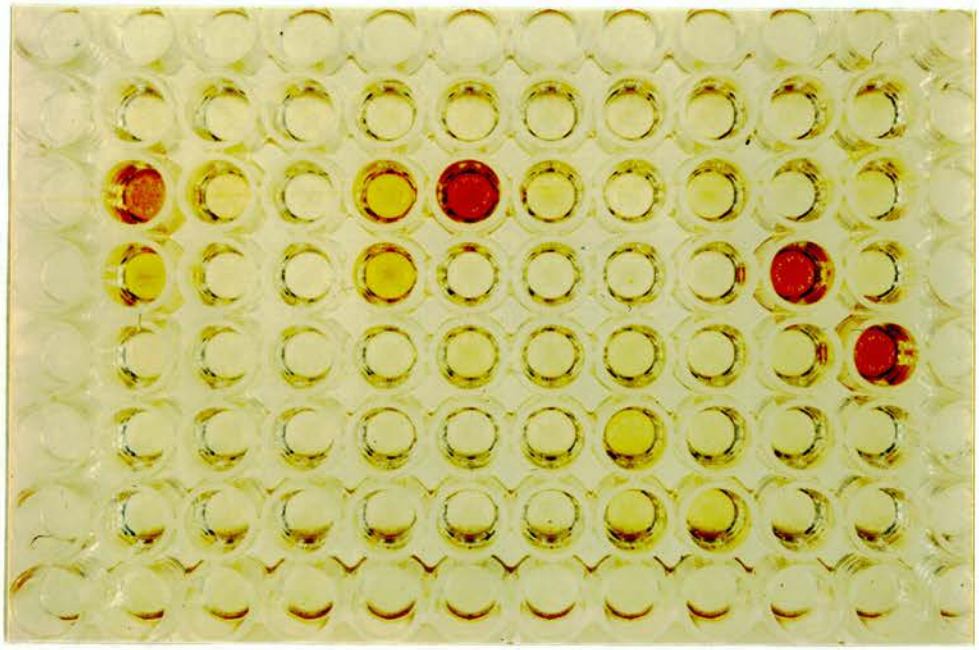


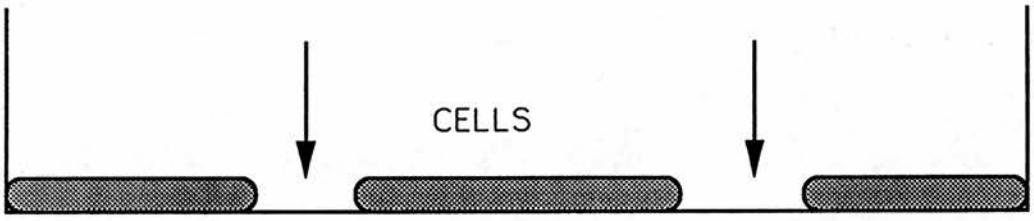
FIG.2.1. Primary screening of fusion growth medium supernatants. Orange precipitate indicates the presence of anti-endothelial cell antibodies.

well of the endothelial test plate. The plates were incubated at room temperature for 60 minutes.

The antibody supernate was removed and the cells rinsed 5 times with PBS. The peroxidase conjugated rabbit anti - mouse second antibody was diluted 1:100 in PBS and 0.1ml was added to each well and incubated at room temperature for 30 minutes. After the second antibody was removed the cells were rinsed 10 times with PBS.

Addition of 0.1ml of 0.04% o-phenlylenediamine (o-PD) / 0.012% H_2O_2 solution to each well initiated precipitation of the o-PD in the presence of the peroxidase labelled antibody, indicating the presence of endothelial cell bound antibody (Fig.2.1). On each plate a set of second antibody plus substrate, and substrate only controls were included.

The second antibody + substrate controls also developed a pale yellow / orange precipitate but at a much slower rate than the antibody positive cells. These controls were used as the basis against which the positive results were judged. Since a photometric plate reader was not available the plates were scored in the following way; the wells which developed orange/brown colour within 1 minute of the substrate being added were scored +++ those within 3 minutes ++ and those within 5 minutes +. After 5 minutes the control wells



DIAG. 2.2 Scratch damaged cell monolayer. Arrows indicate areas denuded of endothelium.

started to turn yellow , only those wells showing a darker colour than the controls were scored, wells at the same colour as the controls were denoted 0.

On a successful fusion only the +++ scoring hybrids were selected for expansion and cloning. With less successful fusions the ++ and + hybrids were used.

This method was also used for the initial screening of the cloning plates.

2.4.3. Secondary Screening - Damaged / Regrowing Cell Model (DAB ELISA).

The primary screening programme picks out a selection of general anti - endothelial cell antibodies by looking at the cell monolayer as a whole. The secondary screening process is designed to look at individual cells and allows selection of specific monoclonal antibodies.

The basic principles of the technique are the same as the primary screening process.

Endothelial cells were grown to confluence in 24 x 16mm plates. Using a glass pasteur pipette with a 1mm diameter end, two scratches were made parallel to each other through the centre of the well (Diag.2.2). Scratches were approximately 5mm apart. This left an area denuded of endothelium with damaged cells along the scratch edge. The plates were either used

immediately for ELISA or were re-incubated to allow the scratch edges to regrow.

After scratching the cell layer was rinsed gently with PBS to remove free cells and fixed in 0.01% glutaraldehyde for 15 minutes at room temperature. The cells were rinsed well with PBS and primary antibody was added in the form of neat supernate for 1 hour at room temperature. After 5 rinses with PBS incubation with peroxidase conjugated second antibody 1:100 in PBS was carried out for 30 minutes at room temperature. After 10 PBS washes, 0.5ml of diaminobenzidine at 0.4mg/ml in Tris buffered saline (TBS) / 0.01% H_2O_2 was added to each well. The cells were checked for colour development under low power light microscopy. Positive cells colour orange to dark brown. Counter staining of negative cells was with Mayer's Haematoxylin.

2.5. MONOCLONAL ANTIBODY PURIFICATION.

The antibodies showing particular staining patterns, which are discussed in detail in chapter 3, were grown up as previously described. The method of isolation depends to some extent on the antibody subtype ,therefore all supernatants of interest were typed by Ouchterlony double diffusion analysis (using

a kit from The Binding Site, University of Birmingham).

2.5.1. Protein Precipitation.

The antibodies grown as bulk cultures were concentrated from the culture supernate by saturated ammonium sulphate (SAS) protein precipitation. This procedure was not necessary for antibody isolation, but by reducing the volume of the antibody it made the affinity chromatography procedure less time consuming.

SAS was prepared by dissolving 900g of $(NH_4)_2SO_4$(BDH)in 1litre of hot distilled water. The solution was filtered through a Buchner funnel while still hot and allowed to cool overnight at room temperature before the pH was adjusted to 7.4 with NH_4OH. The antibody medium was clarified by centrifugation at 10 000 x g for 5 minutes and the pH was adjusted to 7.4. The SAS and antibody solutions were both chilled to $4^{o}C$. The SAS was added slowly to the supernate with gentle stirring until 40% saturation was reached. Precipitation continued at $4^{o}C$ overnight.

The protein precipitate formed was stored with an excess of SAS in a sealed container at $4^{o}C$.

2.5.2. Protein Reconstitution.

The protein precipitate was resuspended in SAS and centrifuged at 10 000xg for 10 minutes. The supernate

was removed completely and the pellet resuspended in a minimal amount of DDW. This was dialysed for at least 36 hours against 200 volumes of 0.05M TRIS / 0.15M NaCl pH8.6 with three changes of buffer.

2.5.3. Protein A - Sepharose Affinity Chromatography.

Work by Goding (1978) has shown that protein A has an affinity for IgG subgroups from various sources. The antibody will bind to the protein A at certain pH's and can be separated from it by altering these conditions.

The following procedure is that of Oi and Herzenberg (1980).

Protein A - sepharose , 1.5g was swollen in an excess of 0.05M Tris / 0.15M NaCl / 0.02% NaN_3 pH8.6 for 15 minutes at room temperature. A column of bed volume of 6ml (1.6 x 5cm) was prepared and washed with 200ml of pH8.6 buffer.

The antibody in the form of reconstituted protein was run through the column at a slow flow rate (approximately 1ml per minute). The column was then washed with pH8.6 buffer until the A_{280} had returned to baseline and all unbound protein was washed out.

Stepwise elution of the bound antibody was carried out under altered pH conditions. The following buffers were used in order :

pH 7.0 ; 0.05M phosphate / 0.15M NaCl

pH 5.5 ; 0.05M citrate / 0.15M NaCl

pH 4.3 ; 0.05M acetate / 0.15M NaCl

pH 2.3 ; 0.05M glycine / 0.15M NaCl

In each case the A_{280} was monitored and the buffer flow continued until the baseline had returned to zero. The fractions which showed absorbance were pooled and neutralised using the pH 8.6 buffer and stored at $4^{o}C$ with 0.02% NaN_3. All antibodies eluted in the pH 5.5 or 4.3 fraction.

Column regeneration was carried out by washing with the pH 2.3 buffer after each run and equilibrated with pH 8.6 buffer.

2.5.4. Hydroxylapatite Affinity Chromatography.

A number of the antibodies produced were of IgM subtype. IgM antibodies from murine origin have been reported not to bind to protein A (Ey et al 1978). IgM does however have some affinity for hydroxylapatite. The following method was based on that of Stanker (1985).

COLUMN BUFFER : 0.01M sodium phosphate/ 0.02% NaN_3 pH 6.8

ELUTION BUFFERS : 0.1M, 0.2M, 0.35M, 0.4M sodium phosphate pH 6.8

COLUMN REGENERATING BUFFER : 0.5M sodium phosphate pH 8.6

Hydroxylapatite was hydrated in column buffer overnight at room temperature. A column with a bed volume of 75 ml (2.5cm x 15 cm) was prepared. The antibody was prepared as in section 2.4. If the antibody was in ascites form. then it was clarified by filtration (0.22u Millipore filter) and added directly to the column. The unbound protein was washed through with column buffer before stepwise elution with the elution buffers (starting with the lowest salt concentration buffer).

All fractions were monitored at A_{280} and those containing protein were tested for antibody activity by ELISA (2.3.2). Positive fractions were pooled, concentrated by ultrafiltration and stored at $4^{o}C$ with 0.02% NaN_3.

2.6. MICROSCOPY AND PHOTOGRAPHY.

A Leitz Diavert inverted microscope with a Cosina camera and attachments were used for all photomicrography. All photographs were taken at 100 x magnification unless otherwise stated, with phase contrast optics and a blue filter. Photomicrographs were taken on Kodacolour Gold 400 or 200 ASA film.

CHAPTER 3

IMMUNOHISTOLOGICAL STAINING PATTERNS OF MONOCLONAL ANTIBODIES ON ENDOTHELIAL CELLS IN VITRO.

3.1 INTRODUCTION

The role of endothelial injury in vascular thrombosis and atherosclerosis is well recognised and has been discussed (1.6). Most of the evidence for this comes from experiments in which the endothelium is deliberately damaged and the consequences examined (Hirsch and Robertson 1977, Ramsay et al 1982, Groves et al 1979, Moore 1973, Clowes and Karnovsky 1977).

Evidence for non-deliberate damage to endothelium in vivo is difficult to obtain and comes from morphological studies on excised vessels (Davies et al 1988), known disease models such as homocysteinemia (Starkebaum and Harlan 1986) and as the result of therapeutic interventions such as heparin-associated thrombocytopenia (Cines et al 1987), and balloon angioplasty (Block et al 1981).

Monoclonal antibodies to damaged endothelium may make it easier to detect such damage in vivo. In the following experiments the techniques used to produce in vitro endothelial damage were based on techniques which have been used to produce endothelial damage in vivo, such as mechanical abrasion, air drying and heat. As a comparison a range of mechanisms which have been suggested to lead to naturally occuring endothelial damage were also tested. These include bacterial endotoxin, hydrogen peroxide, thrombin and hyperoxia.

3.2.ANTIBODY SCREENING AND SELECTION .

A total of 25 fusions were done from which 137 colonies of hybridoma cells producing anti-endothelial antibody were chosen for expansion and cloning, on the results of the two screening methods in section 2.3, Of this 137, 27 were cloned giving rise to a total of 90 cloned cell lines. The reproducibility of the method for each screening was maintained by using cloned endothelial cell lines in their second or third subculture 24 hours after reaching confluence. If secondary screening was negative, the ELISA was repeated using a primary cell culture. Only a few hybridoma clones could be investigated in any detail, the rest are kept in liquid nitrogen storage. After expansion and before cloning the antibodies were tested on both non-damaged and scratch damaged cells with diaminobenzidine (DAB ELISA) procedure (2.3.3), give a more detailed staining pattern.

Hybridomas producing antibodies with interesting staining patterns were cloned and screened using the DAB ELISA method. Many of these cell lines proved to be unstable and stopped producing antibody after a short time in culture. However a panel of clones were chosen for further investigation according to their in vitro stability and the staining pattern they produced on endothelial monolayers. Four major staining patterns

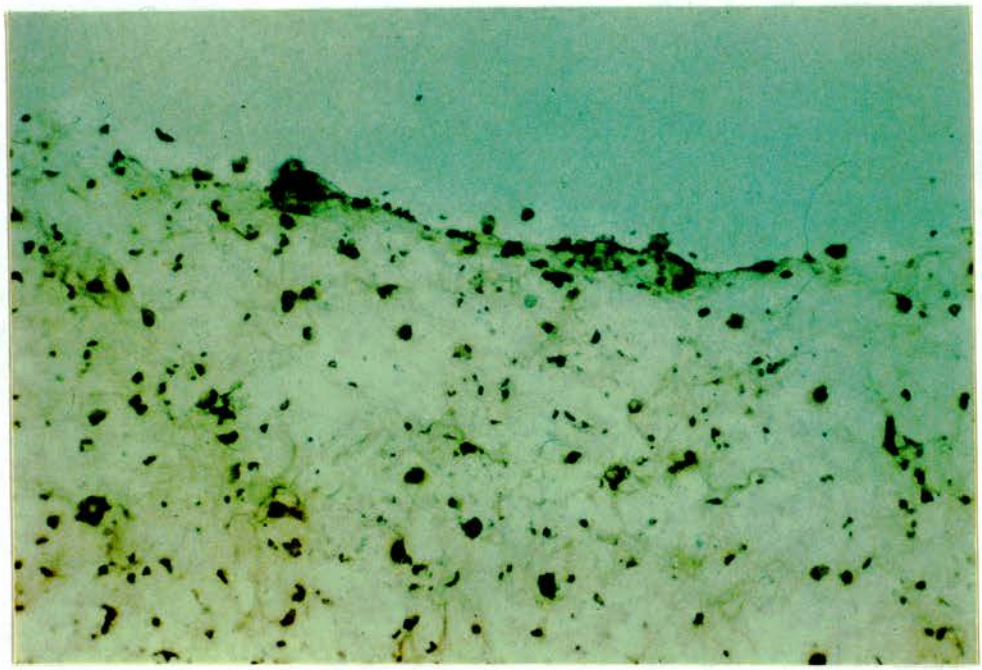


FIG.3.3. Diffuse "speckly" staining pattern of B9B8 on human umbilical vein endothelium.

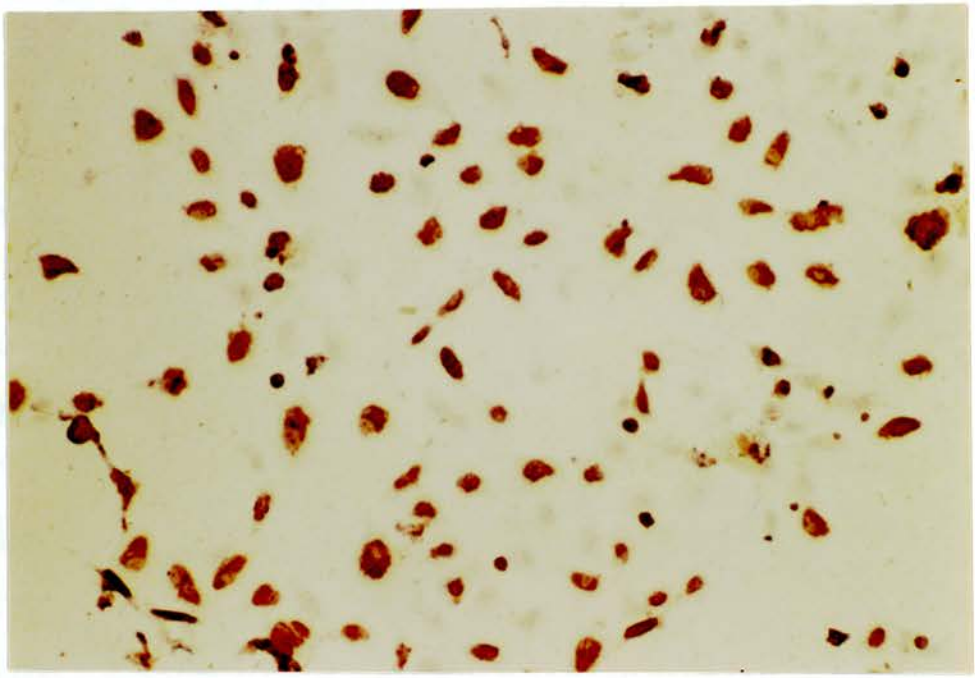


FIG.3.1. Diffuse staining pattern of D6F1 on bovine aortic endothelium. Mayer's haematoxylin counterstain.

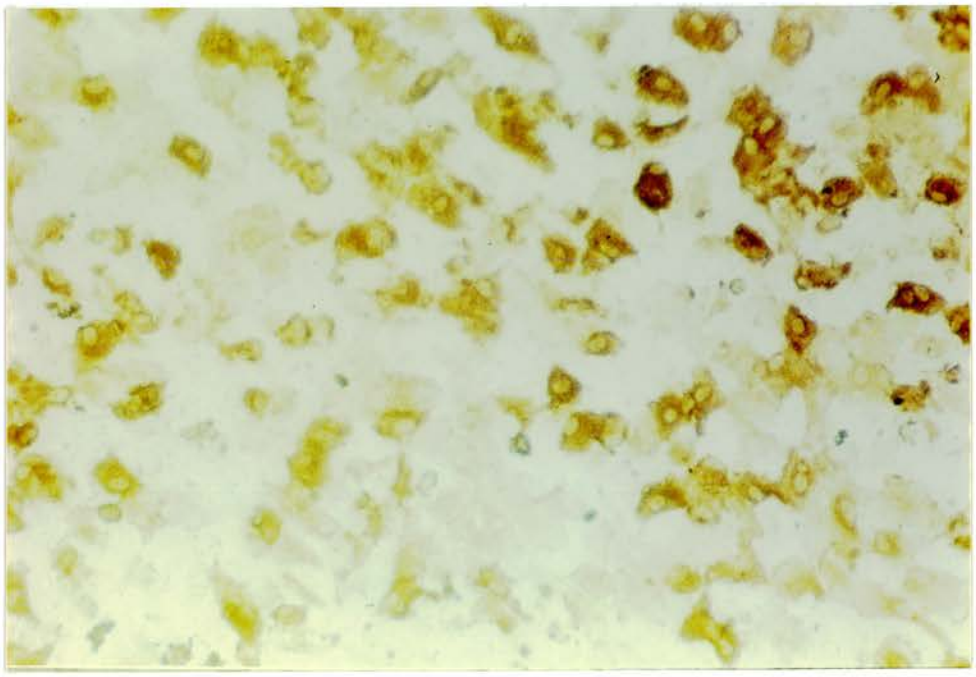


FIG.3.2. Diffuse staining pattern of B9G6 on human umbilical vein endothelium.

were observed and are described in the following sections.

3.3. DIFFUSE STAINING.

The antibodies in this group bound to non - deliberately damaged endothelial cells giving a general non-specific staining pattern. Fig. 3.1 shows antibody D6F1 on bovine aortic endothelial cells. Fig.3.2 shows antibody B9G6 on human umbilical vein endothelium. This antibody stained the cytoplasm of all the cells in the monolayer but with varying intensity. Fig.3.3 shows antibody B9B8 on human umbilical vein endothelium. This antibody gave a "speckly" diffuse staining pattern and may in fact be due to cell alteration or damage
There appears to be no increase in staining at the scratch edge with this antibody.

Of the antibodies producing a diffuse staining pattern only D6F1 was produced in quantity for further investigation. The others were either unstable in culture or are still in liquid nitrogen storage awaiting further investigation.

3.4. NEGATIVE STAINING.

Two antibodies, B10A10 and B10C10, clones from the same cell line appeared to stain the plastic around the cells in a culture of HUVEC where some cell detachment

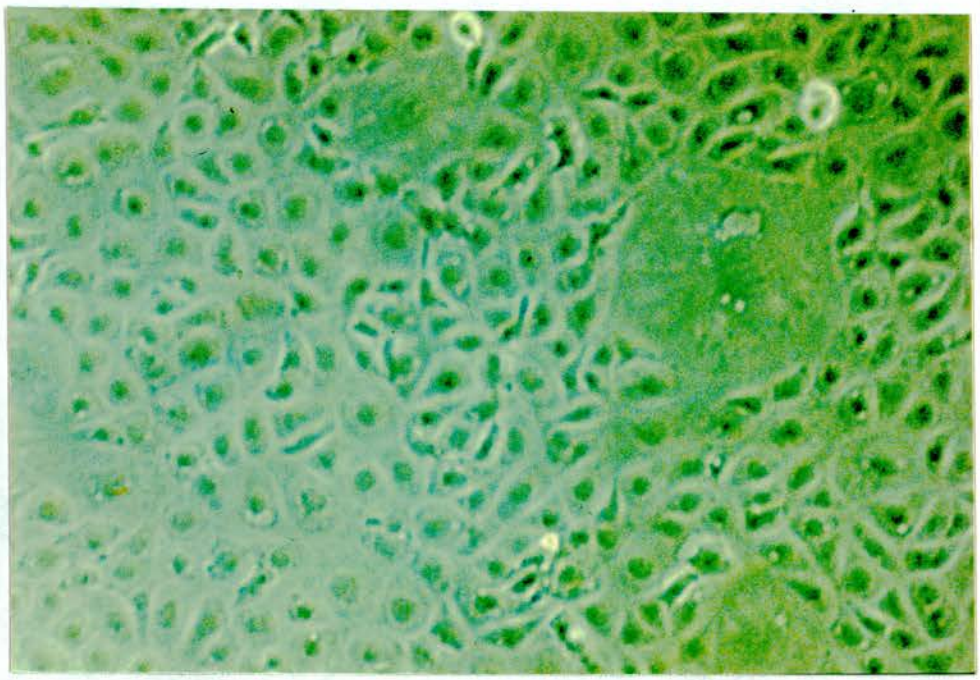


FIG.3.5. Bovine aortic endothelial cell monolayer showing numerous giant cells.

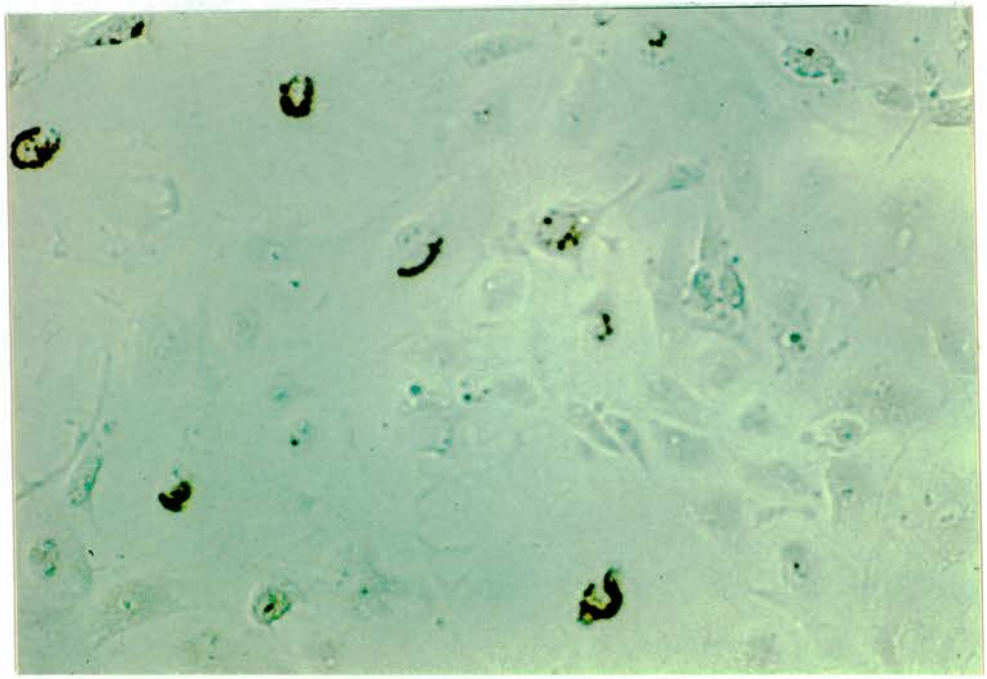


FIG.3.6. Perinuclear staining of giant cells by C9F8. Adult human saphenous vein endothelium.

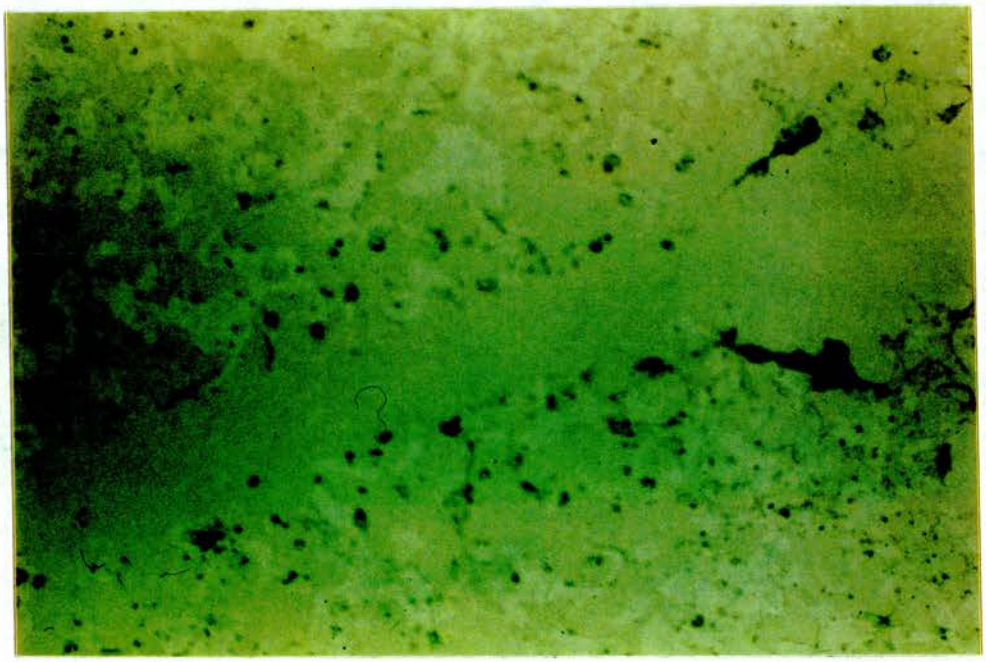


FIG.3.4. Negative staining pattern of B10A10 on human umbilical vein endothelium.

had occured. Very little cell staining was apparent (Fig.3.4). Control experiments show that the antibodies did not bind to untreated plastic or the fibronectin or gelatin surface on which HUVEC were grown.

3.5. GIANT CELL / PERINUCLEAR STAINING.

In vitro cell senescence is typified by the presence of giant multinucleate cells (Haudenschild 1975, Rosen et al 1981). Fig.3.5 shows a control bovine aortic cell monolayer with numerous giant cells. Fig.3.6 shows perinuclear staining of these giant cells on adult human saphenous vein endothelium (SAVEC) with antibody C9F8. Similar staining patterns were also seen with antibodies C14H11 and C11D7. This type of perinuclear staining was only seen with antibodies directed to SAVEC.

3.6. DAMAGED CELL STAINING.

Narrow scratch damage has been used as a method of looking at endothelial repair after injury in vivo (Reidy and Schwartz 1980). Here a similar in vitro scratch damaged / regrowing model was used for screening monoclonal antibodies. Scratches were made with a glass pasteur pipette the end of which was approximately 1mm in diameter. This produced denuded areas of approximately 10 to 15 cells wide. Two

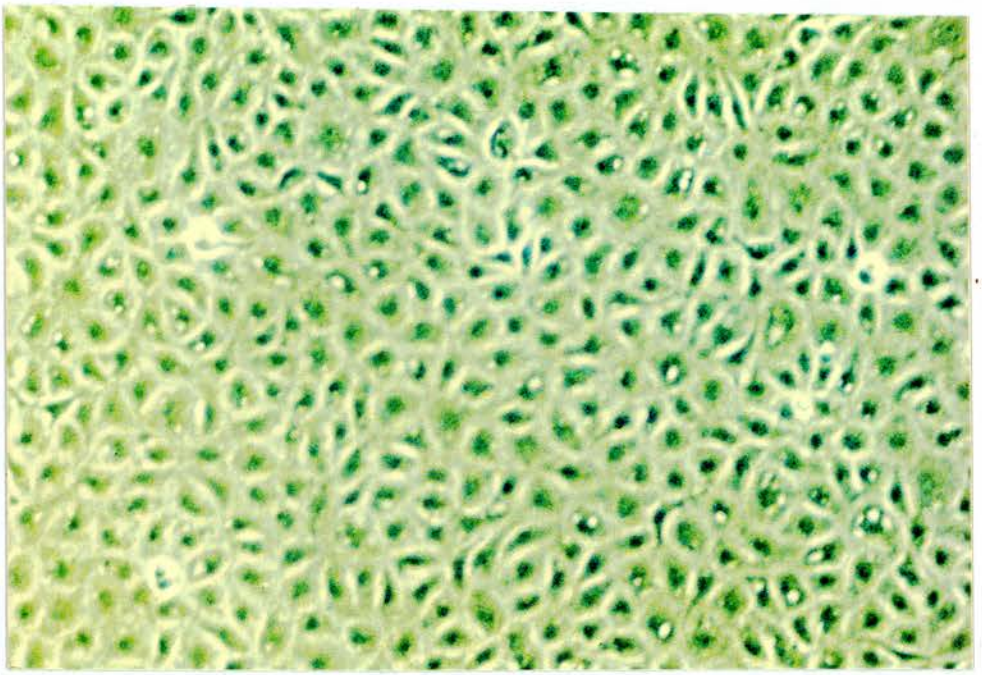


FIG.3.7. Confluent monolayer of bovine aortic endothelial cells.

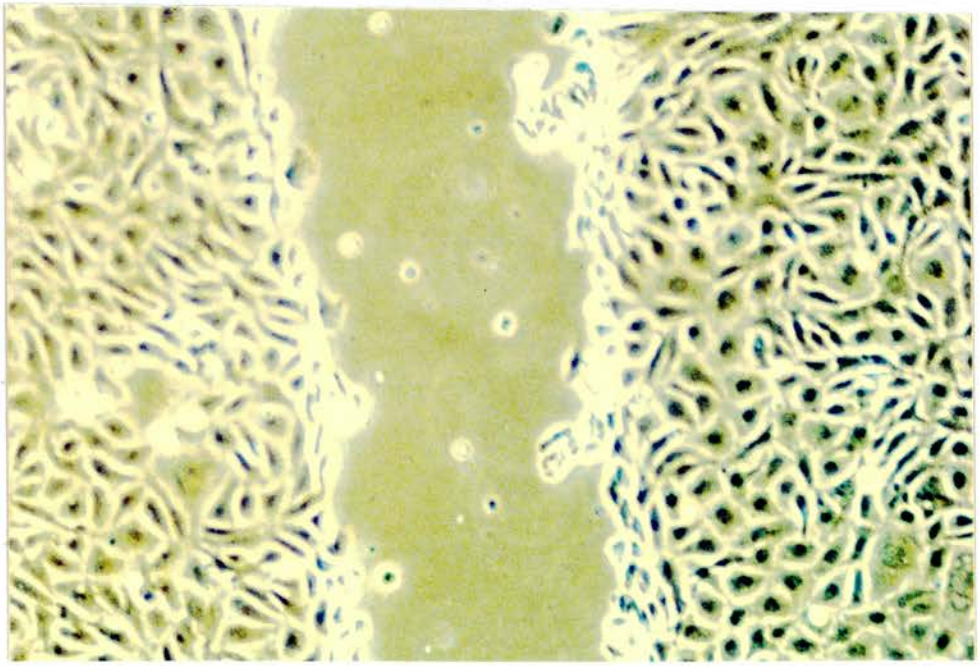


FIG.3.8. Scratch damaged monolayer of bovine aortic endothelial cells.

scratches were made approximately 0.5cm apart across the centre of each well. Figs. 3.7 and 3.8 show a confluent monolayer of bovine aortic endothelial cells before and after scratch damage.

Antibodies which reacted with scratch damaged cells were tested on cells damaged by other methods. Cell damage was also assessed by the non - immunological cytochemical stains trypan blue and acridine orange / ethidium bromide (AO/EB). Damaged plasma membrane allows entry of 0.1% trypan blue into the cytoplasm where it binds to intracellular proteins. With AO/EB (one-part-per-million solution of acridine orange and ethidium bromide in PBS. One part cell suspension was mixed with one part AO/EB solution) live cells metabolise the dye and fluoresce bright green whereas dead cells fluoresce brown/orange.

Table 3.1 shows the antibodies staining damaged cells. Antibody subtype was determined by Ouchterlony double diffusion analysis (Mouse Monoclonal Typing Kit, The Binding Site Ltd., University of Birmingham).

3.6.1 Non-specific staining of damaged cells by IgG.

Investigations by Hansson et al (1979 and 1980) show the accumulation of IgG in injured endothelial cells . It has been suggested that IgG binds by the Fc region of the molecule to the vimentin component of

ANTIBODY	SPECIES OF E.C.	Ig SUBTYPE	IMMUNISATION PROTOCOL
A3E10	BAEC	IgM	1
*B7H5	HUVEC		1
*B8D2	HUVEC		1
B10F1	HUVEC	IgM	1
*B17B11	HUVEC		1
B17E4	HUVEC	IgM	1
*D5G2	BAEC		2
D6G11	BAEC	IgM	2
D8G8	BAEC	IgM	2
P13C1	PAEC	Not known	1
P14G11	PAEC	IgG2a	1

TABLE 3.1. Monoclonal antibodies to scratch damaged endothelium. Cell lines * were unstable in culture and stopped producing antibody after 2 to 3 subcultures.

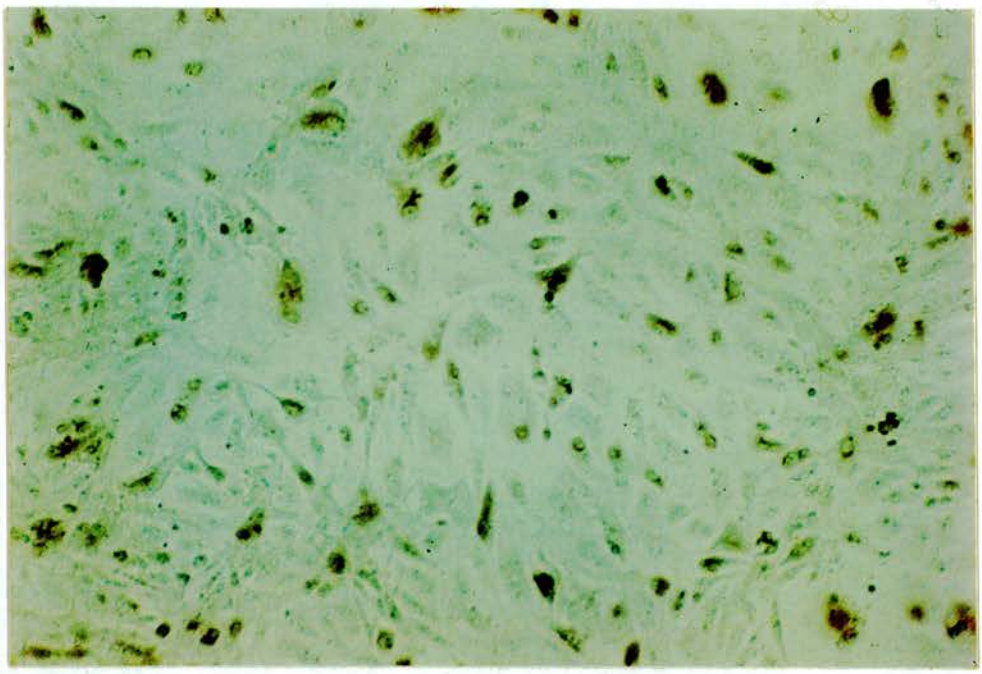


FIG.3.9. Non-specific staining of fixed bovine aortic endothelial cells by mouse IgG.

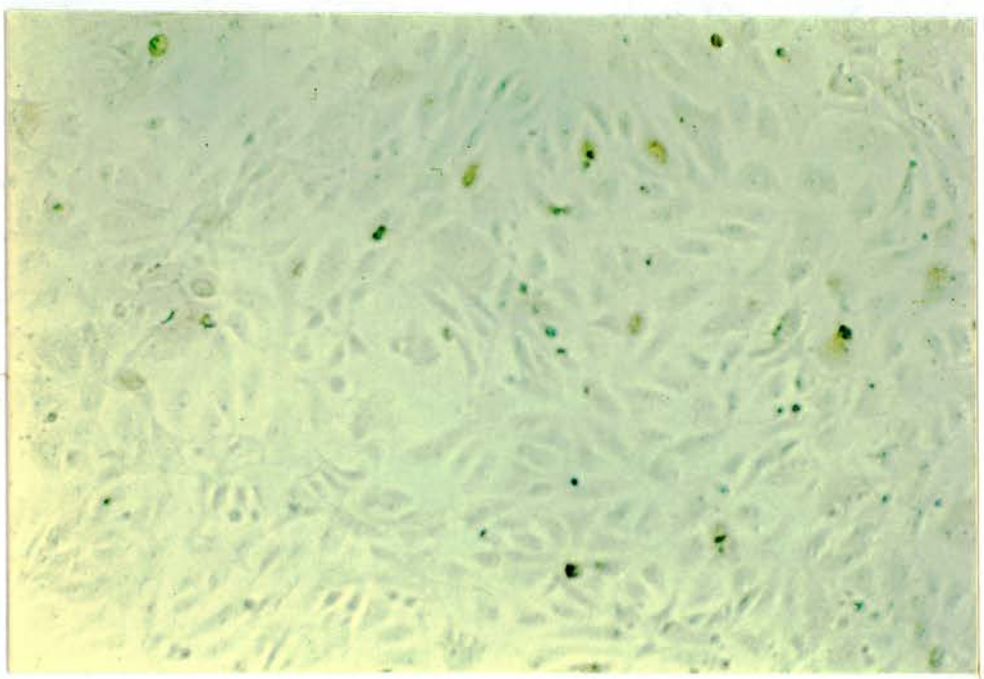


FIG.3.10. Pre-incubation of bovine aortic endothelial cells with autologous IgG blocks non-specific binding of mouse IgG.

cytoplasmic intermediate filaments (Hansson and Schwartz 1984).

Changes in the plasma membrane permeability of damaged cells permits access of IgG molecules to cytoplasmic proteins. Fig.3.9 shows the presence of mouse IgG in bovine endothelial cells at the monolayer edge. These cells had not been deliberately damaged in any way prior to fixation and it was assumed that damaged had occured during the fixation process (0.025% glutaraldehyde). (see section 3.7). The cells at scratch edges also showed IgG accumulation.

Incubation of fixed and non-fixed cells with an excess of autologous serum or IgG, in this case neat newborn calf serum (NBCS), prior to mouse IgG incubation and staining, blocks the non-specific binding sites (Fig.3.10). The peroxidase conjugated second antibody used in the staining procedure is directed to mouse immunoglobulins and therefore does not recognise the bovine IgG on the non-specific binding sites. Therefore, in all following in vitro investigations with monoclonal antibodies the cells were preincubated with serum or non-mouse IgG prior to monoclonal antibody staining.

In conjunction with the cytochemical stains damage to the cells was assessed by the presence of IgG.

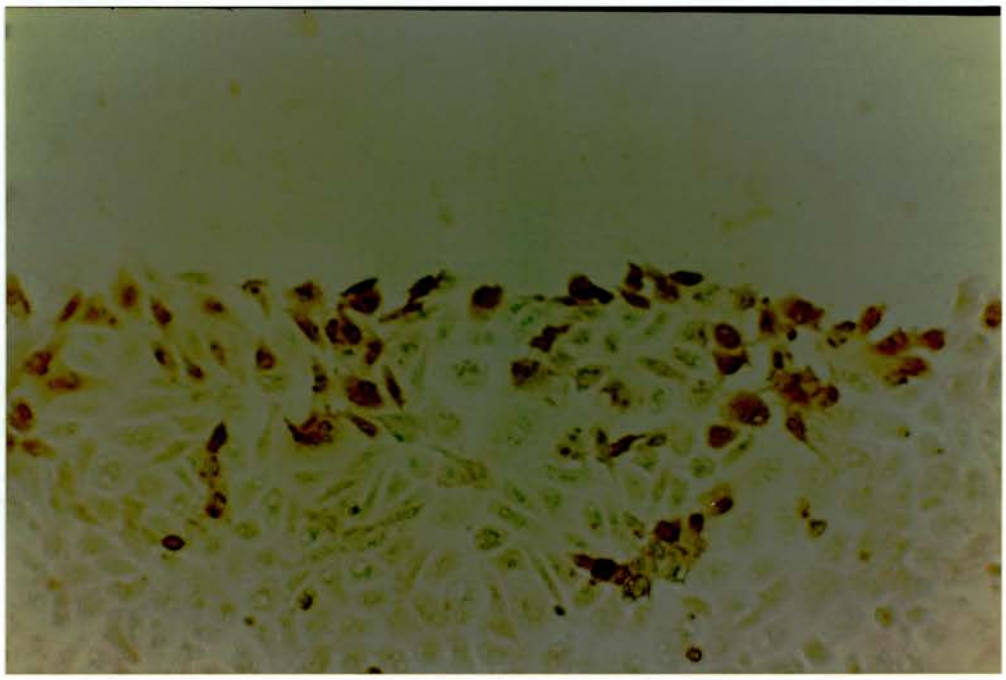


FIG.3.13. P14G11 stained scratch damaged bovine aortic endothelial cells, showing damaged cells away from the edge of the scratch.

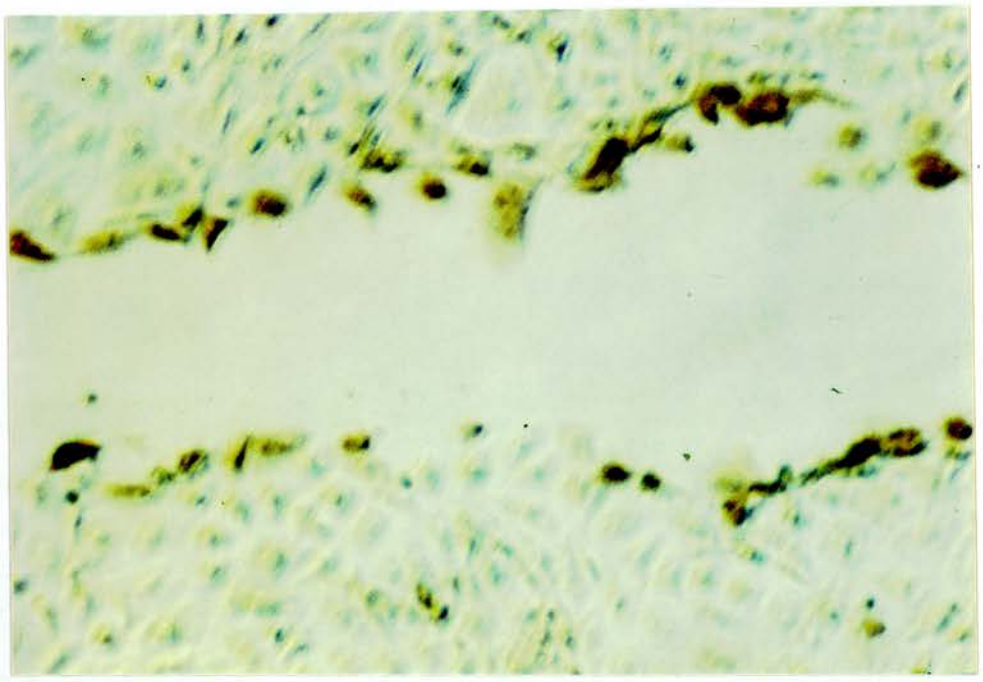


FIG.3.11. P14G11 stained scratch damaged pig aortic endothelial cells.

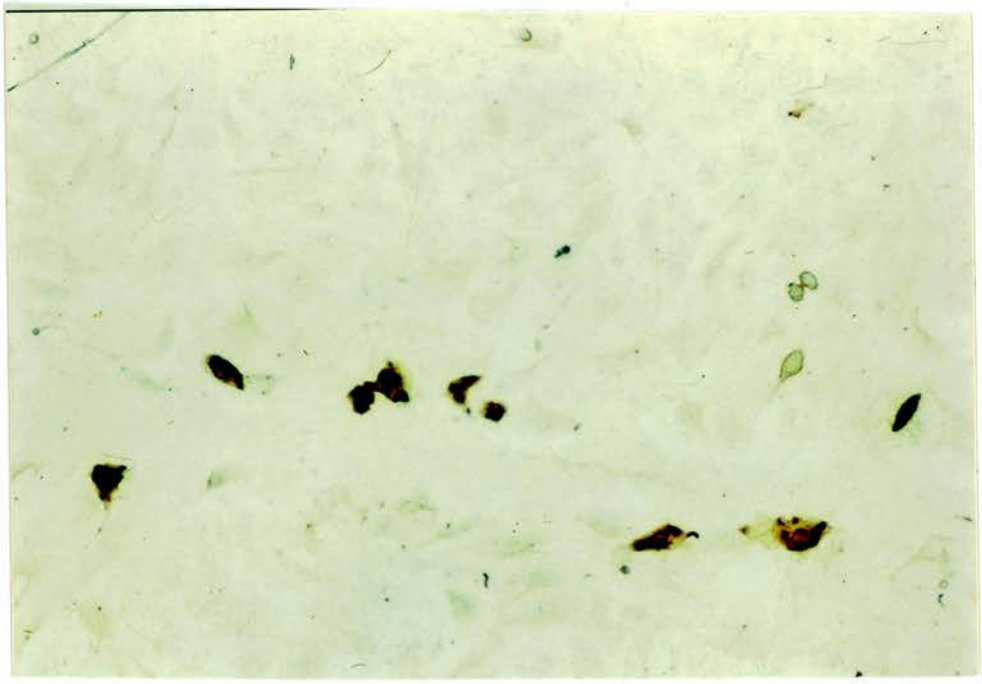


FIG.3.12. D8G8/B8 stained scratch damaged bovine aortic endothelial cells.

3.6.2. Scratch Damage.

On secondary screening , a group of antibodies were found to stain the cells along the scratched edges of otherwise normal endothelial monolayers (Fig.3.11 and 3.12). These antibodies are listed in Table 3.1. Hybridomas from the adult human saphenous vein endothelium immunised mice did not produce antibodies to scratch damaged cells.

In most instances the antibody bound to cells immediately adjacent to the scratch edge, as seen in Figs.3.11 and 3.12. However, in some cases, especially those monolayers damaged by wider scratches, damaged cells were evident up to 10 cells in from the scratch edge (Fig.3.13). Antibody stained cells were still physically attached to the plastic surface. Cells which had been removed from the scratch area and were floating free in the medium also stained intensely. Trypan blue and AO/EB staining of these cells confirmed that they were not viable. Not all the cells at the scratch edge were damaged, as some excluded trypan blue. This was also apparent on antibody staining (Fig.3.12).

3.6.3. Regenerated scratch damaged cells.

Scratch damaged cell monolayers were allowed to regrow completely before fixing and staining. A scratch

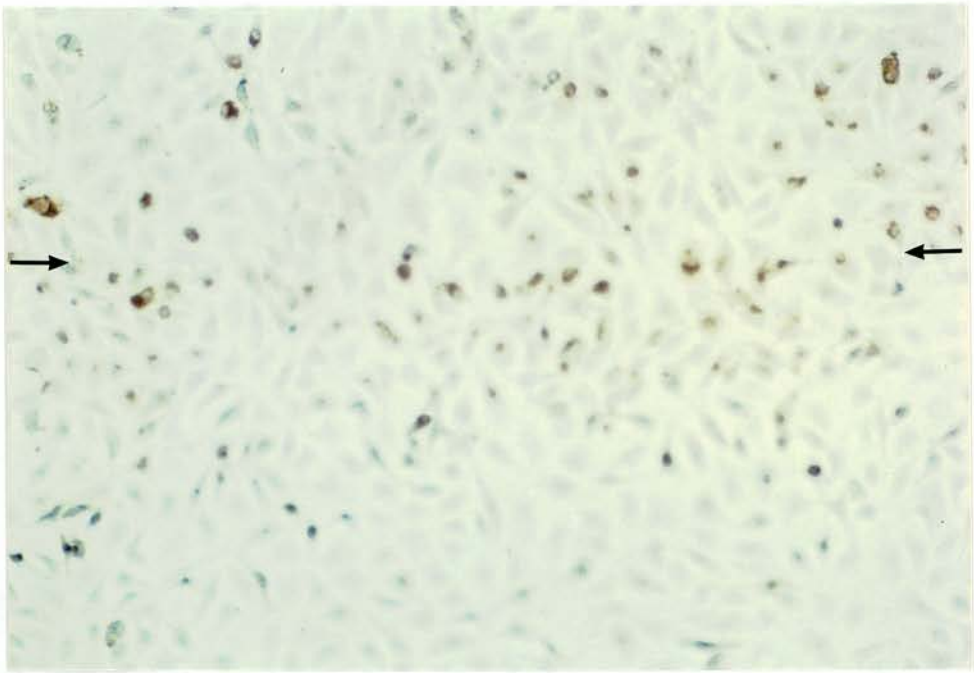


FIG.3.14. Regrown scratch (arrows) damaged bovine aortic endothelial cells. D8G8/B11. Mayer's haematoxylin counterstain.

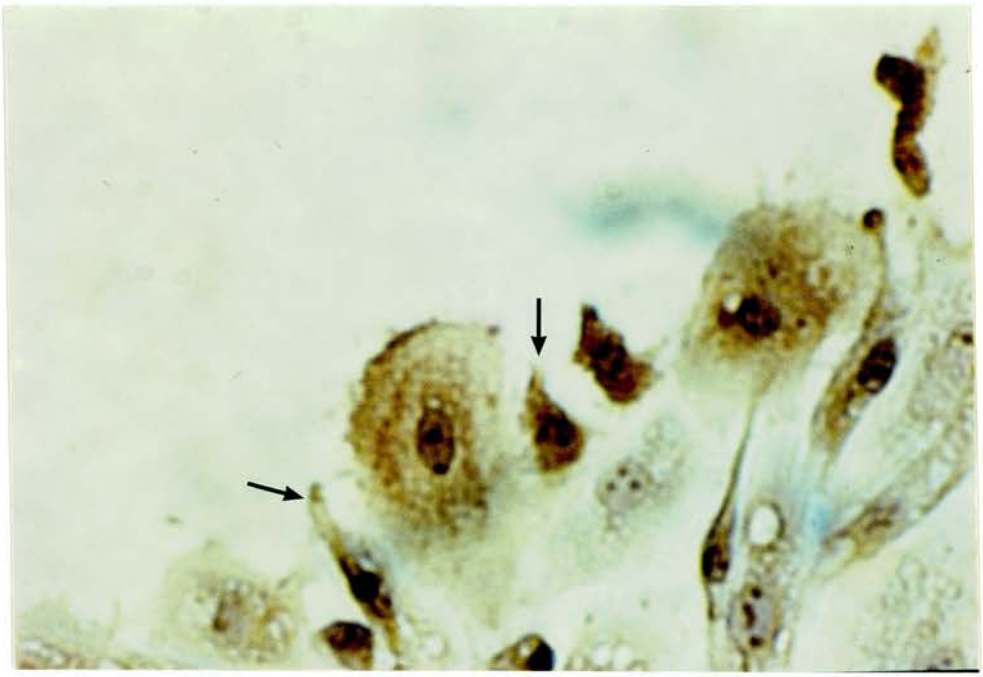


FIG.3.15. Regenerating cells at a scratch edge two hours after scratching. D8G8/B11. Magn.x400. Mayer's haematoxylin counterstain.

of 5 to 10 cells wide regrows completely in approximately 24 hours. Fig.3.14 shows a regrown scratch area (indicated by arrows). Antibody D8G8/B8 bound to cells in this area of regrowth making the line of the scratch quite visible even after monolayer regeneration (approximately 24 hours after initial damage). Fig.3.15 shows at high magnification, cells at the scratch edge stained with antibody D8G8/B8 one hour after scratching. The stained cells show signs of regeneration, elongation in the direction of growth and cytoplasmic lamellapodia (arrows) as the cells migrate into the denuded area.

3.6.4. Air Dried Damage.

Air drying has been used as a model of damage in in vivo studies (Clowes and Karnovsky 1977).

Growth medium was removed from confluent monolayers and cells were allowed to dry out at 37^{o}C. At intervals of 5 minutes up to 30 minutes samples of cells were fixed in 0.025% glutaraldehyde, treated with autologous serum or non-mouse IgG to block non-specific binding sites and treated with antibody in the standard screening procedure . At times up to 20 minutes little damage was apparent with trypan blue and antibody screening. After 20 minutes air drying extensive staining is seen with both antibody P14G11 and trypan

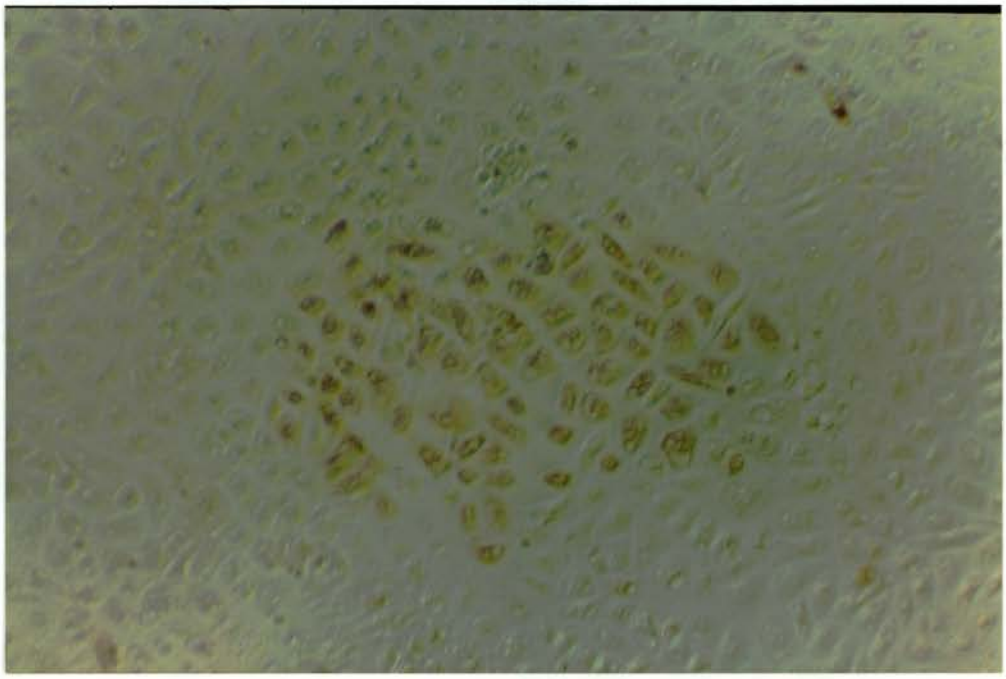


FIG.3.18. Heat damaged bovine aortic endothelial cells. A3E10/D11.

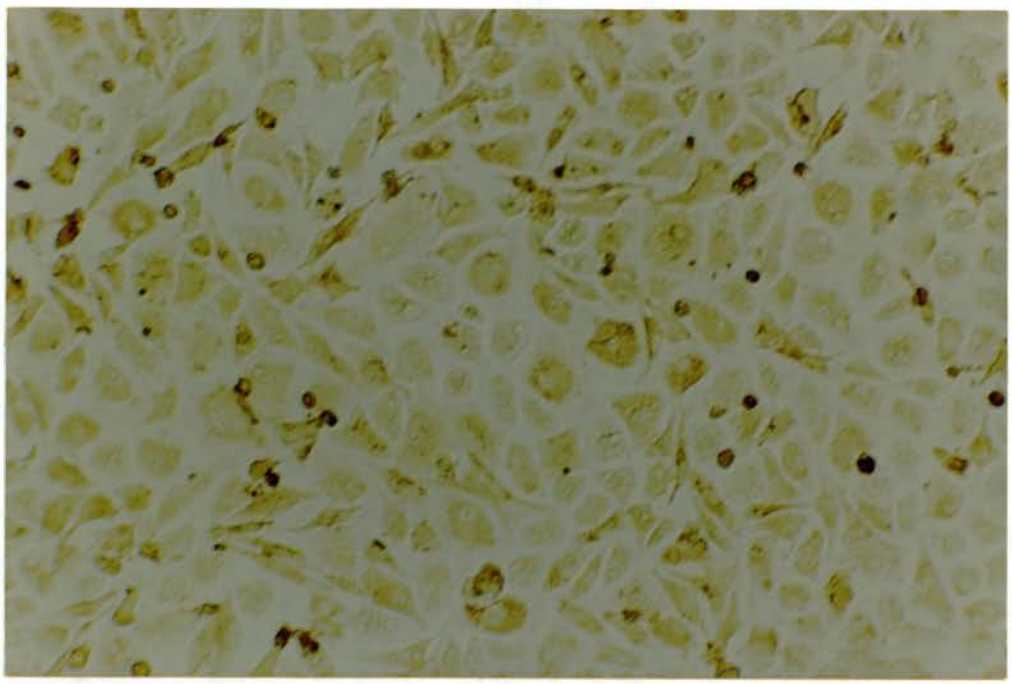


FIG.3.16. P14G11 stained air dried damaged bovine aortic endothelial cells.

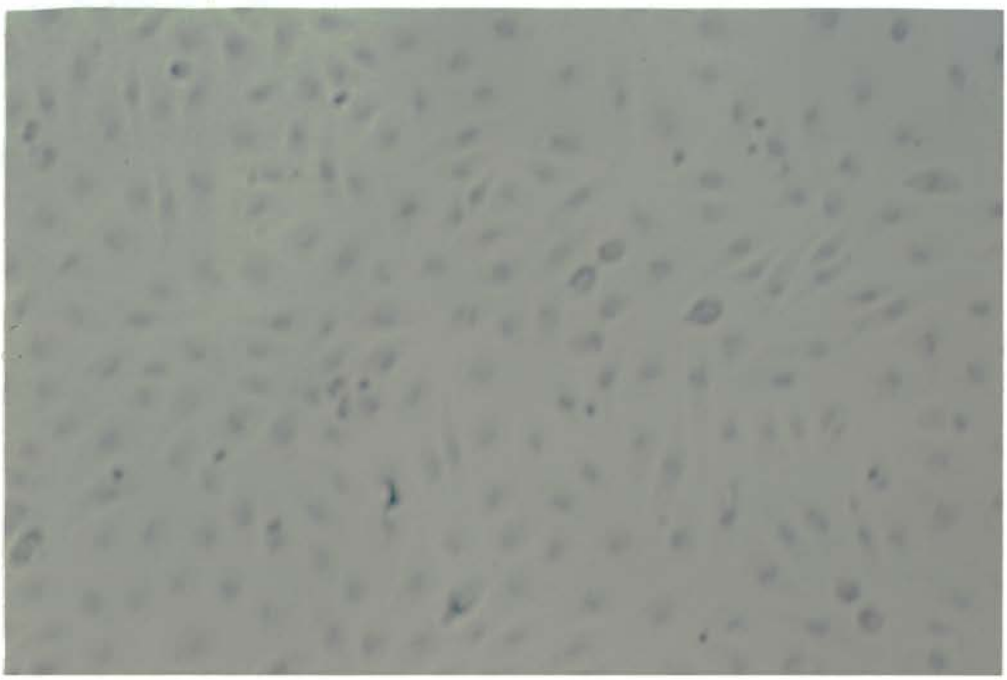


FIG.3.17. Air dried damaged bovine aortic endothelial cells showing trypan blue uptake.

blue. (Figs.3.16 and 3.17).

3.6.5. Heat Damage.

This effect was noticed as an aside to the scratch damage method. The scratches are made with a glass pasteur pipette which was heated in a bunsen flame to bend the tip to a 90^{o} angle, to allow easier access to the cell surface in the 24 well plates. On screening with antibody a small round patch of cells at the start of the scratch stained heavily . This patch of cells corresponded in size to the pipette tip and in position to where the pipette was introduced to the cell surface. The procedure was repeated without scratching the monolayer or any physical contact with the cells and before the pipette tip was completely cool. On staining for antibody (A3E10/D11) a group of cells corresponding to the hot pipette tip was apparent (Fig.3.18). It was assumed that heat had caused the damage to the patch of cells.

3.6.6. Hyperoxic Damage.

Prolonged exposures to high partial pressures of oxygen can result in alterations of structure and function of many organs (Clark et al). Various theories have been proposed as to the mechanisms of O_2

toxicity. The production and action of free radicals being the favoured theory at present time.

Investigations into the effect of hyperoxic conditions on cultured endothelial cells have shown increases in lactate dehydrogenase (LDH) release and decrease in 5-hydroxytryptamine (5-HT) uptake which are both indicative of cell injury. These studies have also shown a decrease in catalase (CAT) activity and an increase in superoxide dismutase (SOD) activity. (Block et al 1985) These changes in antioxidant enzyme activity suggest an increase in generation and a decrease in elimination of hydrogen peroxide (H_2O_2) in endothelial cells exposed to high O_2 levels. This would in turn lead to an increase in in the intracellular production of hydroxal radical which has also been implicated in cell injury (Varami et al 1985).

Cells were grown as normal until confluent and changed to a normal growth medium saturated with pure oxygen (>95% O_2). The cells were placed in a modular incubator chamber (Flow Laboratories) which was flushed with oxygen for 10 minutes and then sealed and incubated at 37^{o}C. Fresh O_2 was added as above every 12 hours. At 48 and 96 hours the cells were fixed in glutaraldehyde and stained with monoclonal antibody. The results are shown in Table 3.2.

Very little staining if any, was seen after 48

ANTIBODY	CELL DAMAGE	
	48 hours	96 hours
A3E10/D11	-	+++
D6G11/E12	-	++
D8G8/B8	(+)	++
D8G8/B11	(+)	++
IgG CONTROL	(+)	++
NBCS CONTROL	-	-
PER.AB CONTROL	-	-
DAB CONTROL	-	-

TABLE 3.2. Hyperoxic damage as detected by the monoclonal antibodies above. (+) indicates some staining of cells around the well edge only. ++ moderate staining, +++ strong staining. PER.AB - peroxidase conjugated second antibody.

ANTIBODY	CELL DAMAGE	
	48 hours	96 hours
A3E10/D11	(+)	++
A3E10/D11*	-	-
D6G11/E12	(+)	++
D6G11/E12*	**(+)**	-
D8G8/B11	+	+
D8G8/B11*	-	-
CONTROL	-	-
CONTROL*	-	-

TABLE 3.3. Inhibition of hyperoxic damage by ascorbic acid. * indicates cultures treated with ascorbic acid prior to incubation in oxygen. (+) indicates staining at the well edges only.

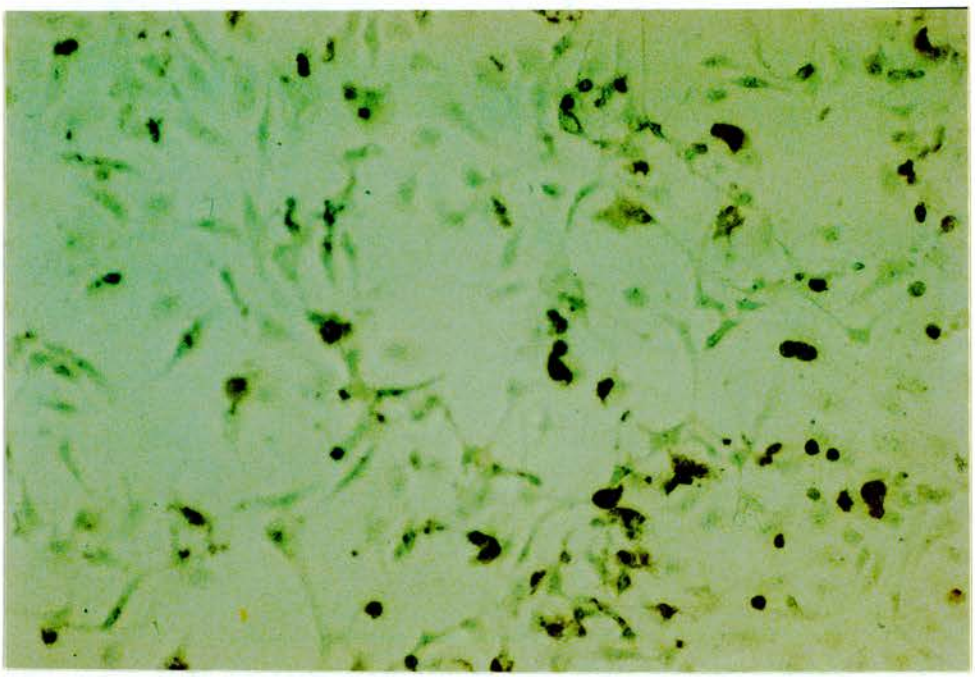


FIG.3.19. Oxygen damaged bovine aortic endothelial cells stained with D8G8/B8.

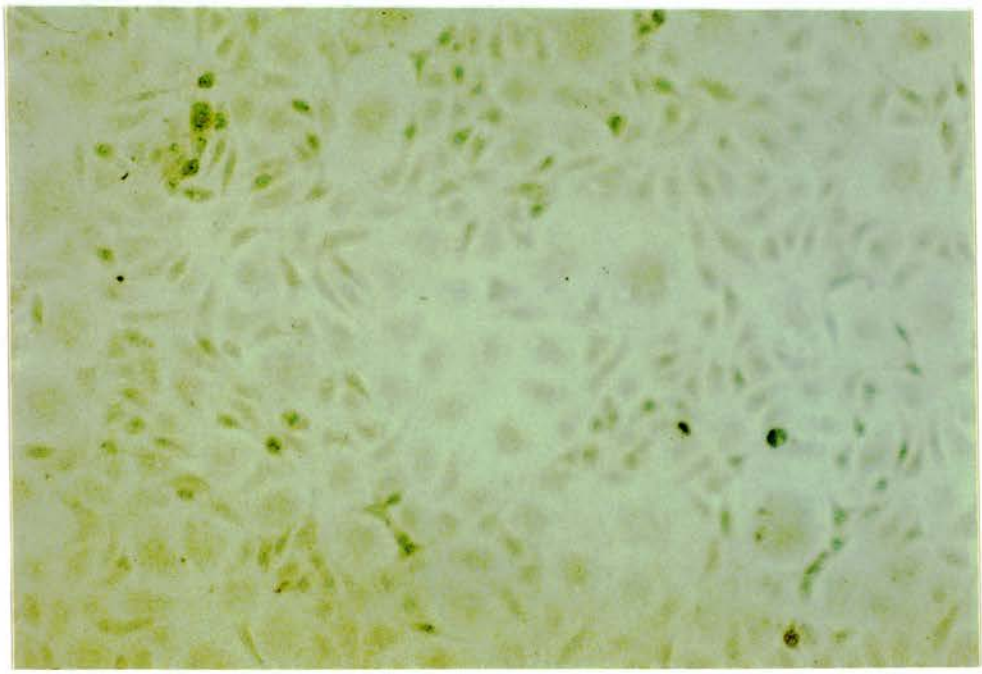


FIG.3.20. Non-damaged bovine aortic endothelial cells grown in 5% CO_2 in air. D8G8/B8.

hours, however extensive staining was present after 96 hours in a high oxygen atmosphere. Fig.3.19 shows bovine aortic endothelial cells stained with D8G8/B8 after 96 hours in hyperoxic conditions. The monolayer configuration has been disrupted and cells look spindly and straggly. Fig.3.20 shows a control bovine aortic endothelial cell culture grown in a normal 5% CO_2 in air atmosphere and treated with antibody D8G8/B8. Cell morphology looked normal and no antibody staining was apparent.

This experiment was repeated in the presence of 1mM ascorbic acid as an antioxidant. The results are shown in Table 3.3. Ascorbic acid appears to inhibit the damage caused by hyperoxa to some extent.

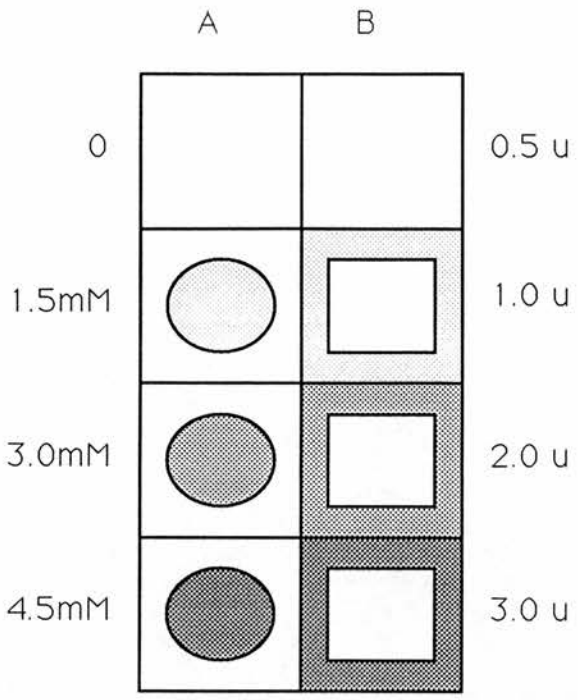
Control cells were treated with the same panel of antibodies after incubation in a 5% CO_2 in air atmosphere, with and without 1mM ascorbic acid . Results are shown in Table 3.4, Fig.3.20. No damage had occurred in these control cells.

3.6.7. Hydrogen Peroxide Damage.

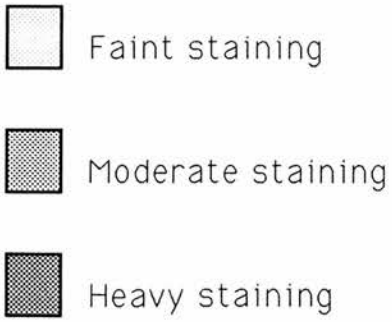
Hydrogen peroxide (H_2O_2) has been implicated in endothelial cell injury in a number of situations such as in hyperoxia (section 3.6.6.), homocystinuria (Starkebaum and Harlan 1986) and with the respiratory burst associated with granulocyte activation which

ANTIBODY	CELL DAMAGE	
	48 hours	96 hours
A3E10/D11	(+)	(+)
D6G11/E12	-	-
D8G8/B8	-	-
D8G8/B11	(+)	-
IgG CONTROL	(+)	-
NBCS CONTROL	-	-
PER.Ab CONTROL	-	-
DAB CONTROL	-	-

TABLE 3.4. Assessment of damage to cells grown in 5% CO_2 in air atmosphere, control results to hyperoxic damage. (+) indicate staining of cells at well edge only. Control cells treated with 1mM Ascorbic acid showed the same results.



DIAG. 3.1 Diagramatic representation of cell staining after damage by hydrogen peroxide (A) and thrombin (B). Only the cells at the well edge stained with thrombin, the cells in the centre of the well remained undamaged.
Cells at the well edge were lost after treatment with hydrogen peroxide leaving stained damaged cells in the centre of the well.



occurs at the vessel wall during the inflammatory response. (Weiss et al 1981, Harlan et al 1981, Ager et al 1984).

Cells were grown to confluence and the growth medium removed and replaced with varying concentrations of H_2O_2 in serum free medium 199 (M199). During incubation of the cells at 37^{o}C their condition was checked every 5 minutes to make sure that detachment had not taken place, as this would have meant loss of cells during the staining procedure. After 30 minutes incubation the cell surface looked very patchy with ill - defined cell borders, therefore the medium was removed and the cells fixed and stained with P14G11. The results are shown on Table 3.7. The staining was different here from the other experiments in this group. All cells were stained in the centre of the well. The cells around the well edge were lost during fixing and washing stages. This and the obvious difference in degree of staining with increasing peroxide concentration was easily seen when the plate was viewed with back illumination. (Diag.3.1.)

3.6.8. Endotoxin Damage.

Endotoxin, a gram - negative bacterial lipopolysaccharide (LPS) is known to be pyrogenic and

is associated in the pathogenesis of septic disseminated intravascular coagulation (Bradley 1974). It produces a number of direct effects on endothelial cells such as pyknosis, release of lactate dehydrogenase and an increase in prostacyclin production (Meyrick et al 1986, Sage et al 1986).

Cells were grown as normal until confluent. The growth medium was removed and replaced with 0.5 to 2.0 ug/ml of endotoxin (E.Coli serotype 055:B5 - Sigma) diluted in serum free M199. The cells were incubated with the endotoxin at 37^{o}C for 2 hours. At the end of this time the medium was removed and the cells rinsed gently with PBS. They were then fixed and stained with antibody P14G11. The results are shown in Table 3.5. Very little staining was seen except at the cells around the well edge. There was no noticeable difference in the the amount of staining with increasing endotoxin concentration.

3.6.9. Thrombin Damage.

Thrombin has been implicated in increased permeability of endothelium during inflammation as shown by uptake of Evan's Blue (Killackey et al 1986). It has also been shown to cause contraction and cell damage as indicated ^{51}Cr release in intact, isolated blood vessel segments (Lough and Moore 1975). In

LPS CONC.(ug/ml)	CELL DAMAGE
0.5	-
1.0	+
1.5	+
2.0	+

TABLE 3.5. Endotoxin damage to bovine aortic endothelial cells as detected by antibody P14G11.

THROMBIN CONC. (I.U)	CELL DAMAGE
0.5	-
1.0	+
2.0	++
3.0	+++

TABLE. 3.6. Thrombin damage to bovine aortic endothelial cells as detected by antibody P14G11.

$H_2 O_2$ CONC.(mM)	CELL DAMAGE
1.5	+
3.0	+++
4.5	++++

TABLE 3.7. Hydrogen peroxide damage to bovine aortic endothelial cells as detected by antibody P14G11.

studies with cultured endothelial cells, thrombin at low concentrations has induced endothelial injury (Barnhart and Chen 1978).

Cells were grown to confluence. The growth medium was removed and replaced with varying concentrations of thrombin in serum free M199 and incubated at 37^{o} C for 2 hours. Cells were then rinsed, fixed and stained with antibody P14G11. The results are shown in Table.3.6. This was difficult to assess under the microscope, however when the plate was examined with back illumination a marked difference could be seen in degree of staining with increasing thrombin concentration. The staining was confined to the cells at the edge of the well, the area of which increased with increasing amounts of thrombin. (Diag.3.1).

3.7. CELL STAINING AS A RESULT OF NON-DELIBERATE DAMAGE.

When DAB ELISA screening antibodies to scratch damaged monolayers was carried out in 24 x 16mm well plates, secondary staining was apparent which was not associated with the scratch damage. An area of cells around the well edge stained intensely either as single cells or as small patches of cells. The area of staining was very consistently confined to a ring of cells approximately 10 cell widths from the edge of the

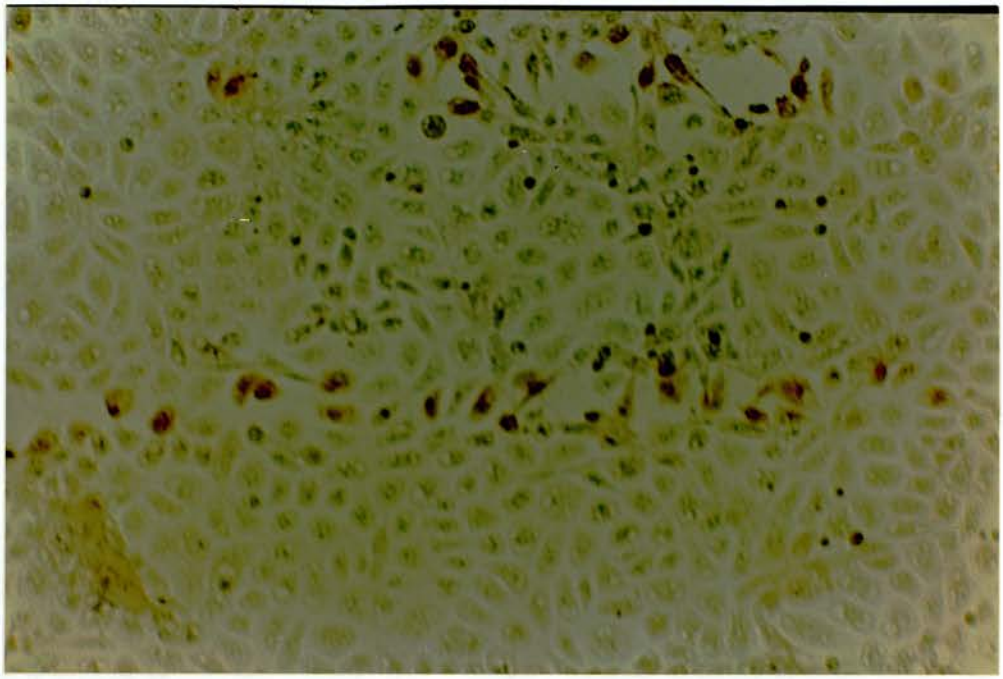


FIG.3.23. P14G11 stained bovine aortic endothelial cells at the well edge. Non-deliberate damage.

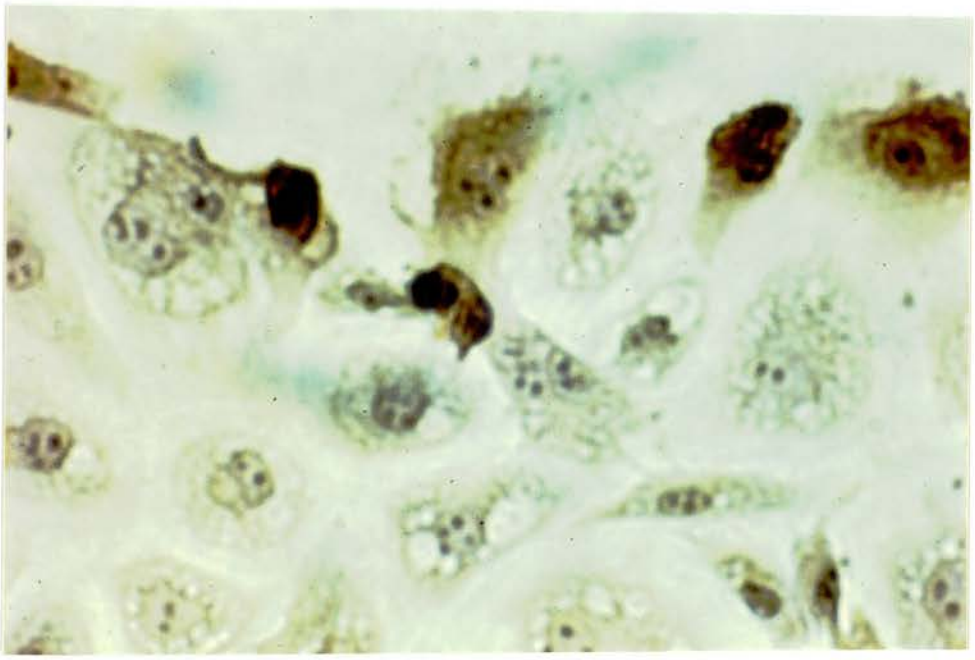


FIG.3.24. High magnification (x400) of fig.3.24. Mayer's haematoxylin counterstain.

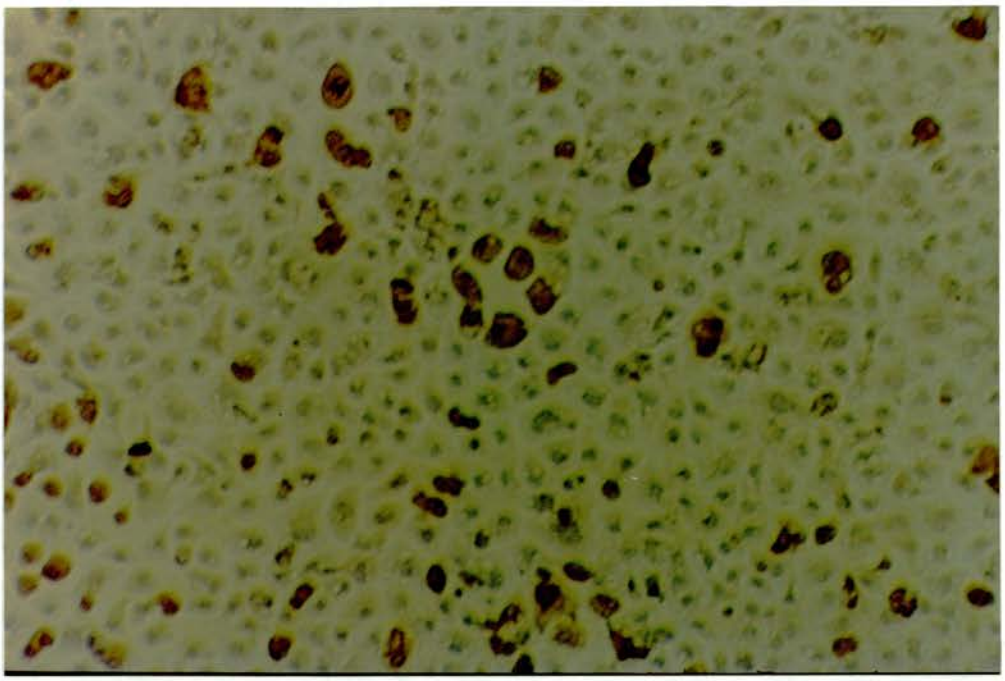


FIG.3.21. D8G8/B11 stained bovine aortic endothelial cells at the edge of the culture well. Mayer's haematoxylin counterstain.

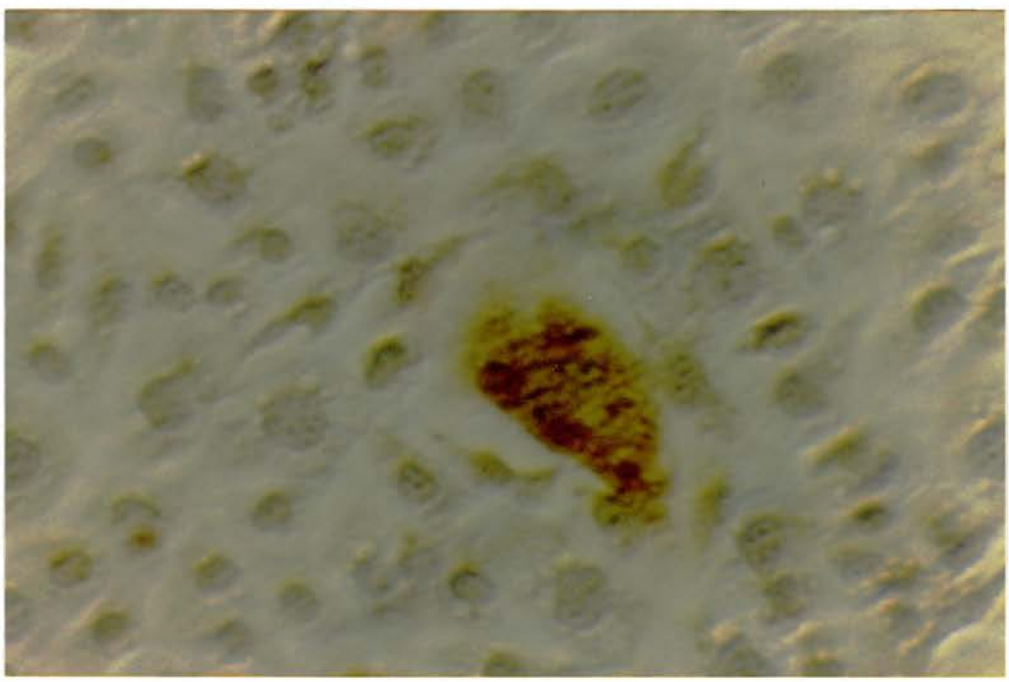
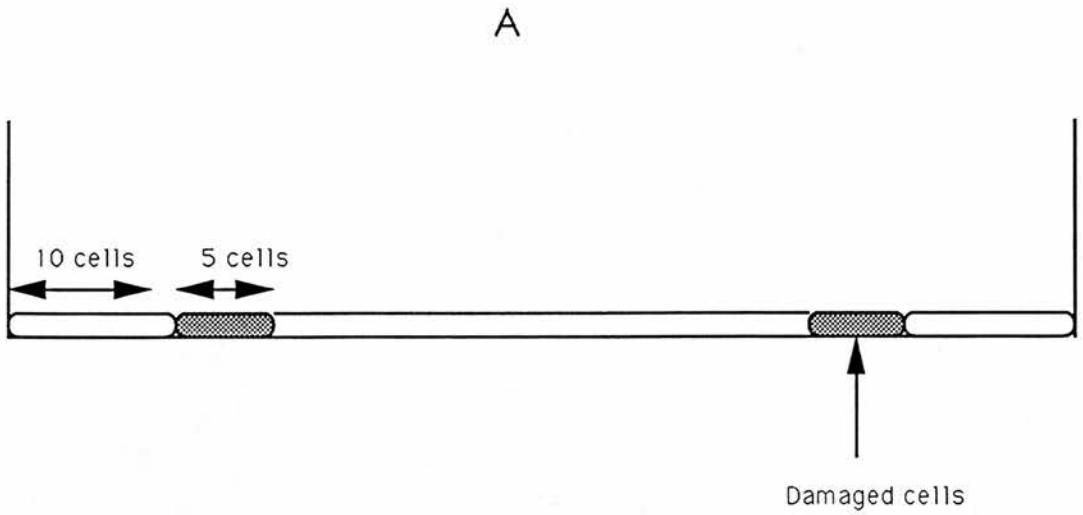
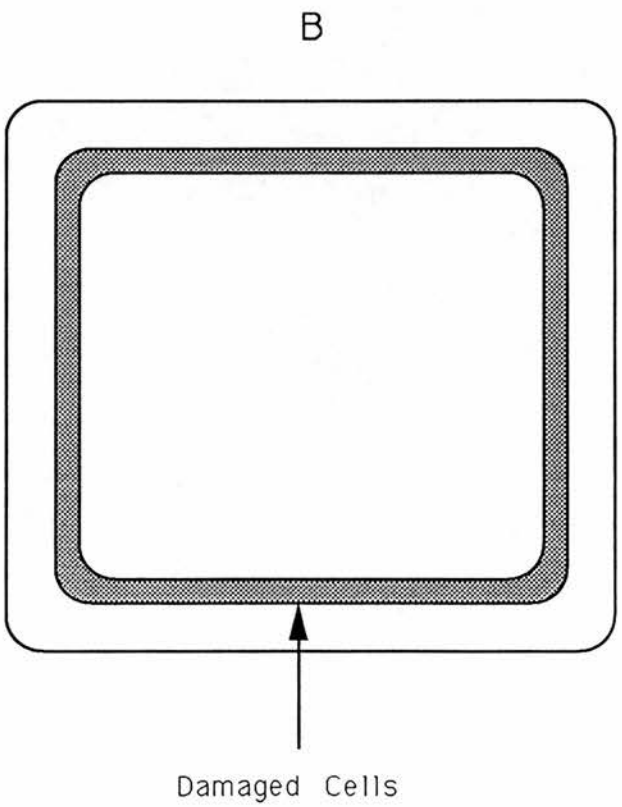


FIG.3.22. High magnification (x400) of one area of FIG.21. Mayer's haematoxylin counterstain.





DIAG. 3.2 Cross sectional (A) and top (B) views of damaged cells at the well edge of a confluent monolayer.

cells approximately 10 cell widths from the edge of the well and 5 to 10 cells wide (Diag.3.2). These cells stained with trypan blue and showed accumulation of IgG, confirming their damaged status. On examination of confluent monolayers under low power light microscopy before staining, these cells appeared normal with no significant difference to the cells in other parts of the culture well. In some older cultures this area of the monolayer contained a higher proportion of giant cells which also stained intensely. On closer examination after staining with antibody these cells did however have a more rounded shape and monolayer confluence was disrupted in some areas with loss of cell to cell contact (Figs.3.21 to 3.24).

This staining was was also present in control intact non - scratched monolayers. Therefore making the the possibility of this being a distance effect of scratch damage unlikely. Variations in degree of staining was noticed with the length of time the cells were maintained in these culture plates. Young cells which were fixed and stained as soon as confluence was reached, approximately 24 to 36 hours showed very little edge staining . Whereas cultures which had been maintained in these wells for longer than 48 hours after becoming confluent showed much more staining.

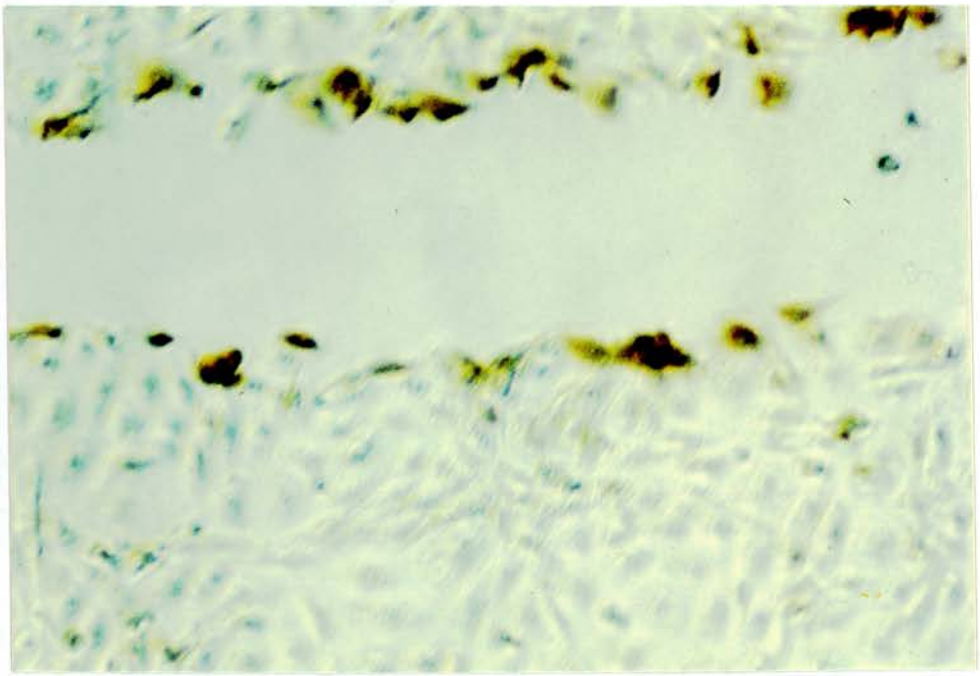


FIG.3.27. P14G11 stained, fixed scratch damaged pig aortic endothelium.

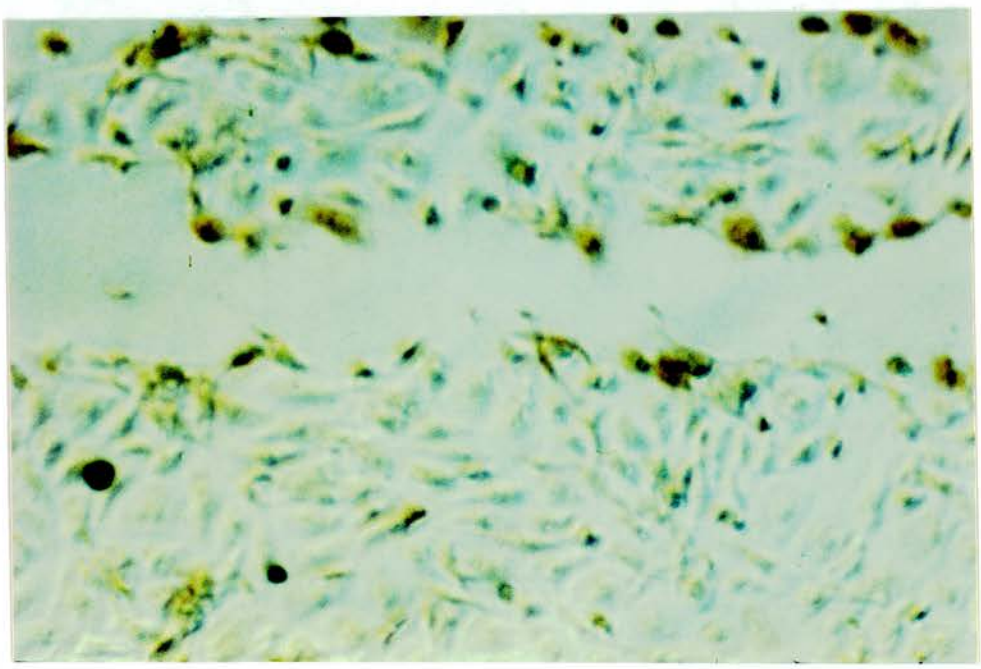


FIG.3.28. P14G11 stained, non-fixed scratch damaged pig aortic endothelium.

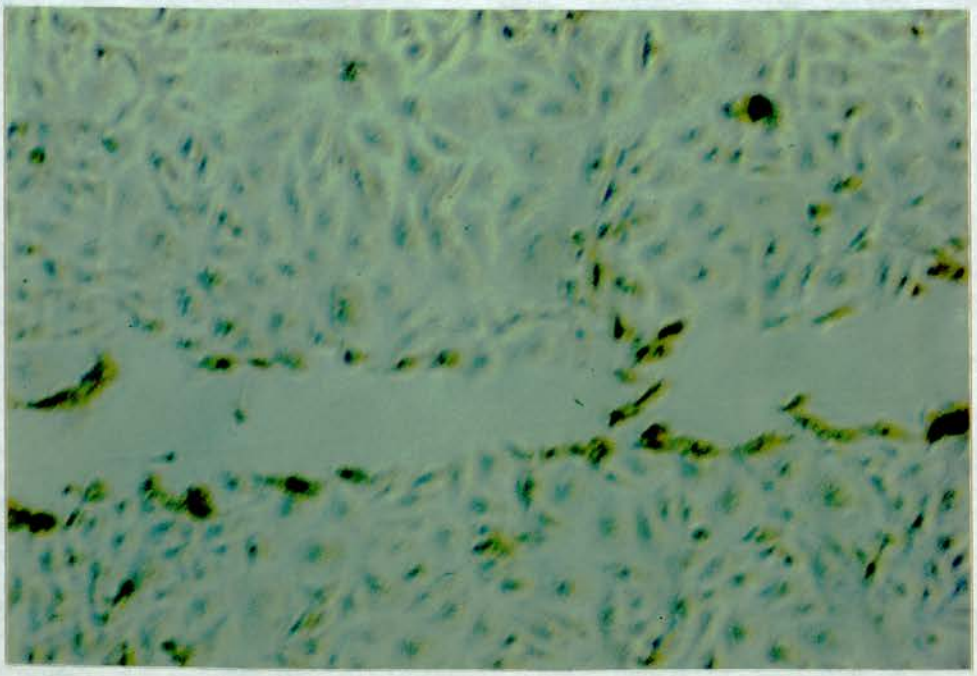


FIG.3.25. A3E10/D11 stained, fixed scratch damaged bovine aortic endothelium.

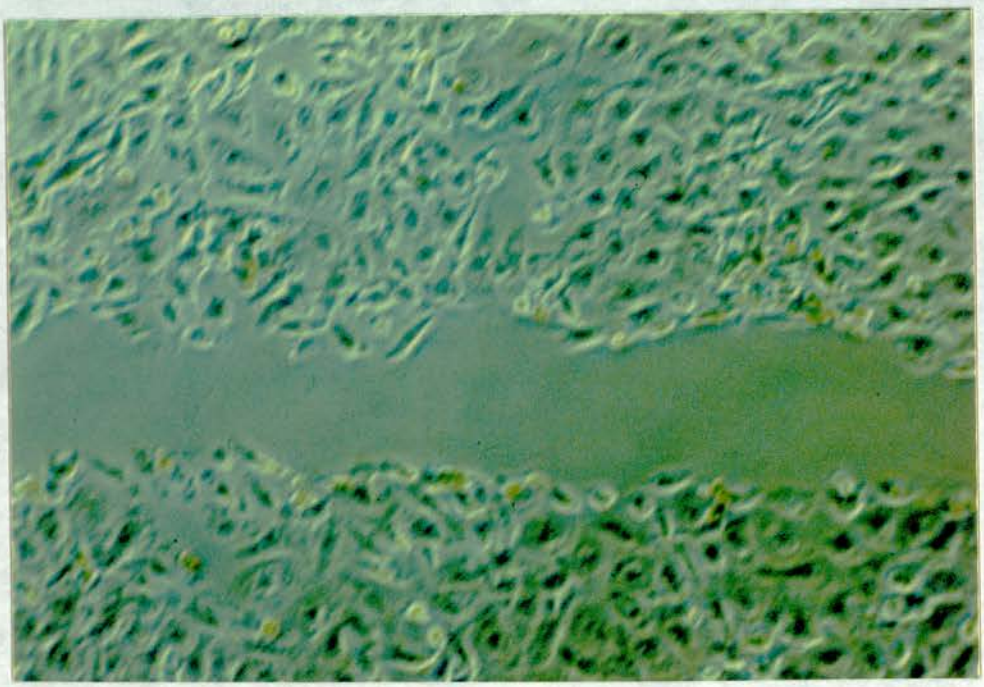


FIG.3.26. A3E10/D11 stained, non-fixed scratch damaged bovine aortic endothelium.

3.8. BINDING OF ANTIBODY TO FIXED AND NON-FIXED CELLS.

All primary and secondary screening procedures were carried out on glutaraldehyde fixed cells. This procedure itself causes some degree of membrane disruption . Therefore antibody incubations were repeated on non-fixed cells. The results for the non-specific binding of IgG remained the same and the blocking of the binding could be achieved in the same way. However D8G8, D6G11 and A3E10 antibodies did not bind to unfixed damaged cells (Figs.3.25 and 3.26). P14G11 on the other hand was found to bind to both fixed and non-fixed damaged cells (Figs.3.27 and 3.28). The cells were fixed in 0.025% glutaraldehyde after primary antibody incubations and washes and before the peroxidase labelled second antibody incubation. Non-fixed cells were sensitive to the second antibody incubation stage and were lost from the plate on subsequent washes. This screening procedure was much more difficult with non-fixed HUVEC. Large numbers of cells were lost from the plate during initial incubations and washing steps, making interpretation of the results difficult.

The results are summarised in table 3.8.

ANTIBODY	FIXED	NON-FIXED
A3E10	++	-
B10F1	+	-
C11D7	+	+
C18B5	+	+
D5D2	++	-
D6G11	++	-
D8G8	+++	-
P14G11	+++	+++

TABLE 3.8. Antibodies staining fixed and non-fixed endothelial cells.

ANTIBODY	BAEC	PAEC	HUVEC	SPINDLES
A3E10	(++)	-	+	-
B9B8	-	+	(++)	?
B10A10	+	+	+	+
B17E4	+	?	(++)	?
D6F1	(++)	?	?	-
D8G8	(++)	++	++	++
D6G11	(++)	++	++	++
P14G11	++	(++)	++	-

TABLE 3.9. Cross - reactivity of monoclonal antibodies with endothelial cells from different sources.
The results in parenthesis denote the endothelial type against which the antibody was raised.
? : Not known

3.9. CROSS-REACTIVITY OF MONOCLONAL ANTIBODIES.

Mice were immunised with endothelial cells from four sources, cow aorta (BAEC), pig aorta (PAEC), human umbilical vein (HUVEC) and adult human saphenous vein (SAVEC) with a view to raising monoclonal antibodies specific to each type of cell. When monoclonal cell lines were established which produced stable antibodies with consistent staining patterns on homologous endothelial cell lines, their reactivity with cells from other sources was investigated.

Saphenous vein endothelium was not used in these tests due to problems in growing and maintaining stable cultures of this particular cell at the time.

Cells were grown to confluence in 24 x 16mm plates, scratched, fixed and stained with antibody. The results are shown in Table 3.9.

3.10. STAINING OF FROZEN SECTIONS WITH SELECTED MONOCLONAL ANTIBODIES.

Antibodies which reacted well with cultured endothelium were tested on acetone fixed frozen sections. Sections of normal lung tissue were kindly provided by the Department of Pathology, Edinburgh University.

Sections stored at -70^{o}C were thawed at RT slowly. Endogenous peroxidase activity was inactivated by

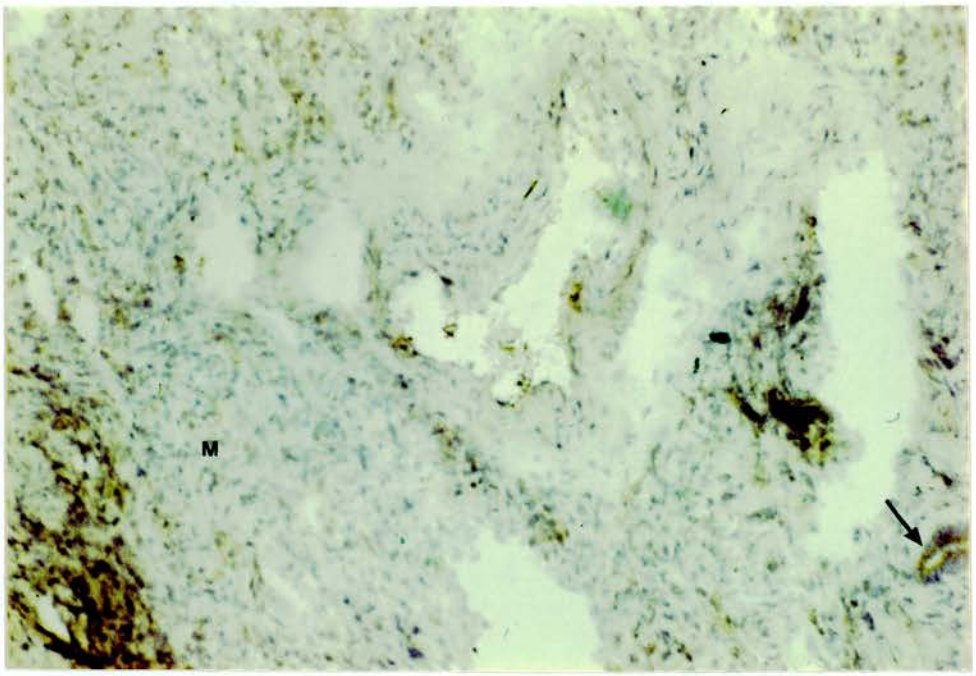


FIG.3.31 Lung. D8G8. Very little obvious staining of endothelium. There may be some small vessel staining, arrow bottom right. Some mesothelial staining (M).

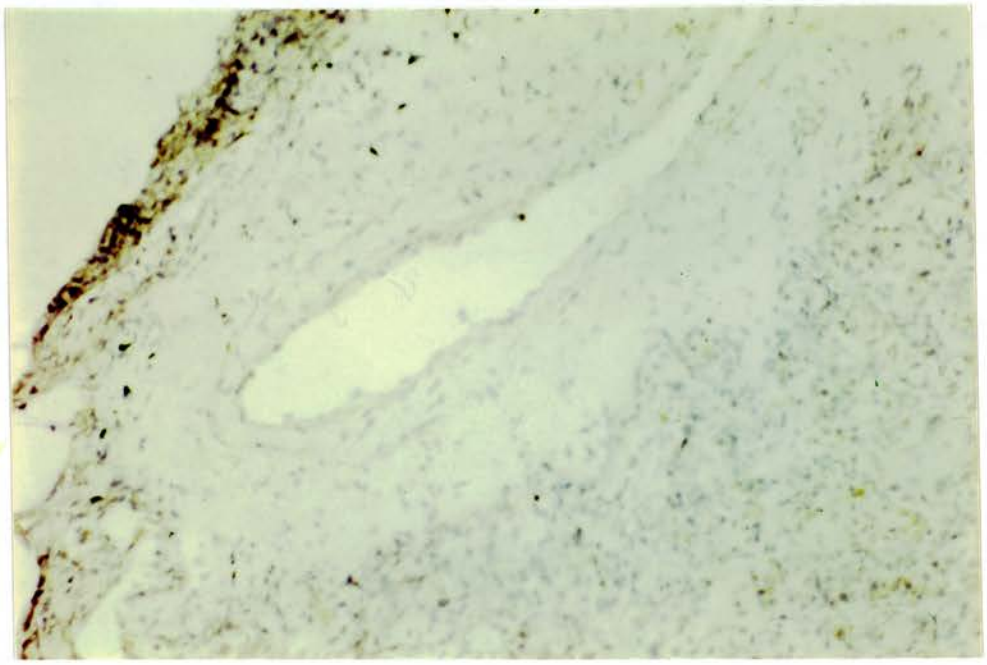


FIG.3.32. Lung. B10A10. No endothelial staining

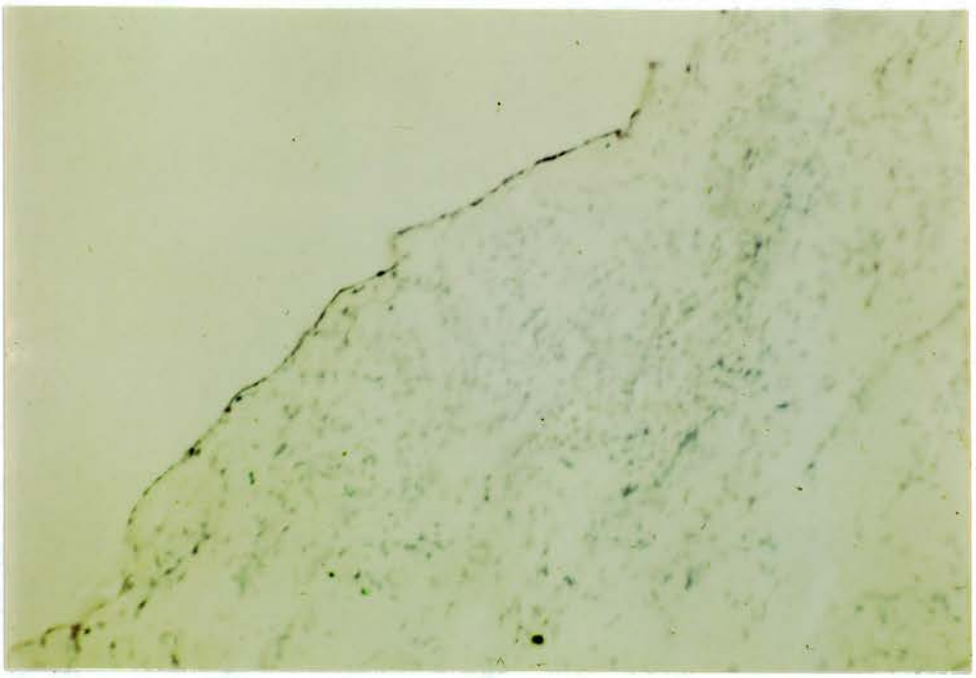


FIG.3.29. Lung. Mayer's Haematoxylin stained control.

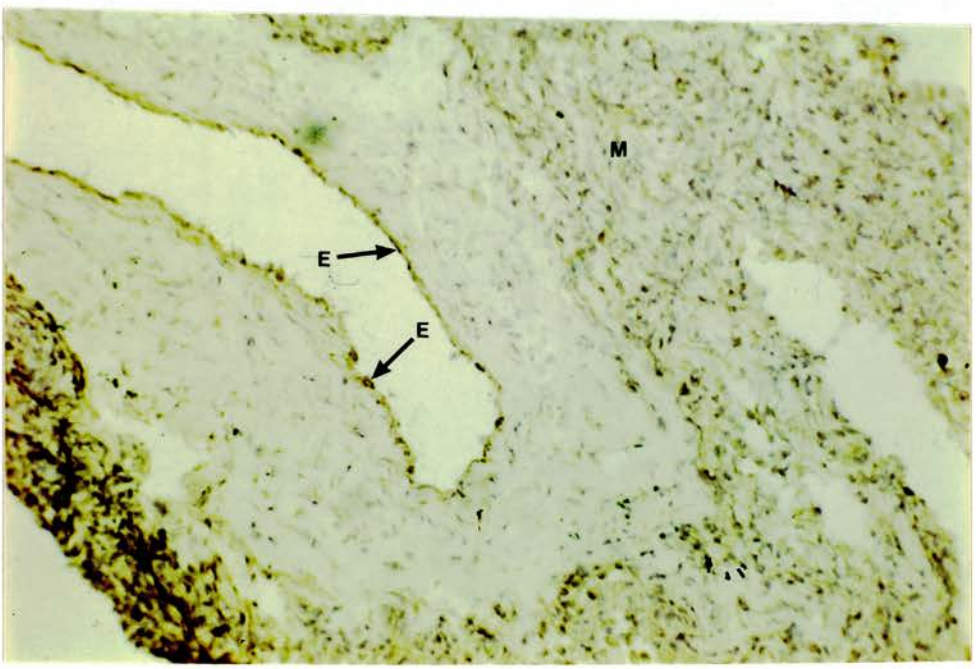


FIG.3.30. Lung. D6F1 staining endothelium (E) of large vessel and some staining of mesothelium (M).

incubation with methanol containing 0.3% H_2O_2 for 1 hour at RT. Non-specific binding sites were blocked by incubation with autologous serum for 15 minutes at RT. The sections were stained with DAB (section 2.3.3), using 100ul of neat antibody containing growth medium supernatant. After the final DAB staining step the sections were rinsed in tap water and counterstained with Mayer's Haematoxylin for 5-10 minutes. Colour was developed by rinsing in tap water and sections were dehydrated through 50%, 75%, 95% and absolute ethanol for 5 minutes each step. After clearing in toluene for 5 minutes sections were mounted in DPX and examined by low power light microscopy

Antibody D6F1 which produced a diffuse staining pattern on cultured endothelial cells, stained large vessel endothelium in lung sections. There was no smooth muscle cell staining but there was some mesothelial staining (Fig.3.30.).

D8G8 which stains only damaged cultured endothelial cells, showed very little staining on lung sections with no distinctive endothelial staining (Fig.31). Some staining of the mesothelium was apparent and there may be some small vessel staining (Fig.3.32). B10A10 which gave a 'negative' staining pattern on cultured cells, did not show any endothelial cell staining on lung sections as was expected (Fig.3.32). P14G11 (Fig.3.33)

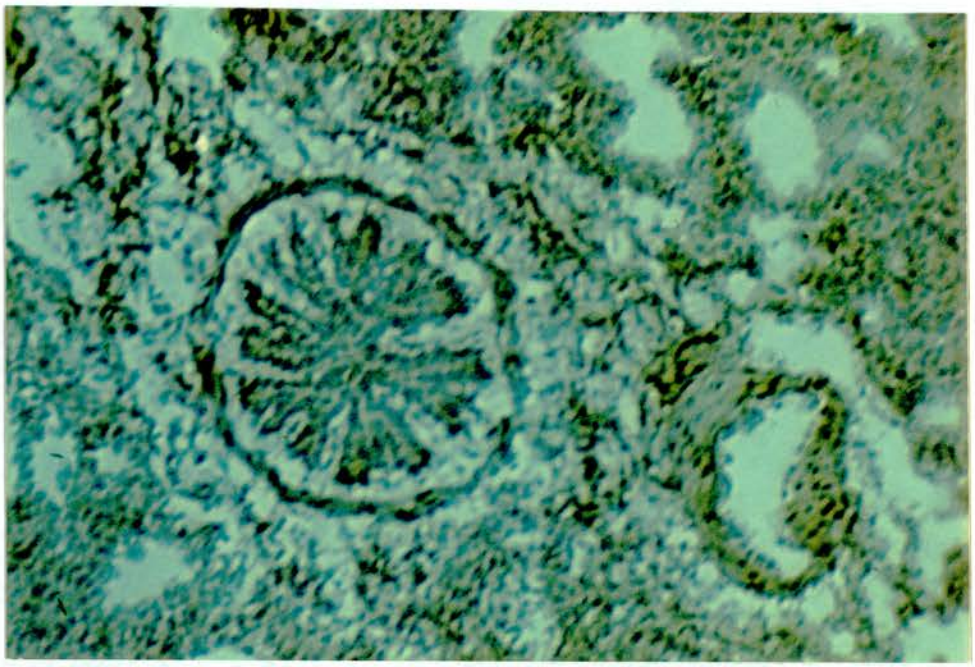


FIG.3.33. Lung. P14G11. Heavy non-specific staining.

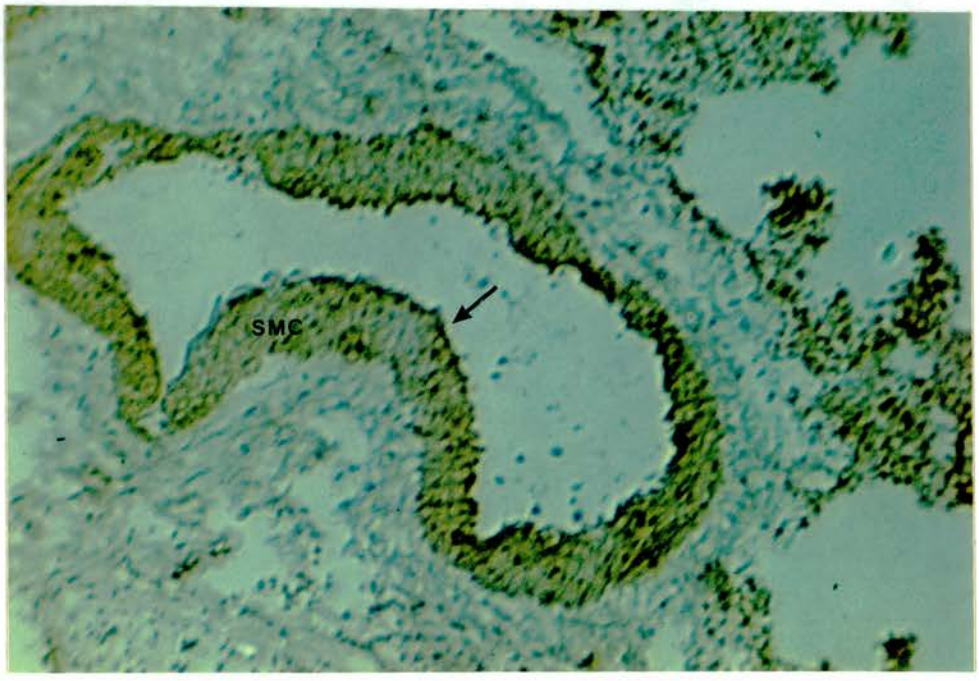


FIG.3.34. Lung. B17E4. Endothelial (E) and smooth muscle cell (SMC) staining.

produced heavy non-specific staining. B17E4 (Fig.3.34) stained both endothelium and smooth muscle cells distinctively.

3.11. DISCUSSION.

The antibodies produced by the four immunisation groups were placed in categories according to the staining patterns found.

Diffuse staining was defined as general non-specific staining of non-damaged endothelium. Only two antibodies were found to produce such staining, D6F1 and B9G6. D6F1 was directed against bovine aortic endothelial cells. The mice in this group (D) were immunised with immunogen 2, spleen cells pre-incubated with BAEC (2.2.2). It stained all the cells of a scratch damaged monolayer of cloned endothelium without preference for damaged cells. It also shows the same staining pattern on non-damaged endothelial monolayers. In non-confluent cultures not all the cells stain as shown in Fig.3.1. D6F1 was the only antibody effective in non-specific staining of endothelium in tissue sections (Fig.3.30).

B9G6 (Fig.3.2) was raised against HUVEC. The mice in this group (B) were immunised with immunogen 1 (whole cell preparations of endothelial cells). In an

uncloned HUVEC culture B9G6 stained all the cells, but with varying degrees of intensity. This would suggest that the epitope recognised by B9G6 antibody is produced by cells to varying amounts, perhaps indicating different stages of growth or metabolism of individual cells. The epitopes recognised by these antibodies are most probably membrane components since intracellular access of IgM molecules would be highly unlikely in undamaged cells.

B9B8 (Fig.3.3) is included in the diffuse staining group although it gave a "speckly" staining pattern. This may in fact be a variation in the damaged cell staining with the antibody recognising cytoplasmic components extruded through damaged plasma membrane.

Two antibodies B10A10 and B10C10 from the same cell line gave a negative staining pattern. These were directed against HUVEC but cross-reacted with BAEC and PAEC and spindle cell cultures. Plastic, fibronectin and gelatin controls proved to be negative. It seems likely that these antibodies recognise a component of the endothelial extracellular matrix which is present only on the apical surface of the cell and is only accessible to the antibody when the cell has been removed non-enzymatically . These components may be cell attachment proteins such as vitronectin or collagen.

Perinuclear staining of giant cells occurred only with antibodies directed against SAVEC. Giant cells are a typical feature of senescing cell cultures from all sources. SAVEC cultures tend to have greater numbers of giant cells in primary culture and can very rarely be grown for more than one or two subcultures. This may indicate that these cells have already reached the end of their replicative life span in situ and hence the difficulty in maintaining these cells in culture. The source of SAVEC in this laboratory is from coronary artery bypass patients and the difficulty in maintaining endothelial cells from such a source may be a reflection of the pre-existing vascular disease in such patients. Staining was confined to what appeared to be intracellular granules or vesicles surrounding the nucleus of giant cells. Cells showed varying degrees of staining. In Fig.3.6 the cells in the top left and bottom right of the picture have nuclei completely surrounded by stained granules. These cells are unmistakably giant cells with characteristically large quantities of stringy cytoplasm. The cells in the centre of the picture have less staining and have the morphologic appearance of normal cells. The presence of such granules or vesicles may be an early indication of cellular senescence. If so, such antibodies may have a potential role in the assessment of suitable vessels

for grafting.

The negative and perinuclear staining effects could not be investigated in any detail due to instability of the hybridoma cell lines producing these antibodies. Cells either died or stopped producing antibody after two to three days in culture or could not be revived from liquid nitrogen storage.

A number of antibodies screened on scratched damaged monolayers stained the cells lining the scratch edge. These cells showed trypan blue uptake and accumulation of IgG confirming their damaged status.

Staining was usually confined to the cells immediately adjacent to the scratch edge, indicating that damage was also confined to the cells at the scratch edge (Figs3.11 and 3.12). However in some instances staining occured in cells up to 10 cells away from the scratch edge (Fig 3.13). This may indicate that scratch damage has a distance effect. Schwartz et al (1978) have shown that cells not immediately associated with areas of damage exhibit changes in thymidine uptake and DNA synthesis associated with regeneration. The effect seen in Fig. 3.13 may in fact be associated with cell regeneration, the stained cells at the scratch edge indicating regeneration rather than damage. Cell monolayers were therefore scratched and allowed to regrow before antibody treatment (Section

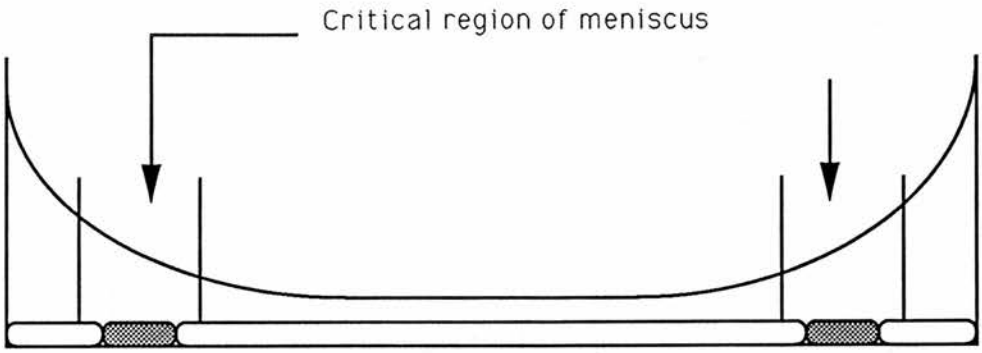
3.6.3). The site of the scratch was still evident as a line of stained cells although the cells were completely confluent (Fig.3.14). It may be that these cells would eventually be pushed off the monolayer by regenerating cells from behind and that in this case healing of the wound is not in fact complete. However when scratched damaged cells were examined after two hours of regrowth, antibody stained cells did show tentative signs of regeneration i.e. elongation and alignment in the direction of movement. However, without further work on the metabolism of these cells it is not possible to deduce from the staining pattern alone whether these cells are damaged or regenerating cells. Trypan blue uptake would indicate that they are non-viable, however trypan blue really only shows membrane damage. There is no indication that the damage is irreversible and it may be sublethal. However without further investigation it is impossible to say that these are regenerating cells.

Cells damaged by air drying, heat and hyperoxic conditions did stain with antibodies D8G8, P14G11 and A3E10. The cells damaged by hyperoxic conditions did have gross monolayer morphology changes. However the heat and air damaged cells did not look any different to normal cells on examination under low power light microscopy. These antibodies may have a potential

application in detecting sublethal or early non-denuding endothelial injury.

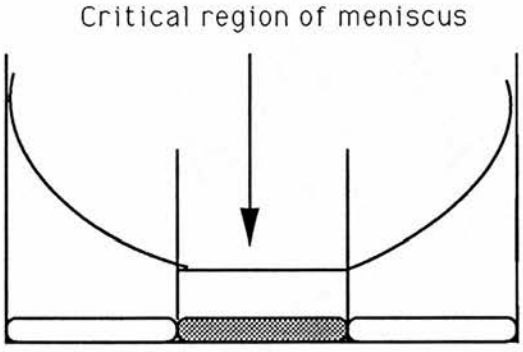
Endotoxin damage was not detected by these antibodies. The concentration of LPS used was in the range of that used by Sage et al 1986 to induce damage. No macroscopic morphological changes were observed with these concentrations and very little cell staining occured even with increased concentrations of LPS. Some staining of the cells at the well edges did occur but was considered non-specific. Treatment of the cells with thrombin and hydrogen peroxide produced interesting staining patterns. Thrombin at 0.5 U caused no antibody detectable damage. At 1.0 U staining of cells around the edge of the monolayer was apparent. This staining increased in intensity with increasing concentrations of thrombin without staining cells in the centre of the well. The concentration of hydrogen peroxide used gave cell staining in the centre of the well which increased in intensity with increasing concentration. The cells around the edge of the well which had stained intensely after thrombin treatment were lost from the well after hydrogen peroxide treatment. The cells at the edge of the culture well appeared to be more susceptible to lethal damage by thrombin and by hydrogen peroxide.

Staining of cells around the well edges was an



A

Areas of damage in monolayers grown in 24 well plates.



B

Area of damage in monolayers grown in 96 well plates.

Damaged cells

Non-damaged cells

DIAG. 3.3 Areas of non-deliberate damage in monolayers grown in 24 and 96 well plates appear to coincide with what may be critical points of the meniscus in terms of surface tension effects.

effect seen with all the anti-damaged cell antibodies screened. Staining varied in intensity according to how long the cells had been maintained in culture in 24 well plates. Investigation of cells grown in other sizes and types of vessel, revealed that monolayers in $25cm^2$ and $75cm^2$ flasks did not show this staining pattern. However, monolayers grown in 96 well plates showed cell damage in the centre of the well but not around the edges. The areas of damage in both the 24 and 96 well plates appear to coincide with critical regions of the meniscus of the culture medium in the individual wells (1.0ml and 0.1ml respectively). Diagram 3.3 attempts to explain the association. Three possible explanations of this effect exist.

1. These are areas where gas diffusion is least efficient. In the 24 well plates this a plausible explanation of cell damage. However, the cells immediately adjacent to the vertical walls of the well do not appear to be damaged (no trypan blue uptake or non-specific staining by IgG). The damaged cells in the 96 well plates occurs in the centre of the wells where gas diffusion would be optimal. On these plates, damage at this region could be due "drying out" effects.

2. Surface tension forces at the gas - fluid interface may be acting on the cell surface. The formation of a meniscus however ensures equal surface pressures on

both sides of the interface , ruling out pressure forces as a cause of damage. Newly plated cells, i.e. cell suspensions before cell to plastic adherence during routine subculturing procedures, tend to aggregate in the centre of the 24 plate wells and around the edges of the 96 plate wells. This effect was not unique to endothelial cell suspensions but was also noted with the hybridoma cells, which were also grown in 96 and 24 well plates with 0.1ml and 1.0ml of medium respectively. This would suggest that some other physical forces may be acting on both cell suspensions and cell monolayers at these areas of fluid - plastic interface or the fluid - air interface.

3. Accumulation of toxic waste products. In static culture toxic waste product accumulation may become localised at specific sites. These sites in turn may be determined by physical forces and fluid mechanics. Without frequent medium changes these waste products could cause localised cell damage. It was noted that non-deliberate cell damage became more pronounced with length of time in culture. This type of damage was very slight in cultures which reached confluence and were used within 24 hours.

All of the antibodies screened except one (P14G11) bound only to fixed cells. P14G11 appeared to bind with equal affinity to both fixed and non-fixed cells.

P14G11 is an IgG type antibody whereas the others are IgM's. The size and conformation of these molecules differs greatly and differences in binding between fixed and non-fixed cells can be explained in terms antibody and antigen "size" and plasma membrane damage. This is explained in detail in Chapter 4 (Section 4.4., Diags.4.1, 4.2, 4.3.).

CHAPTER 4

SEPARATION OF ENDOTHELIAL CELL PROTEINS BY SDS - POLYACRYLAMIDE GEL ELECTROPHORESIS AND IMMUNODETECTION OF WESTERN BLOTS WITH MONOCLONAL ANTIBODIES.

4.1. INTRODUCTION.

The work described in this chapter uses the techniques of polyacrylamide gel electrophoresis (PAGE) and protein transfer (Western Blotting) to investigate the reactivity of endothelial cell proteins with the monoclonal antibodies developed in the laboratory,and aims to provide a framework for discussing the mechanisms of the diverse staining patterns produced by the antibodies as discussed in chapter 3.

The method of separating and identifying polypeptides by polyacrylamide gel electrophoresis in the presence of the detergent sodium dodecyl sulphate (SDS) has proven to be reliable and reproducible (Maizel 1966, Shapiro et al 1966, Vinuela et al 1967, Fairbanks et al 1971). By this technique, molecular weights can be estimated quickly and simply (Shapiro et al 1967, Weber and Osborn 1969). Further analysis of separated proteins is facilitated by electrophoretic transfer onto nitrocellulose or nylon based membranes (Towbin et al 1979, Burnette 1981).

SDS - PAGE and immunoblotting techniques have been widely used in investigating aspects of endothelial cell behaviour under conditions of normal growth (Vlodavsky et al 1979, Ketis et al 1986, Dickinson et al 1986,) and in conditions of stress (Sage et al 1986). The recent boom in monoclonal antibody

production has also led to immunolological identification of specific endothelial proteins (Darnule et al 1983, Kaplan et al 1983, Arvieux et al 1986).

There are two main limitations of SDS-PAGE which must be considered when interpreting the results of the following experiments. First, separation is only on the basis of effective molecular weight and two different proteins with the same molecular weight will not be resolved. Second, it implies that antigen is both stable and soluble under these conditions.

The initial experiments investigate the general reproducibility of protein patterns during electrophoresis and compare the differences and similarities of these patterns in cells from different species. Once a reproducible technique of separating the proteins had been established their antigenicity was investigated using the monoclonal antibodies described in Chapter 3.

4.2. METHODS.

4.2.1. Preparation of Cells for Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

The cells were prepared for electrophoresis according to the method of Vlodavsky et al (1979).

Confluent monolayers of cells were gently rinsed 3 times with PBS and resuspended by incubation in 10mM sucrose/ 1mM EDTA in 20mM HEPES buffered PBS at 37°C until the cells were detached (section 2.1.6). The cell suspension was then rinsed 3 times in serum free EBS with 15 second mixes on a vortex mixer at each rinse.

Cell pellets of 10^5 to 10^6 were resuspended in 1 to 2ml of lysis buffer : 15% glycerol

2% SDS/ 75mM Tris-HCl (pH6.8)

2mM PMSF

2mM EDTA

1mM N-ethylmaleimide

1mM iodoacetic acid

1 unit of DNAse I per ml of buffer.

This was incubated for 30 minutes at 37°C in an HNO_3 washed glass tube with shaking. A further 2 hour incubation at 37°C was carried out after the addition of 2-mercaptoethanol to a final concentration of 5%. Immediately before use, dithiothreitol was added at a final concentration of 0.1M and the samples were boiled for exactly 2 minutes and immediately cooled on ice. Samples were spun at 10 000 rpm for 5 minutes before the addition of the glycerol and bromophenol blue tracking dye.

4.2.2. SDS - PAGE.

The Laemmli (1970) buffer system was used throughout all the electrophoresis experiments.

ELECTRODE BUFFER : 0.025M Tris ; 0.192M Glycine ; 0.1% SDS pH8.3

GEL BUFFER : 0.75M Tris ; 0.2% SDS
Resolving gel buffer pH8.8
Stacking gel buffer pH6.8

ACRYLAMIDE STOCK : 22.2% acrylamide ; 0.6% NN'-bisacrylamide, made up in double distilled water (DDH_2O) and filtered through Whatman No.1 paper and stored in a dark bottle at $4^{o}C$ and discarded after 2 weeks.

AMMONIUM PERSULPHATE STOCK : 1.5% ammonium persulphate in DDH_2O and made up fresh each time.

	ACRYLAMIDE CONC. IN GEL			
	10%	7.5%	5%	3.3%*
ACRYLAMIDE STOCK	18	13.5	9	3
WATER	--	4.5	9	6
BUFFER	20	20	20	10
AMMONIUM PERS.	2	2	2	1
TEMED	0.06	0.06	0.06	0.03

The figures given in the table are the quantities in ml of each component of the gel required for one 1.5mm thick slab gel of the appropriate concentration of acrylamide. The 3.3% gel denoted by * was used only as a stacking gel and the volume given above was enough to provide a 1cm layer on two slab gels. Each component of the gel was mixed in the above order adding the TEMED immediately before pouring the gel and mixing in each solution very gently as it was being added. All solutions were at room temperature before mixing to avoid formation of gas bubbles which would prevent the gel from setting.

Samples of 20 - 50ul of cell lysate were carefully applied to each well using a Hamilton syringe and molecular weight standards (Sigma) were included in each run according to the manufacturers instructions. Electrophoresis was carried out in an LKB 2001 Vertical Electrophoresis Unit at 50mA constant current until the samples were through the stacking gel and then at 70mA until the sample front was 1cm from the bottom of the gel. Gels were then prepared for protein transfer or stained in Coomassie Blue R250 and or silver stained.

4.2.3. Staining of Polyacrylamide Gels.

Coomassie Blue Stain.

Coomassie Blue (CB) R250 (0.25%) was dissolved in 95% ethanol, stirred for 1 hour and filtered through Whatman No 1 paper. Gels were stained overnight in a 50/50 mixture of stain concentrate and 10% acetic acid. Destaining was carried out in the following solutions in the order listed :

200ml ethanol + 300ml 5% acetic acid for 1-2 hours

(150ml ethanol + 350ml 5% acetic acid for 1-2 hours) x 2

100ml ethanol + 400ml 5% acetic acid for 2 hours

150ml water + 350ml 5% acetic acid 1-2 hours

Destained gels were soaked in 48% methanol/ 5% glycerol for at least 2 hours before drying at 80^{o}C for 2 hours.

Silver Nitrate Staining.

The method was that of Dzandu et al (1984). Silver nitrate stain is specific for lipids and sialoglycoproteins, staining them yellow and providing a contrast to Coomassie blue staining polypeptides.

Each slab gel was treated in 500ml of solution unless otherwise stated. The gel was fixed in 40% methanol / 10% acetic acid for 1 hour at room temperature and washed in 10% ethanol / 5% acetic acid for 2 consecutive 30 minute washes. This also removed residual SDS.

Equilibration was carried out in 200ml of oxidiser reagent (6.4mM nitric acid / 6.8mM potassium dichromate) for 10 minutes, which stains the gel pale yellow. The oxidiser reagent was washed with DDH_2O until the background colour cleared. The gel was then stained with 200ml of 20mM silver nitrate for 30 minutes and then washed 3 times for 1 minute each in DDH_2O to remove unbound silver. The presence of unbound silver in the final wash was checked by cloudiness when 1 drop of 1M HCl was added to 1ml of rinse. Rinsing was continued until the gel was free of unbound silver.

The gel was then transferred to 200ml of developer solution at $40^{o}C$ (0.28M sodium carbonate / 0.008% paraformaldehyde). This solution was changed as soon as turned brown in colour, which continued until yellow bands appeared on the gel. It was rinsed with DDH_2O and 10% acetic acid added to enhance the yellow colour of the bands.

Silver stained gels were preserved in the same way as Coomassie blue gels.

4.2.4. Protein Transfer (Western Blotting).

Gels intended for blotting were immediately equilibrated in transfer buffer : 25mM Tris/ 192mM glycine/ 20% v/v methanol/ 0.01% SDS pH8.3 for 15-30

minutes. The immobilising membrane, either nitrocellulose (NC) or Immobilon P (PVDF) and filter papers were also equilibrated in this buffer. The gel was laid on the NC or Immobilon membrane and sandwiched between sheets of filter paper. With 2 or 3 scouring pads on either side to hold the gel and membrane tightly together, they were inserted in a holder with the membrane facing the anode. This was completely immersed in transfer buffer and electrophoresed for 1 hour at 1 amp

4.2.5. Immunostaining of Western Blots.

The NC sheets were cut into strips corresponding to the sample lanes before the paper had dried. The standards and a total protein control lanes were stained in 0.1% amido black/ 25% propanol/ 10% acetic acid for 5 minutes and destained in 25% propanol/ 10% acetic acid, 2 or 3 volumes with a final rinse in cold water before being dried between strips of filter paper. The other strips were incubated with antibody and protein bands detected either by aminoethylcarbazole (AEC) substrate reaction or by immunogold silver staining.

AEC Substrate Reaction : The transfer strips were incubated in 20% BSA solution overnight at room

temperature. This was a quenching step to reduce non-specific background staining. Incubation with primary antibody (monoclonal antibody) was carried out at room temperature for 1 to 2 hours. If the antibody was in growth medium supernate form it was added at a 1:2 dilution , if it was purified antibody then a 1:10 or a 1:100 dilution was used.

The strips were then washed 4 times for 15 minutes each time with PBS/1%BSA and incubated for 1 hour with peroxidase conjugated rabbit anti - mouse second antibody at a 1:100 dilution in PBS/1%BSA. The washes were then repeated 3 times, 5 minutes each.

The 3-amino-9-ethylcarbazole stock solution was made up at 20mg in 2.5ml of dimethylformamide and stored at $4^{o}C$. The substrate incubation solution was 2.5ml of stock solution + 47.5ml of 0.05M acetate buffer pH5.0 and filtered through Whatman No 1 paper. Immediately before incubation with the transfer strips 125ul of 6% H_2O_2 was added to the substrate solution. A red insoluble precipitate characterises the antigen-antibody complex and as soon as this became visible the strips were removed, rinsed with water and dried between sheets of filter paper.

Immunogold Silver Staining : The following method was that recommended by Janssen Life Sciences Ltd for use with their Auroprobe BLplus - Intense II products.

The transfer strips were incubated overnight in 5% BSA / 20mM Tris-HCl / 0.9% NaCl / 20mM NaN_3 pH8.2 at room temperature. All further incubations and washes were done at room temperature under constant agitation. The strips were washed 3 times for 5 minutes each time in 0.1% BSA Tris buffer (as above) and incubated with primary antibody diluted in 0.1% BSA Tris buffer as in AEC substrate reaction section. Again the strips were washed 3 times for 5 minutes as above and incubated for 2 hours with gold labelled second antibody (AuroProbe^{TM}BLplus GAMIgM+IgG) at a 1:100 dilution in 0.1% BSA Tris buffer with 1:20 v/v gelatin. Strips were washed 2 times for 5 minutes each in 0.1% BSA Tris buffer and 2 times for 1 minute each in DDH_2O. Equal volumes of initiator and enhancer solutions from the Intense^{TM}II kit were mixed thoroughly immediately before use. The strips were incubated in this enhancement solution under constant agitation until the required resolution of colour was obtained (10 - 20 minutes). The stained NC strips were then washed in excess DDH_2O several times and dried between sheets of filter paper.

4.3. COMPARISON OF PROTEIN BANDS OF ENDOTHELIAL CELL LYSATES AS DETECTED BY COOMASSIE BLUE AND SILVER STAINS.

The textile dye Coomassie Blue R250 (CB) was first used by Fazekas de St.Groth et al (1963) to stain proteins separated on cellulose acetate strips. In slightly acid conditions the dye anion is electrostatically attracted to the NH_3^+ groups of the protein molecule with Van der Waals forces holding the reactants together. The protein - dye complex is firm but fully reversible under the appropriate conditions of pH. Coomassie blue is the conventional method of staining proteins in polyacrylamide gels (Meyer and Lamberts 1975). It is highly selective for proteins and can detect 0.2 - 0.5ug of protein in a sharp band, but is not quantitative. Different proteins bind coomassie blue to different extents.

A method devised by Dzandu et al (1984) uses silver to detect lipids and sialoglycoproteins in electrophoretically separated cell components. It is complementary to CB staining in that negatively stained regions of silver stained gels stain with CB. It can therefore be used as a double staining technique on the same gel.

In the following experiments the cells were not treated enzymatically prior to electrophoresis so no

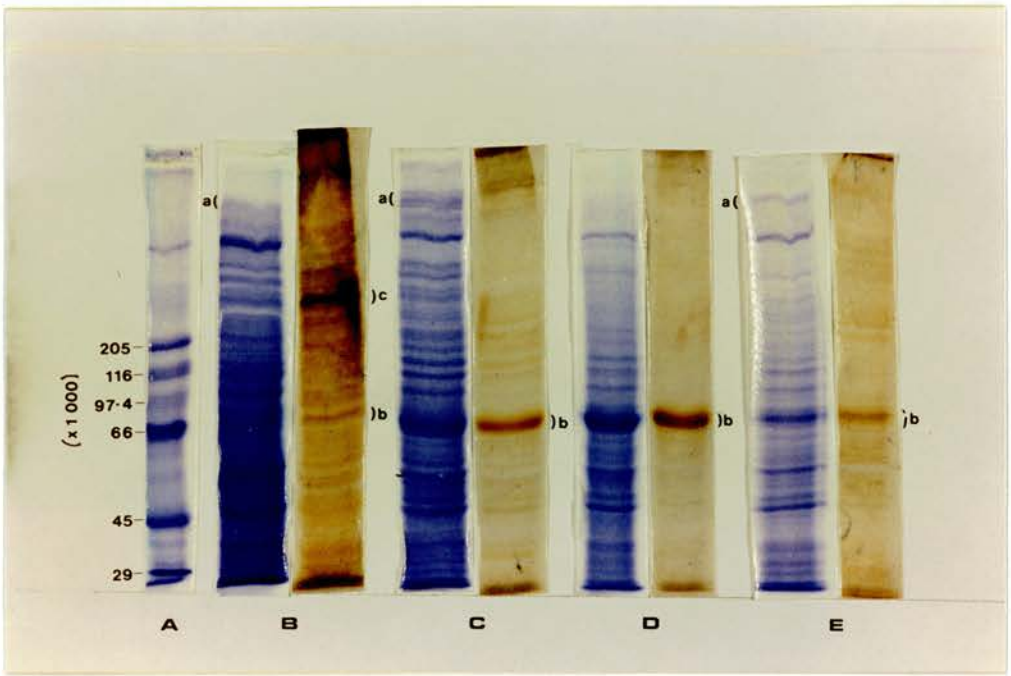


FIG.4.1(a). Silver and coomassie blue stained sections of supernate fraction of bovine, porcine and human endothelial cell lysates.
A: molecular weight standards, B: bovine aortic C: porcine aortic D: adult human saphenous vein, E: human umbilical vein endothelium. 5% acrylamide gel.

distinction between lipids and sialoglycoproteins can be made. However, lipids / sialoglycoproteins as a group, as stained by this method can be compared to the proteins stained by coomassie blue. Two sets of experiments were done with the two staining methods.

4.3.1. Comparison of protein band patterns from bovine, porcine and human endothelium.

The electrophoresis and staining methods are described in section 2.5. Endothelial cell lysates from bovine and porcine aorta, adult human saphenous and human umbilical vein were separated into supernatant and pellet fractions by spinning for 3 to 5 minutes at 7 000 x g and run on 5% acrylamide gels and stained with CB and silver. The results are shown in Fig.4.1(a) The cell lysates were prepared in a Tris buffer which interferes with protein estimation. Although methods are available which exclude such interference (Polacheck and Cabib 1981), it was felt that precise quantitation of protein was not required for these experiments. Cells were counted before lysing and samples were prepared from lysates from equal numbers of cells and in this way the "amount" of protein was kept fairly constant.

Lane B (BAEC) contains too much protein (sample size of 100 ul) and individual bands in the CB stained

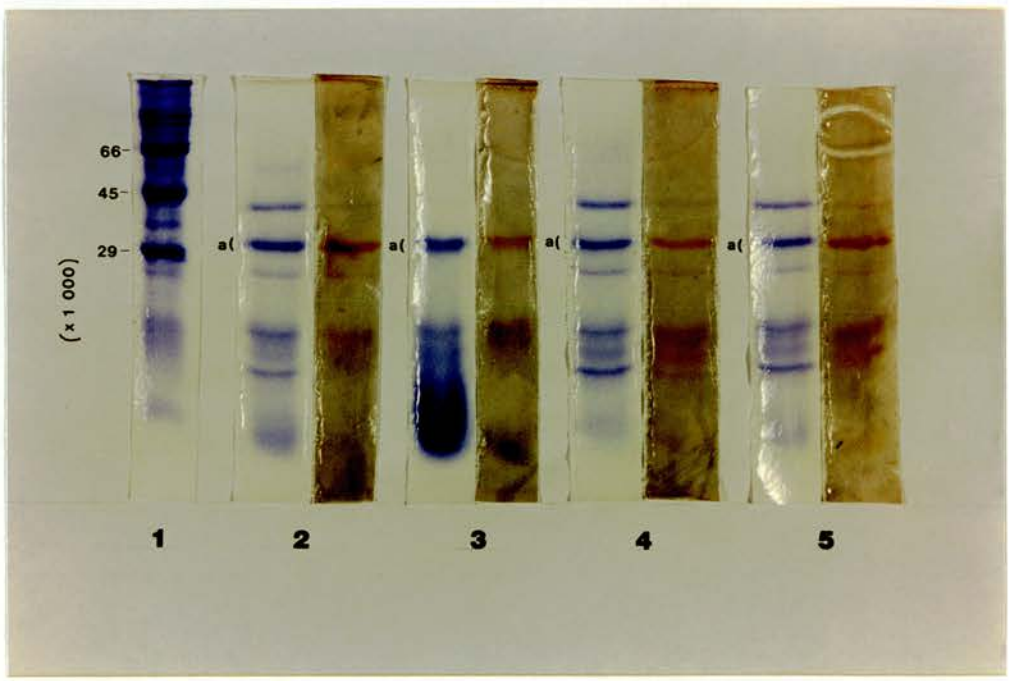


FIG.4.1(b). Silver and coomassie blue stained sections of pellet fraction of bovine, porcine and human endothelial cell lysates.
1: molecular weight standards, 2: bovine aortic, 3: porcine aortic, 4: adult human saphenous vein, 5: human umbilical vein endothelium

lane are difficult to see, especially in the lower molecular weight regions. The other lanes contain less protein (sample size of 50 ul). With total protein staining (CB) there were few major differences in band pattern between bovine, porcine and human . On comparing lanes D (SAVEC) and E (HUVEC), a very high molecular weight protein band (a) is present on human umbilical vein endothelial cells but not on those from adult saphenous vein. This protein is also present on pig aortic endothelium (C) and bovine aortic endothelium (B).

The silver stained gels show only one major band (b) which runs parallel to the 66kD standard. It is very prominent on pig aortic, human saphenous and umbilical vein endothelium but is not so clear on bovine aortic, even although equivalent amounts of sample were run. A heavily stained band with a very high molecular weight (c) is present on BAEC but is not a feature of other cell types.

The pellet fractions of these cell preparations were trypsinised (without this step the samples would not migrate through the gel). This fraction contained fewer proteins and all with low molecular weights up to approximately 45kD (Fig.4.1(b)) Homology exists between the bovine (lane 2), saphenous vein (lane 4) and the umbilical vein (lane 5) cells, however the pig aortic

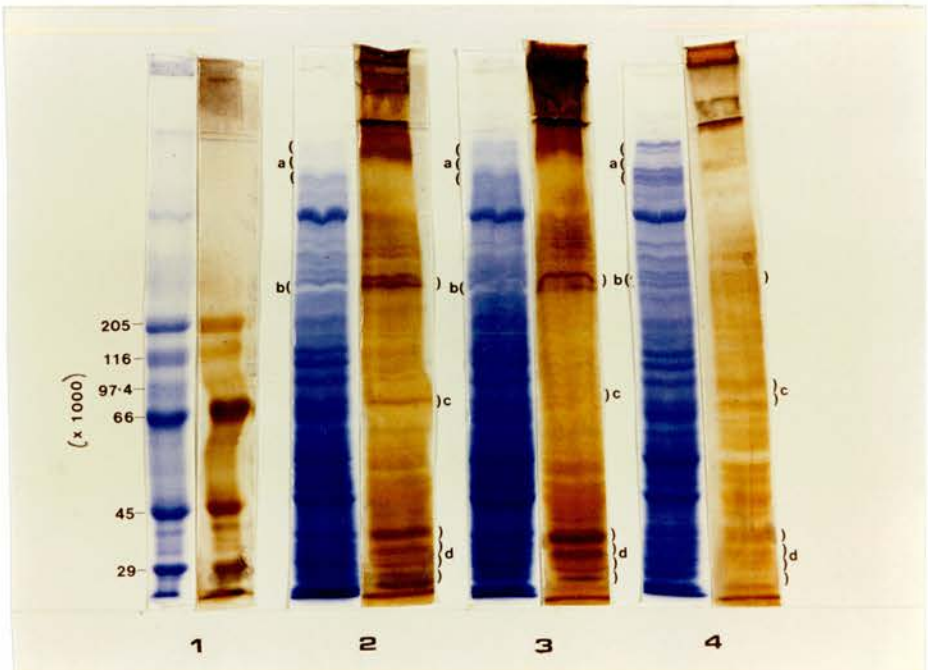


FIG.4.2(a). Silver and coomassie blue stained whole cell lysates of bovine aortic endothelial cells of inreasing subcultures.
1: molecular weight standards, 2: first subculture cells, 3: second subculture cells, 4: fourteenth subculture cells. 5% acrylamide gel.

cells (lane 3) differ in that two bands, one above and one below the major band of approximately 30kD (a) are missing . The silver staining band pattern is similar to that of the CB stained gel.

4.3.2. Comparison of protein band pattern in cells of increasing age.

The bovine cell line B135 was harvested after 1,2 and 14 subcultures. Cells were resuspended using sucrose / EDTA buffer and prepared for electrophoresis as described (2.5). Each sample was prepared in 1ml of lysis buffer and contained protein from 10^6 cells, 100ul of sample was applied to each well of 5% and 10% gels. The results are shown in Fig.4.2(a). The CB total protein stain shows very little difference between the first (2) and second (3) subculture samples. Overloading of the wells has caused some problem in visualising individual bands in the lower molecular weight regions. A cluster of high molecular weight proteins (a) increase in intensity of staining with increasing number of subcultures. This suggests that these proteins increase in quantity as the cells are subcultured. A second region of disparity (b) on the CB stained gel is also seen on the silver stained gel. On lanes 2 and 3 a CB stained band in this region is followed by a negatively stained band which stains as a

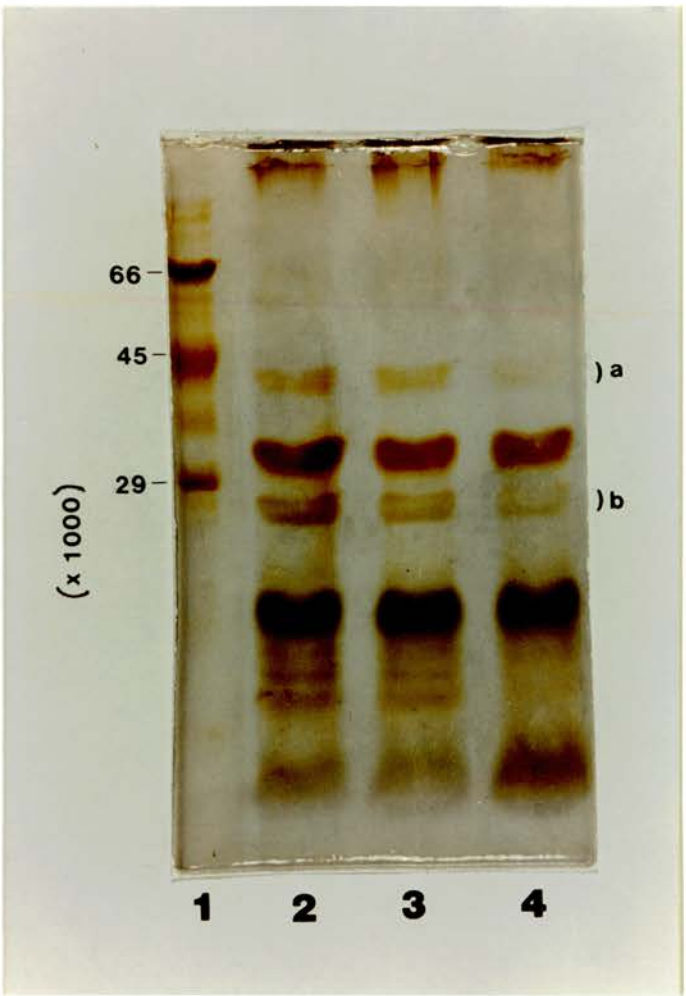


FIG.4.2(b). Silver stained pellet fractions of bovine aortic endothelial cells of inreasing subculture.
1: molecular weight standards, 2: first subculture cells, 3: second subculture cells, 4: fourteenth subculture cells. 10% acrylamide gel.

lipid / sialoglycoprotein on silver staining. This band is not present on the silver stained section of lane 4 and on the CB stained section three bands are present with no negative stained region. Two differences are seen on the silver stained sections only. The first subculture sample (2) shows a band at the 66kD region (c) which is not present on the second or fourteenth subcultures. Two bands are present in this region on lane 4. An area of heavily stained bands of low molecular weight (d) are present on lanes 2 and 3 but are faint in lane 4. These bands are more clearly seen in the pellet fraction of the cell lysate (Fig.4.2(b)). This shows a band (a) just below the 45kD marker which decreases in staining with increasing subcultures. A similar pattern is seen with a band just below the 29kD marker (b) but to a lesser extent. In general, from Figs. 4.2(a) and (b) it would seem that as the cells age the relative amount of lipid / sialoglycoprotein detected by silver staining decreases, compared with total protein as stained by CB. The stacking gels of these samples were retained to show how much lipid / sialoglycoprotein remains at the origin and does not migrate through the resolving gel. To look in more detail at lipid / sialoglycoporotein content a much lower percentage acrylamide resolving gel would have to be used or the cell components broken down into smaller

molecules. No further work was done on resolving the lipid / sialoglycoprotein fraction, for reasons discussed below.

4.4. MOLECULAR WEIGHT ESTIMATION.

From the CB stained gels the molecular weights were estimated by the method of Weber and Osborn (1969). These investigators observed that the mobilities of proteins in polyacrylamide gels were a linear function of the logarithm of their molecular weights. SDS, an anionic detergent, binds to the hydrophobic regions of proteins and separates most of them into their component subunits. SDS binding also imparts a large negative charge to the denatured polypepetides. This masks the charge normally present in the absence of SDS and allows the separation to be based on the size of the molecule. Results are highly reproducible not only for globular proteins but also for helical, rod-shaped protein molecules. However, the linear relationship between mobility and molecular weight does not hold true in some instances. With proteins of high molecular weight (90kD to 200kD and over) curvature of the plots of mobility against log molecular weight have been observed. The linearity is related to the amount of cross linking in the gel matrix. High molecular weight

proteins show a greater degree of linearity if separated on gels with a low percentage of acrylamide and cross-linker, whereas low molecular weight proteins show greater linearity on high percentage acrylamide gels (Hames 1981).

Endothelial cell lysates were prepared and the proteins separated as described. Each gel was run in duplicate, one being used for protein transfer and the other for total protein staining by CB. Molecular weight standard kits, MW-SDS-70 and MW-SDS-200 (Sigma) gave a range of molecular weights from 14kD to 205kD. The length of the gels and the distance of stained proteins from the origin were measured in centimetres and the relative mobility of the protein bands were calculated from the formula :

$R_f = A/C \times B/D$ where:

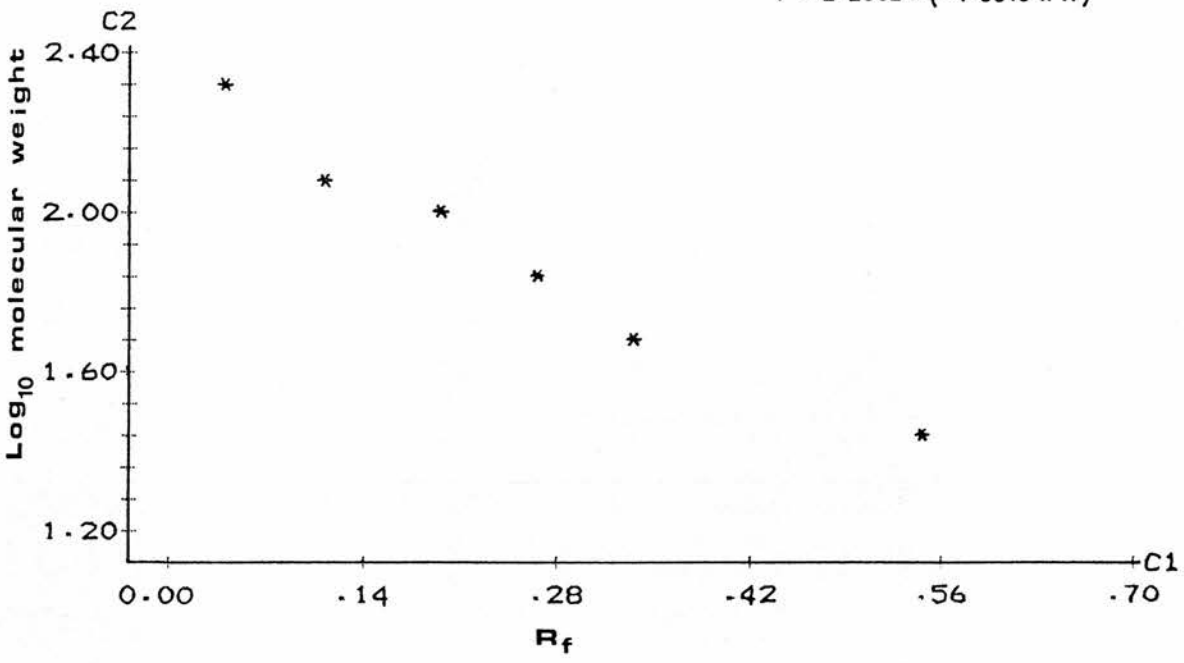
A = distance of protein migration through the gel
B = length of gel before staining
C = length of gel after destaining
D = distance of dye migration i.e. buffer front

The R_f values of the standards were plotted against $\log_{10}$ of molecular weights and least squares regression analysis was used to find the straight line of best fit. The molecular weights of the unknown proteins were

GRAPH 4.1 High molecular weight standards (MW–SDS–200)

$R^2 = 95.4\ \%$

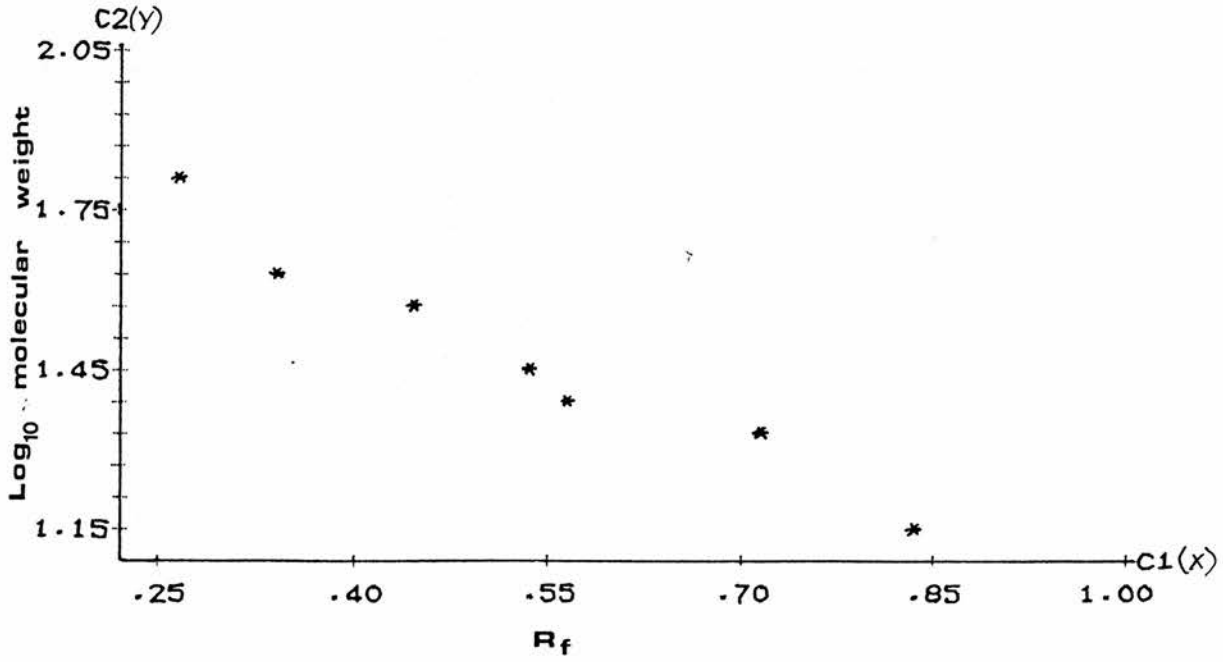
$Y = 2.2932 + (-1.6615 \times X)$



GRAPH 4.2 Low molecular weight standards (MW–SDS–70)

$R^2 = 97.7\ \%$

$Y = 2.0565 + (-1.1025 \times X)$



STANDARD	KNOWN MW(kD)	EST. MW(kD)	DIFFERENCE BETWEEN KNOWN & EST.
MW-SDS-200			
myosin	205	169	36
b-galactosidase	116	129	13
phosphorylase b	97.4	94.9	2.5
bovine albumin	66	73	7
egg albumin	45	53	8
carbonic anhydrase	29	25	4
MW-SDS-70			
bovine albumin	66	59	7
egg albumin	45	48	3
glyceraldehyde-3-phosphate	36	37	1
carbonic anhydrase	29	29	0
trypsinogen	24	27	3
trypsin inhibitor	20.1	18.8	1.3
a-lactalbumin	14.2	13.8	0.4

TABLE. 4.1. Comparison of estimated molecular weights to actual molecular weights of standard kits MW-SDS-200 and MW-SDS-70.

STANDARD	KNOWN MW (kD)	ESTIMATED MW (kD) 1	2	3
MW-SDS-70				
bovine albumin	66	59	62	61
egg albumin	45	48	45	46
glyceraldehyde-3-phosphate	36	37	40	39
carbonic anhydrase	29	29	29	32
trypsinogen	24	27	27	21
trypsin inhibitor	20.1	188	19.4	20.0
a-lactalbumin	14.2	13.0	13.6	14.7
correlation (r^2)		97.7%	98.6%	97.6%
MW-SDS-200				
myosin	205	170	149	169
b-galactosidase	116	117	123	129
phosphorylase b	97.4	105.5	109.6	94.9
bovine albumin	66	79	80	73
egg albumin	45	48	50	53
carbonic anhydrase	29	25	25	25
correlation (r^2)		96.2%	92.0%	95.4%

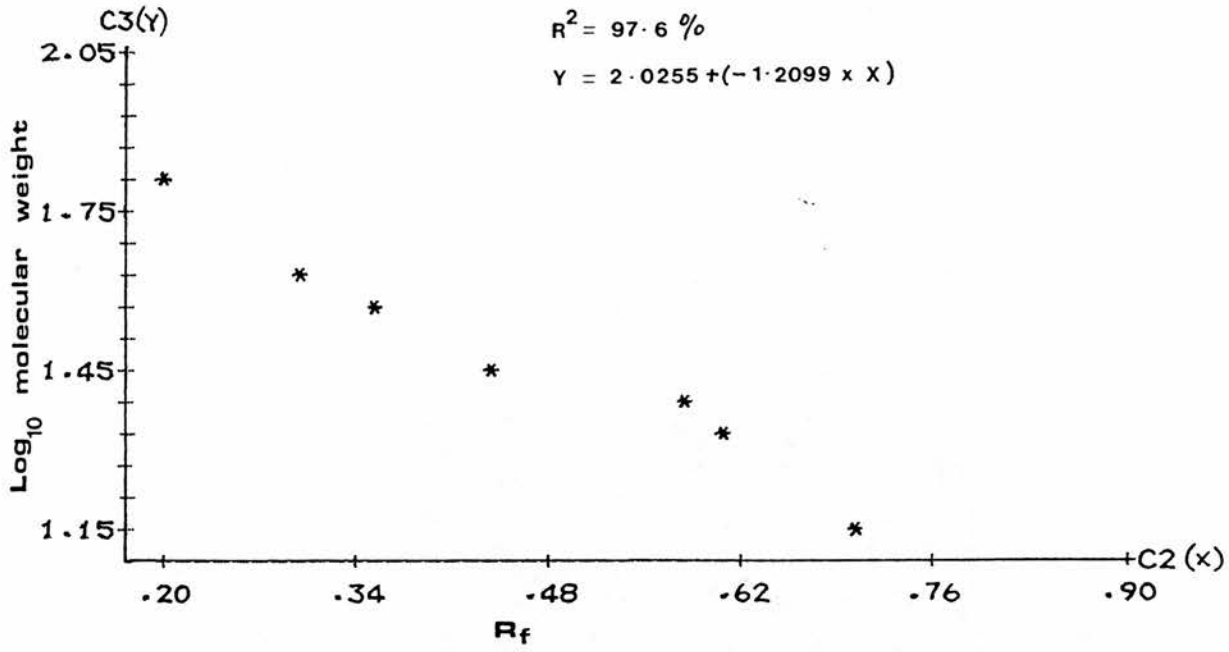
TABLE 4.2. Comparison of estimated molecular weights with known molecular weights of standards from three gels.

calculated from their R_f values and the equation of the line. The calculations were all done on computer using a standard Minitab statistics package.

Molecular weight standards (MWS) from both kits were run on the same 10% acrylamide gel (Fig.4.3) and R values calculated from the above formula. The line of best fit was found for both sets of standards (Graphs 4.1 and 4.2). In theory, the relationship between mobility and molecular weight should be linear. However, as has already been discussed, high molecular weight proteins do not behave so on highly cross-linked gels (10% acrylamide in this case). A comparison of "estimated" molecular weights to known molecular weights was made by applying the R_f values of the MWS proteins to the appropriate line of best fit from graphs 4.1 and 4.2. The results (table 4.1) show quite a difference between estimated molecular weights and known molecular weights. The difference between the known and estimated molecular weights (table 4.1) is smaller with the low molecular weight standards (MWS-SDS-70). From the regression analysis, the correlation, r^2, value was higher for the low MWS, 97.7% compared to 95.4% for the high MWS. This would suggest that the low MWS show a greater degree of linearity than the high MWS under these experimental conditions. These observations were reproducible and based on the results

C3,C2

GRAPH 4·3 MW – SDS – 70 (Gel – Fig. 4.3)



GRAPH 4·4 MW – SDS – 70 (Gel – Fig. 4.4)

C3,C2

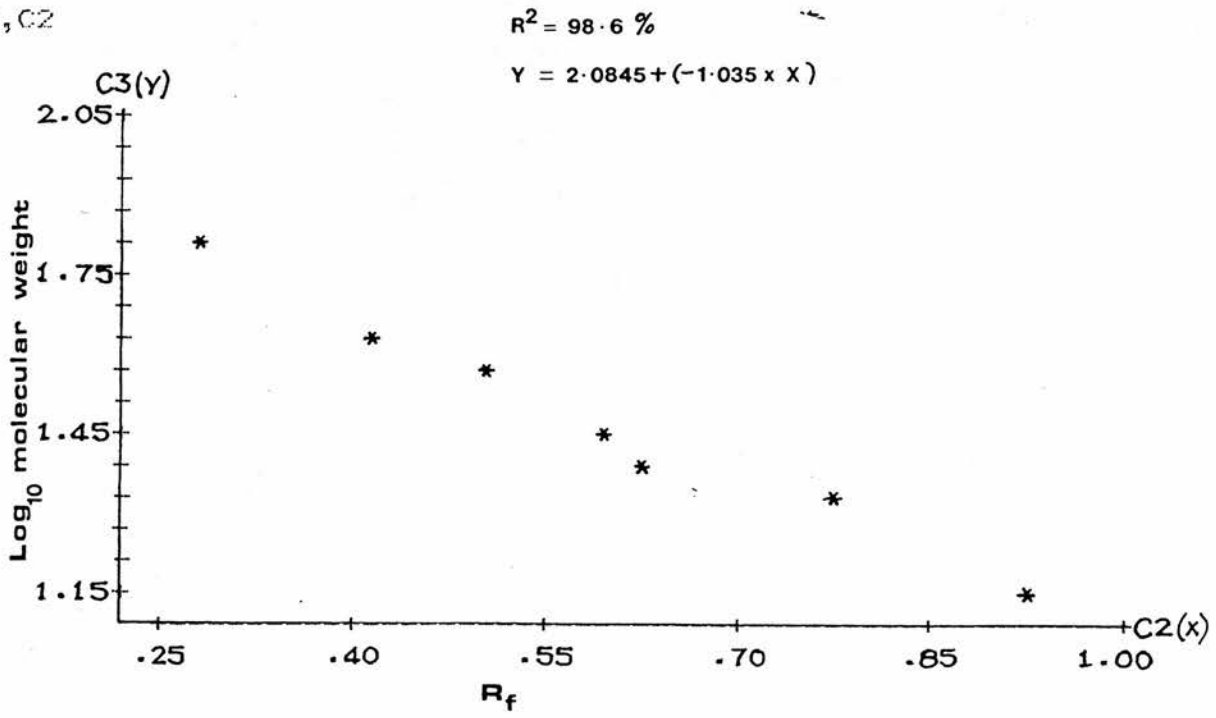


FIG.4.4. Coomassie blue stained 10% acrylamide gel of human umbilical vein (HUVEC) and bovine aortic (BAEC) endothelial cell lysates.
1: molecular weight standards, 2: HUVEC - H150.5, 3: HUVEC - H149.4, 4: HUVEC - H148.1, 5: BAEC - B144.5, 6: molecular weight standards.

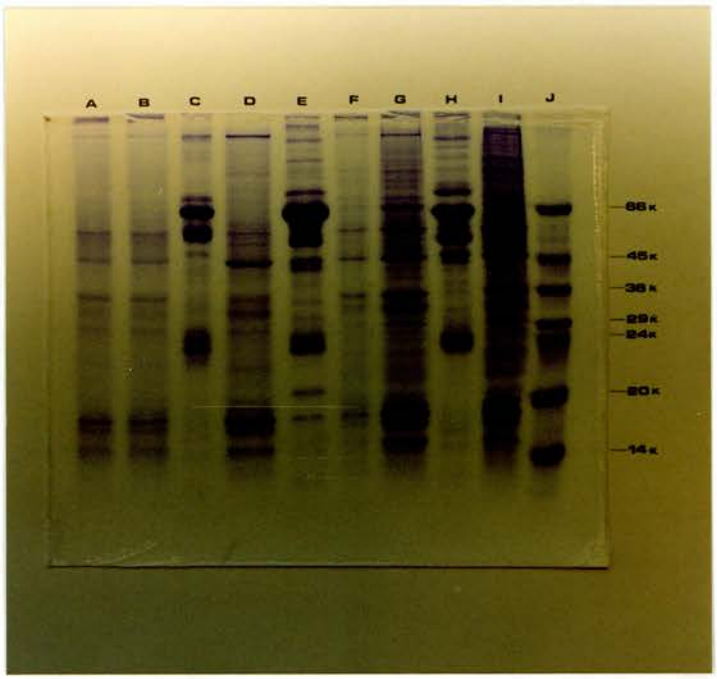


FIG.4.3. Coomassie blue stained 10% acrylamide gel of human (SAPHEC and HUVEC), bovine aortic (BAEC) and porcine aortic (PAEC) endothelial cell lysates.
A and B: HUVEC - H149.1, C: newborn calf serum, D: BAEC - B144.6, E: adult human serum, F and G: SAPHEC - A15.4 and A20.3, H: pig serum, I: PAEC - P53.1, J: molecular weight standards.

STANDARD MW		EST. MW OF HUVEC PROTEIN BANDS		
KNOWN	EST.	H150.5	H149.4	H148.1
		106	106	106
		79	79	79
		70	70	70
		64	64	65
66	62	62	62	61
			57	58
				55
		53		53
		49		49
45	45	47	47	45
				43
				42
36	37	36	36	37
		35	35	34
29	29			30
24	27			29
20	19			
		18	18	18
		16	16	15
		15	15	16
14	14			

TABLE 4.3. Estimated molecular weights of the major protein bands of human umbilical vein endothelial cells.

The molecular weight values are spaced to correspond roughly to the spacing of the bands in relation to each other on the gel.

STANDARD MW KNOWN.	EST.	EST. MW OF PROTEIN BANDS A20.3	P53.1
		98	98
		78	78
66	61	64	64
		56	56
45	46	48	48
36	39	39	39
29	32	36	36
24	21	21	21
20	20	20	20
		19	19
		17	17
14	15		

TABLE 4.4. Estimated molecular weights of the major protein bands of adult human saphenous vein and pig aortic endothelial cells.

from six 10% acrylamide gels prepared and run under the same conditions (table 4.2).

On the basis of these results, molecular weights of unknown protein bands were estimated from the low molecular weight standards. Most protein bands of interest fell within the range of these standards and any bands of interest which were outside this range had molecular weights estimated from the high molecular weight standards.

Endothelial cell proteins were separated on 10% acrylamide gels and the molecular weights of the major bands stained were estimated as described above. The molecular weights of the standards and the unknowns in tables 4.3, 4.4, 4.5 are spaced in such a way as to correspond roughly to the spacing of the bands in relation to each other on the gel. Human umbilical vein endothelium protein bands are shown in table 4.3, graph 4.3 and fig.4.3. Pig aortic and adult human saphenous vein bands are shown in table 4.4, graph 4.4 and fig.4.4. Bovine aortic protein bands are shown in table 4.5. These result were taken from gels shown in figs. 4.3 and 4.4. The mean of the estimated molecular weights were calculated and since these protein bands were also present on human and pig endothlial cells these are the molecular weights referred to in the following results.

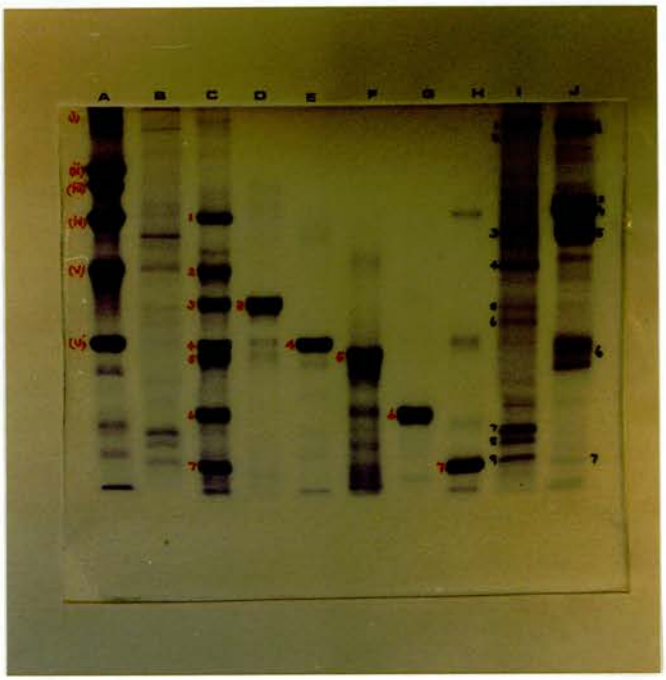


FIG.4.5. Coomassie blue stained 10% acrylamide gel of molecular weight standards.
A: MW-SDS-200 standard kit, molecular weights 205k,116k,97.4k,66k,45k, 29kD. B: bovine aortic endothelium, C: MW-SDS-70 standard kit, moleular weights 66k,45k,36k,29k,24k,20.1k,14.2kD, D: 36kD standard, E: 29kD standard, F: 24kD standard, G: 20.1kD standard, H: 14.2kD standard, I: bovine aortic endothlium, J: newborn calf serum.

STANDARDS MW	STANDARDS Est.	B144 Est.	STANDARDS MW	STANDARDS Est.	B144 Est.	STANDARDS MW	STANDARDS Est.	B144 Est.	B144 MEAN
		100.3			106.6			97.6	101.5
					79			78	78.5
66	59		66	62		66	61		
		53.2			55.3			57.5	55.3
45	48	44.5	45	45	45.7	45	46	51.6	47.3
36	37	34.5	36	37	36.9	36	39	38.9	36.8
		32.0			33.5			36.3	33.9
29	29		29	29		29	32		
24	27		24	27		24	21		
20	19		20	19		20	20		
		17.0			17.6			19.4	18.0
		15.7			16.4			18.9	17.0
		14.2			14.6			16.9	15.2
14	14		14	14		14	15		

TABLE 4.5. Estimated molecular weights of the major protein bands of bovine aortic endothelial cells. The estimates were taken from three different gels and the results varied slightly, the molecular weight standards and their estimated molecular weights are also shown, therefore the mean values of the BAEC molecular weights are given in the far right column.

4.5. IMMUNODETECTION OF PROTEIN BANDS ON WESTERN BLOTS

Acrylamide gels are fragile and difficult to work with. In order to detect reactions between proteins separated by SDS-PAGE and antibodies, it is more convenient to transfer the protein to a more stable material such as nitrocellulose.

Protein blotting is a simple, non-quantitative technique, convenient and fast and provides a stable base for the analysis, concentration or purification of antibodies and other protein ligands (Burnette 1981, Legocki and Verma 1981, Olmsted 1981). A variety of transfer membranes are available. The most commonly used being nitrocellulose (NC) and more recently, nylon based membranes which are more robust than NC.

The binding of monoclonal antibodies to separated, transferred endothelial cell proteins was tested with a selection of monoclonal antibodies discussed in chapter 3. The antibodies chosen were from the most stable cell lines and which gave the most consistent results in fixed cell ELISA. The results are discussed in detail for each individual antibody.

4.5.1. A3E10.

This antibody recognises damaged bovine aortic endothelial cells (BAEC) in fixed cell ELISA tests. It is an IgM subtype and was developed from mice which had

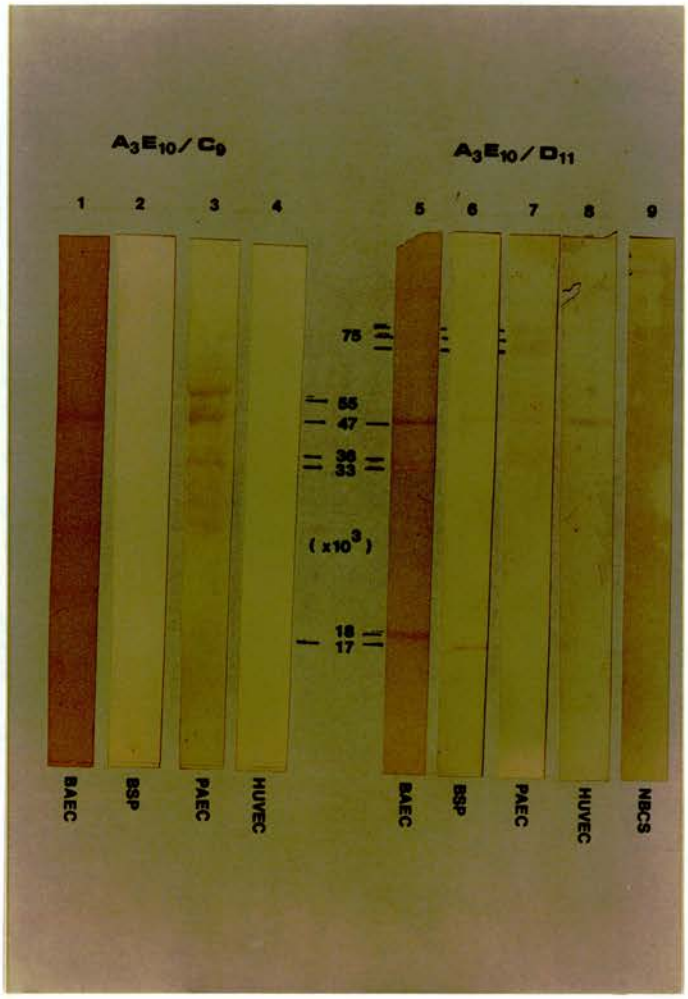


FIG.4.6. Immunoblot of antibody A3E10 on endothelial cell lysates.
The following abbreviations apply to figs. 4.6 to 4.12. BAEC (bovine aortic endothelium) PAEC (porcine aortic endothelium) HUVEC (human umbilical vein endothelium) SAVEC (adult human saphenous vein endothelium) BSP (bovine spindle cells) AHS (adult human serum) NBCS (newborn calf serum) PS (porcine serum)
Molecular weights are shown in kD.

been immunised by protocol 1 (2.2.2). Three clones from this cell line produced stable antibody, C9, D10 and D11. D10 and D11 have the same characteristics, therefore only C9 and D11 are shown in Fig.4.6. The amino-ethylcarbazole (AEC) staining method has produced high backgrounds in strips 1,5 and 9 with some patchiness in strip 9. Strips 3,7 and 8 were stained by the silver enhanced gold method (2.6.5.)

These clones show different cross-reactivity patterns. Clone C9 reacts with bovine aortic endothelial cells (BAEC) and pig aortic endothelial cells (PAEC) but not with bovine spindle cells (BSP) and human umbilical vein endothelial cells (HUVEC). D11 reacts with BAEC, BSP, HUVEC and only very faintly with PAEC. On BAEC it binds to two protein bands with an approximate molecular weight of 47kD. On PAEC it reacts with 3 major bands with molecular weights of 55kD, 47kD and 33kD. Some faintly stained bands can also be seen between the 33kD and the 47kD bands. Clone D11 shows a slightly different pattern. It reacts with two major bands of molecular weight 47kD and 18kD on BAEC. On BSP the same 47kD protein is stained but to a lesser degree and a 17kD protein to greater degree. On HUVEC only the 47kD protein band is stained. On PAEC no definite bands are highly stained but faint bands are present around the 75, 47, 36 and 33kD regions. This

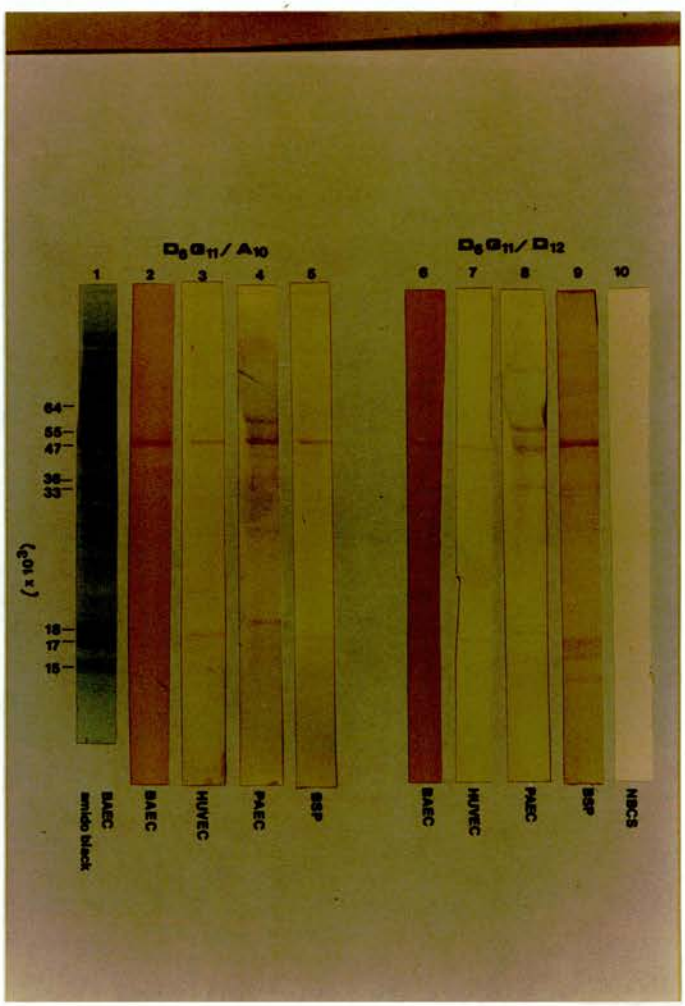


FIG.4.7. Immunoblot of antibody D6G11 on endothelial cell lysates.
Lane 1 is an amido black stain of total protein transfered.

may be non-specific staining by the silver enhancement reagents since the strip was developed a bit longer than usual because of the negative result after the standard incubation time (20 minutes). There is no reaction with either antibody to serum proteins.

4.5.2. D6G11

This antibody recognises damaged endothelium in fixed cell ELISA. It is an IgM subtype and was raised against BAEC from mice immunised by protocol 2. Two stable clones were obtained from this cell line producing antibodies A10 and D12. They both produced identical staining patterns as shown in Fig.4.7. These antibodies cross-react with endothelial cells from all the sources tested. The major protein band stained had a molecular weight of 47kD. Only this band was stained on BAEC. The BAEC lane 6 does not show this band very clearly but it is probably due to bad contact during transfer. On BSP and HUVEC the 47kD protein stains along with an 18kD band and on the BSP lane 9 three bands at 15, 17 and 18kD stain. Lane 9 had been gold/silver stained whereas lane 5 was AEC stained. This would suggest that the gold / silver staining technique is more sensitive. On PAEC these clones, like the A3E10 antibodies, show a slightly a different staining pattern. The major protein detected is the

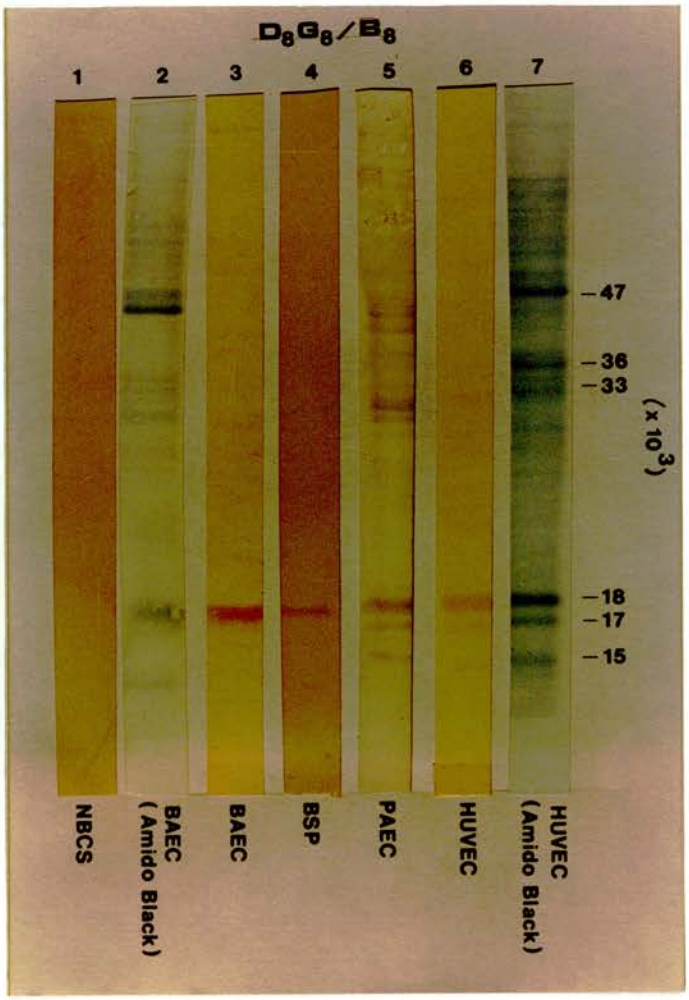


FIG. 4.8. Immunoblot of antibody D8G8/B8 on endothelial cell lysates.
Lanes 2 and 7 are total protein stained.

47kD band, with a more faintly stained band at 18kD. A band around 55kD is heavily stained and faint bands are present around the 33 - 36kD region.

No staining of serum proteins occurs with either of these clones.

4.5.3. D8G8

This antibody like D6G11 is an IgM subtype which was raised against BAEC from mice immunised by the second protocol. It recognises damaged endothelium in fixed cell ELISA. The protein band staining pattern differs from the A3E10 and D6G11 antibodies in that no protein band at the 47kD region is detected on BAEC, BSP and HUVEC (Fig.4.8). Protein bands of 15, 17 and 18kD are stained on HUVEC. Only the 18kD band is stained on BAEC. Two total protein stained control lanes are shown, BAEC (2) shows incomplete transfer of these lower molecular weight proteins , whereas HUVEC (7) shows good transfer. This may explain the presence of only one band in the antibody treated BAEC and BSP. On PAEC D8G8 shows a much greater degree of staining. The three bands at 15, 17 and 18kD are heavily stained along with bands at 33 and 36kD. Faintly stained bands are present at 47 and 55kD. This PAEC lane was gold/silver stained and the others AEC stained. It was also prepared from a gel with a slightly different

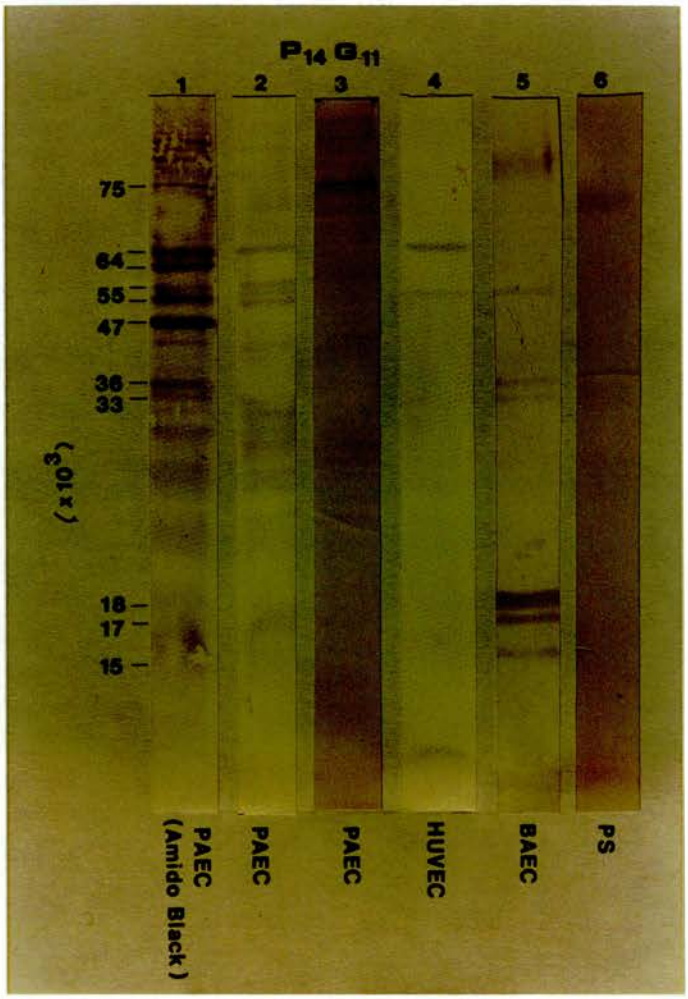


FIG. 4.9. Immunoblot of antibody P14G11 on endothelial cell lysates. Lane 1 is total protein stained.

running time, hence the band pattern is slightly out of step with the control lanes. D8G8 does not react with serum proteins (1).

4.5.4. P14G11

Only one stable clone was raised from this cell line and the antibody is the only IgG2a subtype in all the the monoclonals screened so far. It was raised against PAEC by immunisation protocol 1 and reacts with fixed and non-fixed damaged cells.

Fig. 4.9 shows the results of the immunoreactivity on transferred proteins. All the transfers were gold/silver stained, lanes 1,2 and 4 are Immobilon P membrane the others are nitrocellulose. On Immobilon P, amido black stains protein black, whereas it stains blue on NC.

P14G11 gives conflicting results on PAEC. On lane 2 protein bands at 64kD and three around 55kD stain heavily. There are also faint bands just below the 47 and 33kD areas. On lane 3 the only heavily stained band is at 75kD with very faintly stained bands just above and below. These blots were of cell lysate from a first subculture cell line. The lysate was prepared for electrophoresis and dispensed into 100ul aliquots and stored at $-20^{o}C$. When a gel was bein run a sample was thawed slowly to room temperature and applied directly

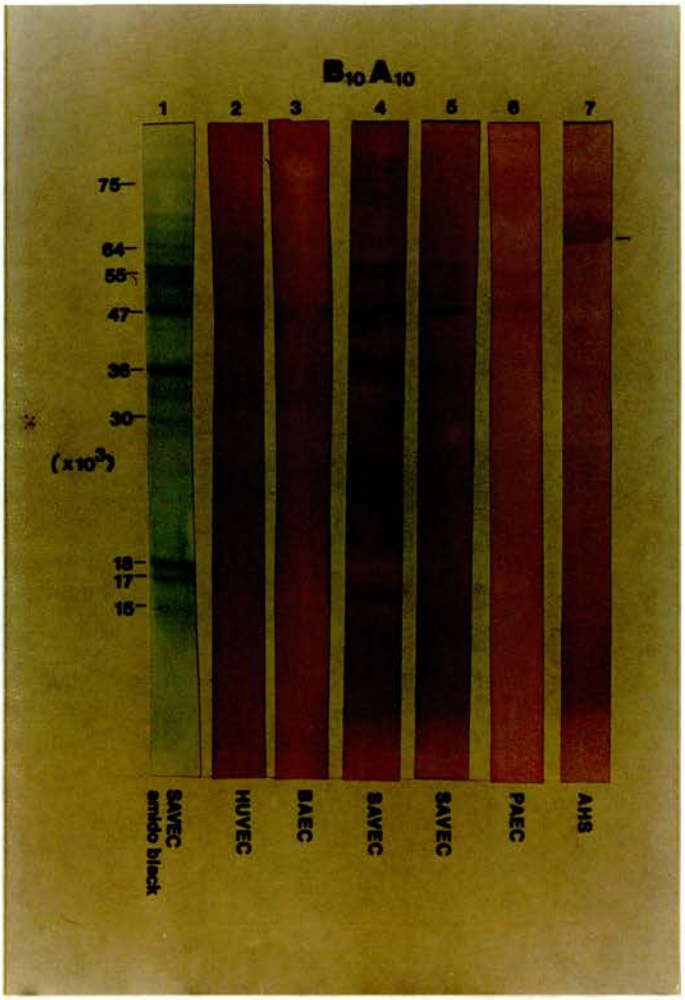


FIG.4.10. Immunoblot of antibody B10A10 on endothelial cell lysates.
Lane 1 is stained for total protein.

to the stacking gel. Lane 3 was from a newly prepared sample, i.e. it had not been frozen. The sample on lane 2 had been stored frozen for one or two weeks. It seems likely that changes had taken place in the lysate during the freezing, storage or thawing processes which would result in the different immunoreactivity with this antibody. Unfortunately this could not be repeated with fresh lysate due to the lack of pig aortic cells.

With HUVEC this antibody detects only two protein bands at 64 and 55kD. On BAEC the strongest staining occurs at 15, 17 and 18kD with fainter bands at 33, 36 and 55kD. On the BAEC (lane 5) there is an area of shadow just above the 75kD region which looks as if it might be an artifact of staining. It is diffuse and not sharply defined and is unlikely to be a protein band.

P14G11 does not react with serum proteins.

4.5.5. B10A10

This antibody was raised against HUVEC from mice immunised by protocol 1. On fixed cell ELISA it produces a diffuse negative stain and is thought to be directed against a component of extracellular matrix.

Fig.4.10. lanes 2,3 and 6 were stained with AEC and lanes 4,5 and 7 with silver enhanced gold.

On HUVEC the antibody stains bands with corresponding molecular weight of 47 and 64-66kD and a

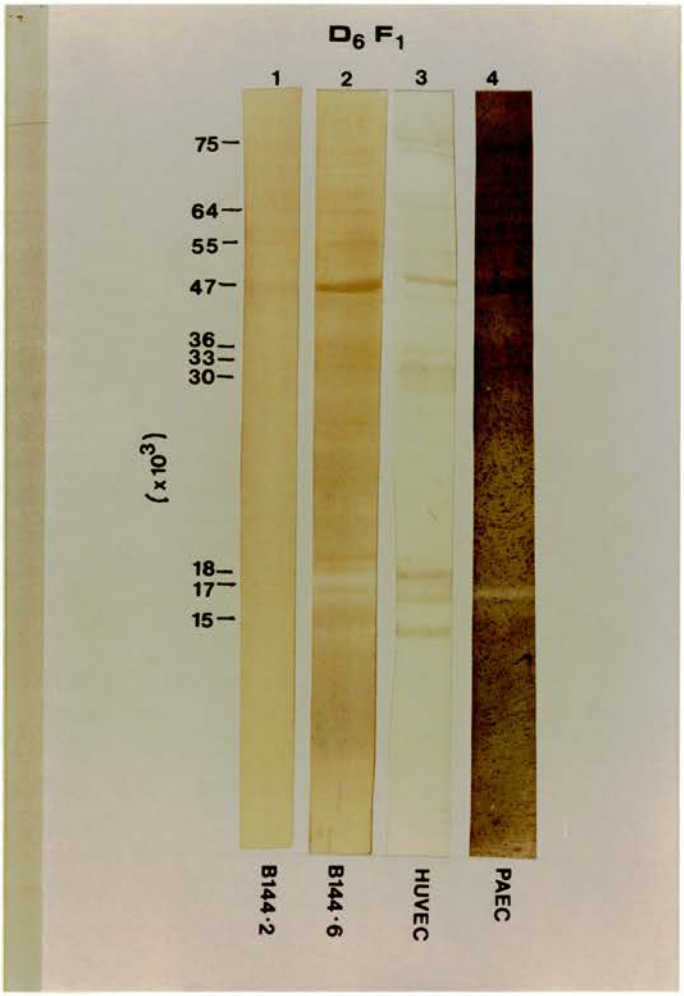


FIG. 4.11. Immunoblot of antibody D6F1 on endothelial cell lysates.

faint band at 55kD. On SAVEC (lanes 4 and 5) 47 and 55kD bands are clearly seen with three bands at the 75kD region faintly stained. On BAEC the 75,66 and 47kD bands are very faintly stained. With PAEC bands at 55,47 and 45kD (very faint) are stained.

On adult human serum (AHS) a diffuse band corresponding to the 66kD marker is faintly stained (marked -). There are faint but sharper bands just above and below this area. On the corresponding gel (Fig.4.5 lane E) these bands are present just above and below the broad 66kD albumin band.

4.5.6. D6F1

This antibody was also raised against BAEC by the second immunisation protocol. Only one clone was kept. In fixed cell ELISA this antibody gives a diffuse staining pattern.

Fig.4.11 lanes 1 and 2 were AEC stained and lanes 3 and 4 silver stained. Lane 1, a first subculture cloned bovine cell line, shows very faint bands at 47 and 55kD. Lane 2, the sixth subculture of the same cell line, shows heavy staining of a band at 47kD with fainter bands at 15,17 and 18kD, 55,64 and 75kD. If the background staining were less perhaps more bands would be seen. On silver stained HUVEC many bands are visible with the major ones at 15,17,18 and 47kD. On silver

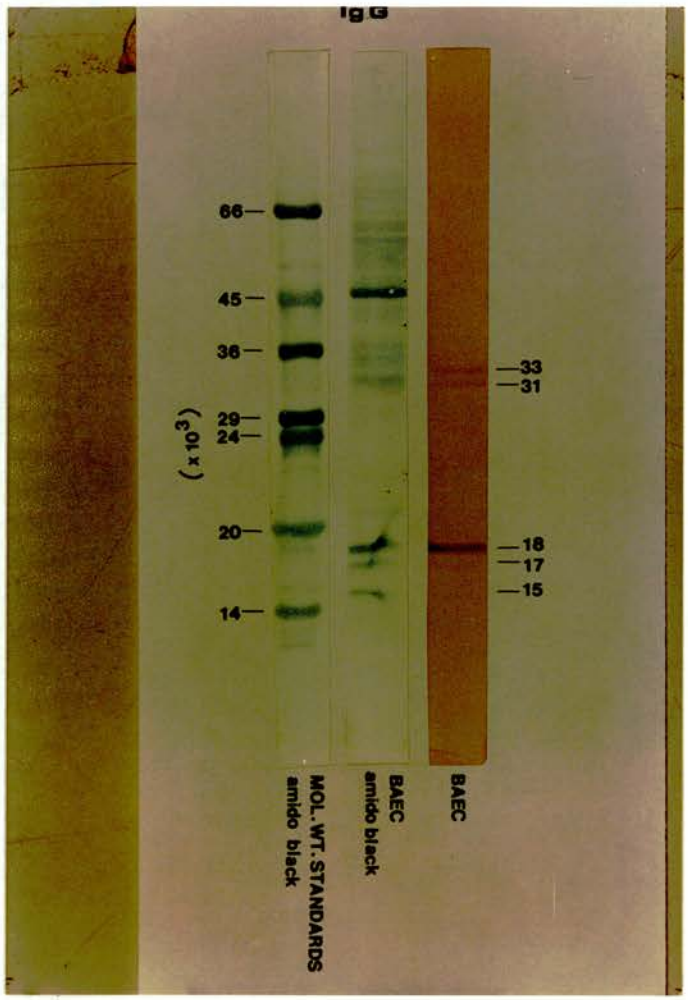


FIG.4.12. Non - specific binding of IgG to endothelial cell lysates.

stained PAEC the background is so heavily stained that no clear bands can be seen at all. It is posible that a preliminary washing step has been missed in the staining procedure of this sample has been missed. This antibody was not tested against serum proteins.

4.5.7. Non - specific IgG binding to transferred endothelial cell proteins.

IgG accumulation is a feature of damaged cells as discussed in section 3.2.1. IgG binds non - specifically to intermediate filaments of the cell cytoplasm (Hansson and Schwartz 1983). The staining pattern on fixed cell ELISA of monoclonal antibodies D8G8, D6G11 and A3E10 is the same as that with IgG. This non - specific effect can be blocked by pre - incubation of the cells with an IgG which cannot be recognised by the second antibody detection system. A protein transfer of BAEC lysate was incubated with a 1:100 dilution of mouse IgG1 and stained by the AEC method. The results (Fig.4.12) shows a strongly stained band at 18kD. Faint bands were present at 15 and 17kD but the colour faded before the blot could be photographed and they are now difficult to see. Faint bands can also be seen at the 31-33kD region.

These are protein bands which are also detected by some monoclonal antibodies described in the previous

sections in particular antibody D8G8/B8.

4.6. DISCUSSION

There are few major differences in band patterns between endothelium from bovine, porcine and human sources in relative protein content. The results of section 4.3.2 show some interesting changes in lipid/sialoglycoprotein band pattern. Highly subcultured cells show extensive decrease in overall content of these components with major loss of particular bands with inreasing number of population doublings. Loss of major functional cell surface proteins such as ACE is known to occur with culture age (Levine et al 1983), making the use of highly subcultured cells for the study of normal cell behaviour questionable. This was taken into consideration in subsequent experiments and hence only cells of up to five subcultures were used. The use of the silver staining technique for detection of sialoglycoproteins/lipids was limited in the experimental protocol of cell lysate preparations and SDS-PAGE used here. The stained gels show that the majority of these components did not migrate through the gel but remained at the origin of the stacking and resolving gels. For this technique to be of more use a

greater degree of molecular breakdown is needed perhaps by enzymatic digestion followed by separation on gels with less cross-linkage. Since none of the antibodies tested by immunoblotting reacted with protein bands corresponding to those of interest on the silver stained gels, this technique was not pursued further.

Further separation and characterisation of endothelial cell proteins could be carried out by separation of the proteins in the first dimension on the basis of isoelectric point and in the second dimension on the basis of molecular weight (Sixma and Schiphorst 1980). This would separate different proteins of the same molecular weight to greater degree. This would be a useful technique in future work on characterisation of endothelial antigens.

The results of the immunoblotting experiments are summarised in table 4.6.

Protein bands of nine different estimated molecular weights were found to react with antibodies tested. The reactivity of these proteins varied between antibodies and endothelial source.

Four protein bands of estimated molecular weights of 47k,18k,17k and 15kD predominate. The 18,17 and 15kD bands ran as a triplet on CB stained gels and in some cases reacted with the antibody as a triplet. Both the 47kD and the 18,17 and 15kD bands were present on all

	BAEC	PAEC	HUVEC	SAVEC	BSP
A3E10/C9	47	55 47 (33)	-	-	-
A3E10/D11	47 18	(75) (47) (36,33)	47	47 17	
D6G11	47	55 47 18 (36,33)	47 18 17 15		47 18 17 15
D8G8/B8	18	18 17 15 (55) (47) (36,33)	18 17 15		18 17 15
P14G11	18 17 15 (55) (36,33)	75 64 55 (47) (33)	64 55		
B10A10	(75) (64) (47)	(55) (47)	(64) (55) (47)	(75) 64 55 47	
D6F1	55 47 18 17 15		75 64 55 47 36 33 18 17 15		

TABLE 4.6. Summary of the proteins bands, by molecular weight, detected by monoclonal antibodies.

endothelium as major protein bands of whole cell lysates.

The 47kD band was not present in any of the silver stained preparations, but instead could be seen as a negative region which corresponded to the CB stained band.

The three lower molecular weight bands did have an equivalent region of silver staining. The most heavily stained being the one with highest molecular weight (Fig.4.2(b)). Although it seems likely that these proteins are glycosylated it cannot be stated with certainty since no equivalent molecular weight standards were run on the gel.

The other protein bands detected by monoclonal antibodies were of estimated molecular weights 75k,64k,55k,36k and 33kD and were also common to all endothelium with the exception of the 64kD band , which was not present on BAEC.

The 47kD protein was the predominant band with which antibodies A3E10, D6G11, B10A10 and D6F1 reacted. D8G8 and P14G11 did not bind to this band in BAEC, HUVEC or BSP and only very weakly with PAEC.

D8G8 reacted strongly with the 18k,17k and 15kD proteins. P14G11 also reacted with these proteins on BAEC but not on PAEC, where 75k,64k and 55kD bands were stained.

Antibody B10A10 which on fixed cell ELISA on sparse cultures showed negative staining, did not react with the low molecular weight proteins at all but weakly with bands at 75k,64k,55k and 47k. On the basis of the fixed cell ELISA results with this antibody it was thought to be directed to extracellular matrix proteins, which are manufactured and excreted by the endothelium. However, the known components of extracellular matrix consist of very high molecular weight proteins such as fibronectin and the collagens.

On fixed cell DAB ELISA it was discovered that false positive results were due to non-specific binding of IgG (from the serum in the growth medium) to damaged cells. This could be blocked by pre-incubation of the cells with an autologous IgG that would not be recognised by the second antibody in the detection assay. Non-specific binding of IgG on immunoblots was examined. Three protein bands of molecular weight 36k,33k and 18kD reacted. The 18k appeared to have a stronger reaction by the more intense staining seen, but it was also present in the control blot in greater quantity than the 36k,33k bands. Non-specific binding of IgG has been shown to occur via the Fc component of the antibody to the vimentin component of intermediate filaments (Hansson et al 1984). This was confirmed on the basis of immunocytochemical studies but not with

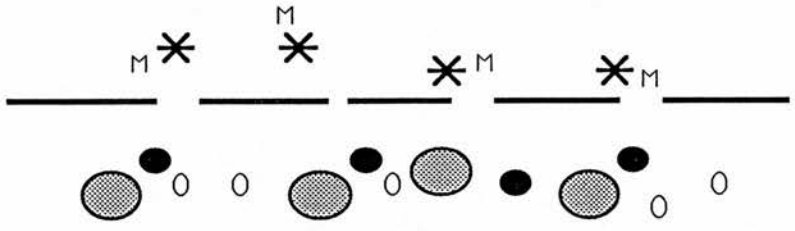
immunoblotting with the isolated protein. Vimentin has a reported molecular weight of 56 - 58kD under reducing conditions (Wu et al 1982 , Ketis et al 1986). The protein bands which reacted with IgG in this study have a much lower molecular weight, from 18kD to 36kD. Until recently it was believed that the intermediate filaments of endothelial cells consisted exclusively of vimentin. It has been demonstrated that endothelial cells of different vasculatures have other intermediate filament components, such as desmin (Fujimoto and Singer 1986). It is possible that intermediate filament associated proteins are present and also take part in non-specific IgG binding, which could explain the results of section 4.5.7.

These low molecular weight protein bands are present in a number of the immunoblots and were generally taken to be due to non-specific binding, except with antibody D8G8. The 18kD (17k,15k) bands were the only proteins to which this antibody reacted. Pre-incubation of cells with IgG to block non-specific binding sites did not block binding of D8G8 (IgM) in fixed cell ELISA. From this information it was concluded that antibody D8G8 was specific for these proteins.

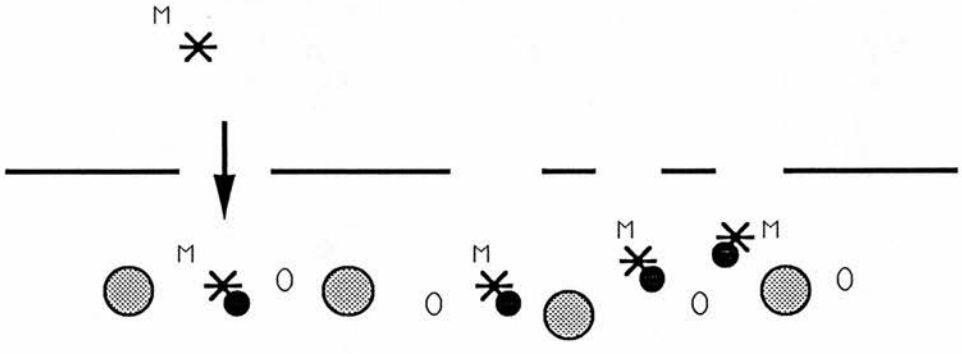
The results of P14G11 are harder to explain. From the results of fixed and non-fixed cell ELISA it is

known that P14G11 cross reacts strongly with BAEC, however the immunoblot results show reactivity with different proteins of BAEC and PAEC. On PAEC the antibody binds to three proteins of high molecular weight and BAEC with the low molecular weight triplet of proteins. The 55kD molecular weight protein is common to both however, although it is very faintly stained in the BAEC lane. This is more likely to be due to differences in quantitative transfer of this protein in BAEC than the behaviour of the antibody. It is proposed that the epitope recognised by the antibody is shared by proteins 75k,64k and 55kD in PAEC. This epitope is also common to proteins 55k and 64kD of HUVEC and 55kD of BAEC (64k protein is not present in BAEC) and the 18k,17k,15k,36k and 33kD bands on BAEC are due to non-specific binding. An alternative explanation for this result could be that P14G11 is not truly monoclonal and two IgG2a antibodies are present, one which reacts with a group of high molecular weight proteins on PAEC and one which reacts with a group of low molecular weight proteins on BAEC.

The findings of these immunoblot experiments may help to explain the reactivity of these antibodies on fixed and non-fixed cells. With the exception of P14G11 none of the antibodies react with non-fixed cells. All the antibodies tested so far are IgM subtype except

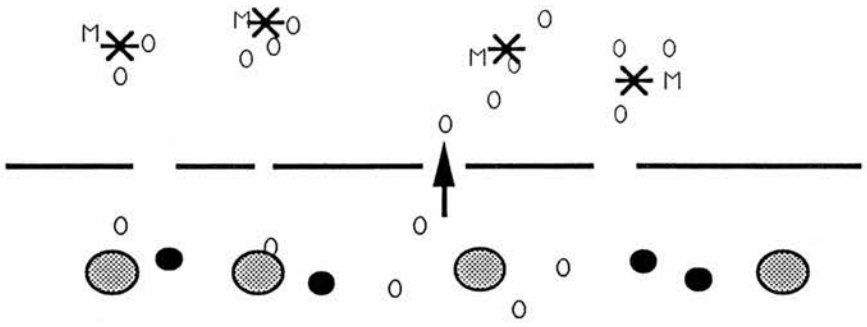


Damaged non-fixed cell membrane does not allow intracellular access of large IgM molecules.

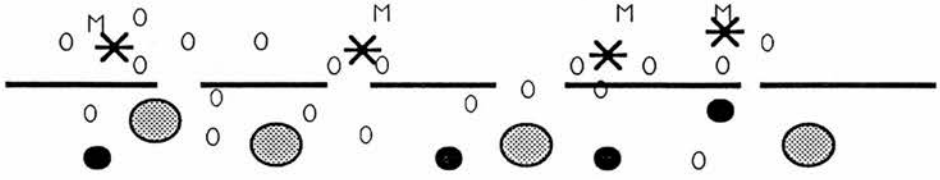


Fixation of damaged cells allows access of IgM molecules to intracellular proteins, through large "holes" in the membrane.

DIAG. 4.2 Possible explanation of differences in IgM antibody staining of fixed and non-fixed cells. Intracellular proteins of varying molecular size are denoted as open and filled circles.



Small intracellular proteins leak from damaged non-fixed cells where Ag-Ab complexes are formed outside the cell and are lost during washing stages of the staining procedure. Therefore a negative result is shown.



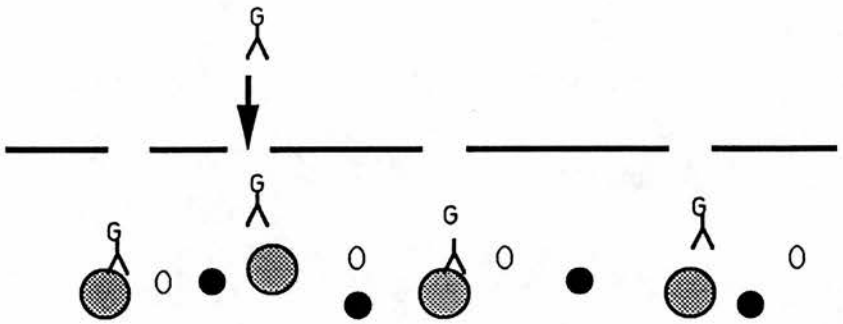
When damaged cells are fixed immediately, these small proteins that leak from the cell are most likely trapped at the cell surface on the outside of the membrane. The IgM molecules therefore bind to the immobilised antigen, giving a positive result on staining.

DIAG. 4.1 Possible explanation of the difference in antibody staining of non-fixed and fixed cells with IgM type antibodies. Intracellular proteins of varying molecular size are denoted as open and filled circles.

P14G11 which is IgG2a subtype. The mechanisms proposed are as follows.

Cell damage invariably results in plasma membrane perturbation allowing entry of extracellular material into the cell and leakage of small intracellular molecules out of the cell. Antibody D8G8 binds to small molecular weight proteins and only stains fixed cell preparations. It is possible that in non-fixed cells the antigen-antibody complex forms outside the cell and is lost in subsequent washing stages of the staining procedure, therefore giving a negative result. When damaged cells are fixed immediately the leaking proteins are probably trapped on the outside of the cell surface. The large IgM antibody molecules therefore have access to these immobilised proteins. (Diag.4.1). This may also be the case for antibodies A3E10 and D6F1 which bind to slightly larger molecular weight proteins. Alternately, fixation may cause formation of large "holes" in the already damaged cell membrane. These holes are large enough for the entry of IgM molecules into the cell where they bind to intracellular proteins, whereas non-fixed damaged cells do not (Diag.4.2).

In the case of P14G11 which was the only antibody to stain non-fixed damaged cells and is the only IgG_{2a} subtype, the cell membrane of these cells probably



DIAG. 4.3 Non-fixed damaged cell membrane allows entry of relatively small IgG2a molecules into the cell. Intracellular proteins of varying molecular size are denoted as open and filled circles.

allows intracellular access of this much smaller and steriochemically different molecule. (Diag.4.3).

The information gained from the immunoblot experiments and the limitations of the immunisation and screening protocols, indicate that the proteins that are antigenic to the monoclonal antibodies described here are normal cell endothelial cell proteins that have been "exposed" by injury. These proteins are present in normal, healthy cultured endothelium and are not due to phenotypic expression or metabolic responses to injury. The mice were immunised with healthy cultured cells which had not been previously exposed to injurious stimmuli. The cells on which the antibodies were screened were damaged by physical means and screened immediately therefore giving a very limited time for phenotypic expression of protein to occur. The antigen - antibody response is therefore indicative of plasma membrane damage.

Many components of the normal endothelial cell have been characterised and identified. Many are highly glycosylated with high molecular weights, vWF subunits of 225kD (Jaffe and Nachman 1975) fibronectin with subunits of 210kD (Stein and Stein 1976), thrombospondin subunits 190kD (McPherson et al 1981), thrombomodulin 115kD (Esmon et al 1982), ACE 130kD (Friedland et al 1981), E92 (Kaplan et al 1983), HEC-1

90kD subunits (Parks et al 1985). These and many more surface proteins can be ruled out as possibilities in identification of the antigens described here on the basis of molecular weight. Antithrombin III has a molecular weight of around 62kD, and a protein of similar molecular weight was detected by antibodies P14G11 and B10A10. However these antibodies did not react with undamaged cells which should express antithrombin III on the cell surface.

A number of culture shock and heat shock proteins have been identified and are proposed as indicators of cell injury (Sage et al 1986, Schorer et al 1985). One of these, a 43kD protein is synthesised and released in response to injury by endotoxin and also by rapidly growing cells in sparse cultures, which suggest it has a general role in cellular function as well. This protein has a molecular weight close to that of the 47k protein recognised by antibodies A3E10 and D6G11 etc.

Darnule et al (1983) characterised two cell surface proteins of molecular weight 18.5k and 66k of which he claims the 18.5k was a surface associated plasma protein. Antibody D8G8 reacts with a group of proteins in this molecular weight region predominantly with a 18k protein. This was not a serum protein associated with the cell surface (the antibody did not react with serum proteins) but may well be a surface antigen or a

cytoplasmic component associated with the plasma membrane. This antibody (and D6G11) was produced by immunisation protocol 2, where the mice were injected with compatible spleen cells which had been incubated with a monolayer of confluent endothelium. Spleen cell lymphocytes adhere to the endothelial surface for up to 5 or 6 hours and then spontaneously detach (de Bono 1977). When they detach from the cell surface it may be that they take with them a fraction of the cell surface and thereby presenting to the mouse immune system a component of the underside of the endothelial plasma membrane or cytoplasmic components associated with the membrane, such as components of the intermediate filaments (Ketis et al 1986). The passage of intracellular molecules between lymphocytes and endothelium during adherence has been demonstrated (Guinan et al 1988). It is therefore possible that the proteins, against which the antibodies from the immunisation protocol 2 react, are also present in lymphocytes. Since the proteins detected by these antibodies are also detected by antibodies from immunisation protocol 1 (47kD, 55kD etc), this explanation seems unlikely. However, further investigation of the cross-reactivity of these antibodies with other blood and lymphatic cells would be useful.

The experiments presented in this chapter were not done with the intention of identifying or characterising the endothelial antigens recognised by the monoclonal antibodies and the work is not within the scope of this thesis. However by identifying the molecular weights of the antigens, possibilties of identity can be excluded on the basis of molecular weight and explanations of the antibody - antigen behaviour can be made.

CHAPTER 5.

RADIOLABELLING OF MONOCLONAL ANTIBODIES

5.1. INTRODUCTION

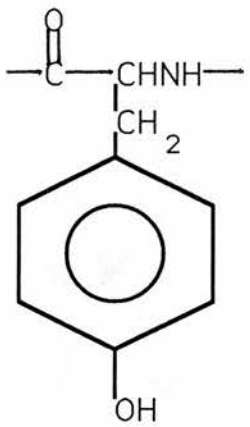
Radiolabelled monoclonal antibodies make effective probes and have been used widely in therapeutic and diagnostic fields (McMichael and Fabre 1982).

Monoclonal antibodies can be radiolabelled in a number of ways. The criteria for the method of labelling and the choice of isotope is based on the lability and stability of the antibody and for what purpose it is being labelled. For imaging and autoradiography, gamma emitors such as ^{125}I, ^{131}I and ^{111}In are required for fast and intense development of X-ray film.

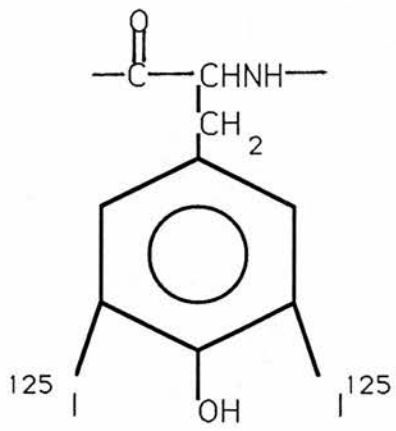
Beta emitting isotopes such as ^{3}H and ^{14}C can be introduced into the antibody molecule by a pre-labelled sugar or amino acid during in vitro biosynthesis. Internal labelling method involves no harsh oxidising procedures and can produce antibodies of high specific activity. The major advantage of beta emitors, in terms of handling and safety, is that it is short range emission and requires less lead shielding. However, because of this their use in imaging and autoradiography is limited.

This chapter describes two labelling procedures and the problems encountered.

TYROSINE

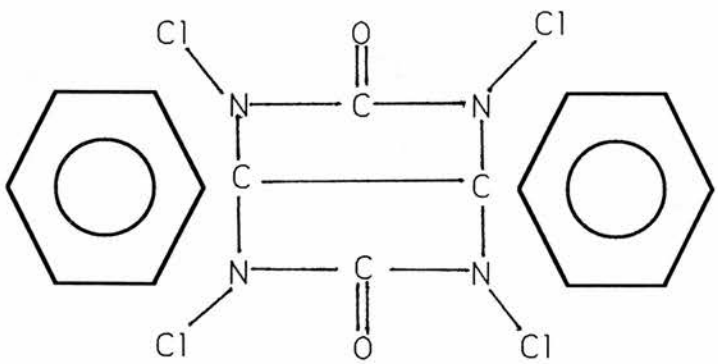


IODOTYROSINE



DIAG.5.1 Position of iodine isotope in the tyrosine molecule

IODOGEN



DIAG.5.2 Structure of Iodogen molecule

5.2. IODINATION OF MONOCLONAL ANTIBODIES D8G8 AND P14G11

Techniques of iodinating proteins all employ oxidising agents to produce an electrophilic iodine species (I^+) from sodium iodide. The radioactive iodine reacts with aromatic ring structures of various amino acids such as tyrosine which is considered to be the predominantly labelled amino acid (Diag.5.1).

Iodogen (1,3,4,6-tetrachloro-3a,6a-diphenylglycouril) (Diag.5.2)(Pierce Chemical Company) is a mild oxidising agent which is very stable and is also insoluble in water. This insolubility allows a solid phase iodination process, whereby the Iodogen acts at the walls of the reaction vessel. This has two major advantages over aqueous phase oxidising reagents lactoperoxidase and chloramine-T; 1. the process can be carried out in an aqueous solution without much direct contact with (and therefore possible damage to the protein by) the oxidising agent, 2. no reducing agents need to be added to stop the reaction, decanting the reactants from the vessel does so (Fraker and Speck 1978, Goding 1986).

Iodogen was used here because of its "gentler" nature and therefore was considered less likely to damage or inactive the monoclonal antibodies to be labelled.

5.2.1. Preparation of Iodogen Tubes

1mg of Iodogen was dissolved in 0.5ml of methylene chloride and diluted to 5ml in methylene chloride. This solution was dispensed into plastic Beckman microfuge tubes in 100ul aliquots. The methylene chloride was evaporated by a stream of dry nitrogen at room temperature, leaving a layer of Iodogen on the inside surface of the tubes. These were stored at -40^{o}C.

5.2.2. Iodination Procedure

The antibody solutions contained a minimum of 500ug/ml of protein in phosphate buffer pH 7.4. To 90ul of antibody solution was added 50ul of 0.05M phosphate pH7.4 / 0.01% v/v Tween 80 and 10ul (37000 kBq) of $Na^{125}I$ in an Iodogen tube. The $Na^{125}I$ was added last and the contents of the tube very gently mixed and incubated for 5 minutes at room temperature.

The contents of the tube were then transferred to a 5ml plastic tube. The reaction tube was rinsed twice with 500ul of T-buffer (0.5M phosphate / 0.5M NaCl / 2.5g/L gelatin / 1% w/v Tween 20 pH7.4)(Dr N Hunter, Scottish National Blood Transfusion Headquarters). The rinses were added to the reactants and incubated for a further 10 minutes at room temperature before gel filtration.

5.2.3. Gel Filtration

Unreacted molecules and free iodine were removed from the reaction mixture by gel filtration on Sephadex G-50 or on Excellulose GF-5 disposable desalting columns (Pierce Chemical Company). Both types of column were equilibrated with T-buffer and protein eluted with T-buffer. 1ml fractions were collected and counted. The labelled protein should elute in the void volume (first count peak) and the free unreacted iodine in the second peak. The labelled antibody was stored in T-buffer at $4^{o}C$.

5.2.4. Counting.

All radioactivity was counted in an LKB Compugamma counter with an efficiency of 75% for ^{125}I.
Counts per minute (CPM) were converted to disintigrations per minute (DPM) as follows;

CPM = DPM x EFFICIENCY

From this radioactivity was calculated in Becquerels (Bq) where 2.2×10^{9} DPM = 37MBq.

The iodinated protein fraction was counted and percentage of radioactivity incorporated in the protein fraction calculated.

5.2.5. Iodination of D8G8/B11

Antibody D8G8/B11, an IgM which was purified from hybridoma growth medium by protein A - Sepharose affinity chromatography (2.5.3.) was iodinated by the above process. The protein concentration was estimated at 2mg/ml by Bradfords (1979) method. A total of 18.5 kBq of $Na^{125}I$ was used in the procedure.

Total Radioactivity Used = 18.5 kBq

The first peak of radioactivity occurred in two fractions (4 and 5). This was assumed to contain labelled antibody.

Radioactivity in Labelled Fraction = 949 cpm
= 1265 dpm
= 0.022 kBq

Percent Radioactivity Incorporated = 0.12%

The amount of labelled protein was much lower than expected. From the results of other laboratories (personal communications) an average of approximately 50% of the radioactivity is incorporated into the protein by this method. There are a number of possible reasons as to why the amount of labelling was so low :

1. The protein estimation of the antibody solution was

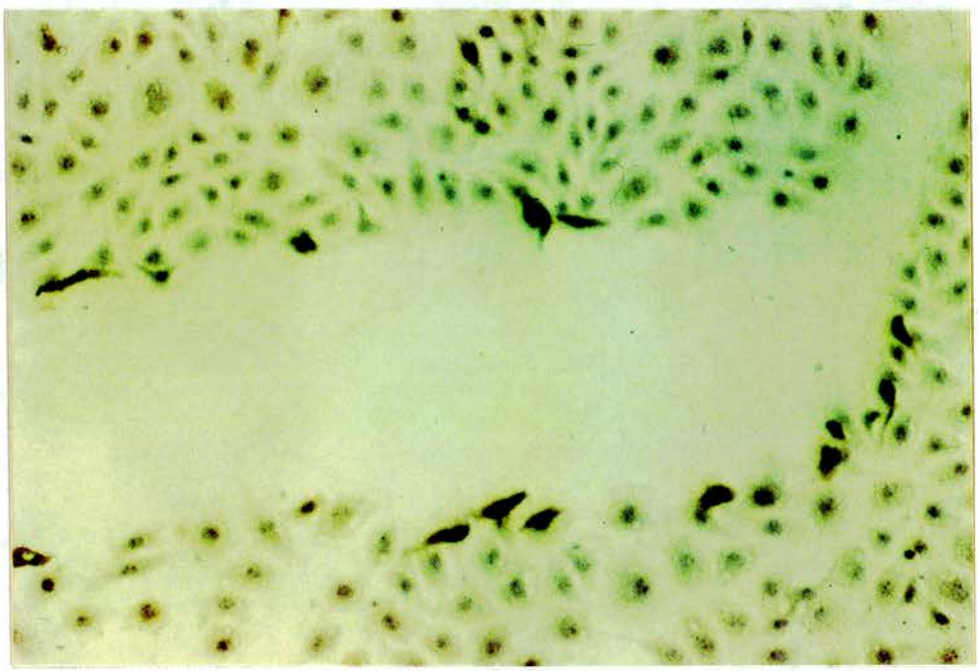


FIG.5.1. ^{125}I D8G8/B11 ON SCRATCH DAMAGED BOVINE AORTIC ENDOTHELIAL CELLS, DAB STAINED. Mayer's haematoxylin counterstained. Unlabelled D8G8/B11 showed exactly the same staining pattern.

wrong and there was much less antibody present than originally thought. 2. Denaturation of the antibody had occured during storage. 3. The Iodogen tube had been in storage for some time and may have become inactive.

The following tests were carried out to determine the nature of the labelled protein fraction.

DAB ELISA.

The screening procedure (2.4.3) was carried out on cultured bovine aortic endothelial cells. A 1 in 10 dilution of the radioactive fraction was used with a 1 in 10 dilution of "cold" D8G8/B11 as a control. The results (Fig 5.1) show that the radioactive fraction contained antibody and that binding activity had not been altered by the iodination process.

Autoradiography of Immunoblots

Bovine cell lysates, SDS-PAGE and protein transfers were prepared as described in section 4.2.

The protein transfer was cut into strips and a control was stained for total protein with amido black. The others were incubated with 20% bovine serum albumin (BSA) solution overnight to block non-specific protein binding sites and then with a 1 in 5 dilution of ^{125}I D8G8/B11 in PBS for 2 hours at room temperature with constant shaking. They were rinsed with PBS / 0.1%

FIG.5.2. AUTORADIOGRAPH OF 18kD PROTEIN BAND, BINDING ^{125}I D8G8/B11. The photograph has been cut above the 17 and 15kD bands which were present on the amido black stained blots but which did not show on the autoradiograph.

BSA until no radioactivity was detected in the wash. The strips were then dried between sheets of filter paper and placed on X-ray film (Agfa - Gevaert, Curix RP1) in a Kodak enhancer cassette and exposed at $-70^{o}C$ for 24 hours. The film was developed on a Fuji automatic developer.

The autoradiograph (Fig.5.2) shows a band equivalent to the 18kD region of the amido black stained section (photograph not shown) which is known to react with D8G8/B11 (section 4.5.3). This would suggest that the antibody D8G8/B11 has been labelled with ^{125}I without loss of antibody activity.

Autoradiography of Scratch Damaged Cells

Bovine aortic endothelial cells were grown to confluence in 5cm diameter petri dishes with tissue culture plastic detachable linings. Scratches were made in the monolayer with a glass pipette, 2 scratches 1cm apart and the cells were fixed in 0.1% gluteraldehyde and incubated for 2 hours with a 1 in 5 dilution of ^{125}I D8G8/G11. Rinsing was carried out with PBS until no radioactivity was detected in the washes. The cell covered plastic lining was removed and wrapped in cling film before being placed in contact with X-ray film and exposed for 24 hours at $-70^{o}C$ in an enhancer cassette. The film was developed as before.

A DAB stained control showed antibody binding to cells at the scratch edge. The results of the autoradiograph were however completely negative.

There are a number of possible reasons as to the negativity of the autoradiograph : 1. The amount of radioactivity on the antibody present at scratch edges was not enough to show up on this type of X-ray film after just 24 hours of exposure. A change of film to a more sensitive type or longer exposure times would perhaps solve this problem if greater incorporation of radioactivity into the antibody cannot be achieved. 2. The radioactive label had "dropped off" the antibody molecule. The stability of the labelled antibody was not known under the conditions in which it was stored (in T buffer pH7.4 at $4^{o}C$). The autoradiograph of the protein blot was done 2 or 3 days after the iodination procedure and the complex appeared to be stable then however the autoradiograph of the scratched monolayers was done approximately 2 weeks later. It is possible that the complex is not stable for this length of time in solution or in this particular buffer and should perhaps be freeze dried as soon after iodination as possible.

At this point all the D8G8/B11 ascites had been used and problems were encountered in raising more from the mice available at that time, so iodination

experiments were switched to another antibody.

5.2.6. Iodination of P14G11 (Ascites Fluid).

Antibody P14G11 recognises damaged cells in both fixed and non-fixed preparations. It also cross reacts with human and bovine endothelial cells (chapter 3). Radiolabelled P14G11 would make possible in vivo investigations of damaged vascular endothelium.

Monoclonal P14G11 is an IgG2a subtype and was raised in quantity in ascites tumours of mice (2.2.10) and purified by protein A - sepharose affinity chromatography (2.5.3).

The first attempt at iodination of this antibody was on an unpurified sample of ascites fluid. Although ascites consists of a mixture of proteins including various immunoglobulins the major component should be the monoclonal antibody. It was decided not to go through the time consuming process of purifying the antibody but to iodinate the whole ascites component and assume that the other iodinated proteins would not interfere with P14G11 binding to damaged cells.

The ascites had an estimated protein content of 29 mg/ml. Iodination of 90ul (2.61mg total protein) of antibody with 37 000 kBq of ^{125}I was carried out as described for antibody D8G8F (5.2.5).

Radioactivity in Labelled Fraction = 556153700 CPM

= 0.74×10^9 DPM

= 12.33 MBq

Total Radioactivity Used = 37 MBq

Percent Radioactivity Incorporated = 33.3%

The percentage of incorporation of label onto antibody P14G11 was much higher than that of the D8G8/B11 iodination, however not all of the labelled protein in this case were antibody.

DAB ELISA

This was carried out as described in section 2.4.3. Bovine endothelial cells were used since no pig endothelial cells were available at the time. A 1:10 dilution of the labelled fraction was used. The cells along the scratch edge stained with DAB indicating the presence of antibody.

Autoradiography of Immunoblots.

Protein transfer was performed as described previously (section 4.2). Transfers were incubated with 1:100 and 1:1 000 dilutions of labelled fraction and prepared for autoradiography by the same method as described for D8G8. The developed film showed very high

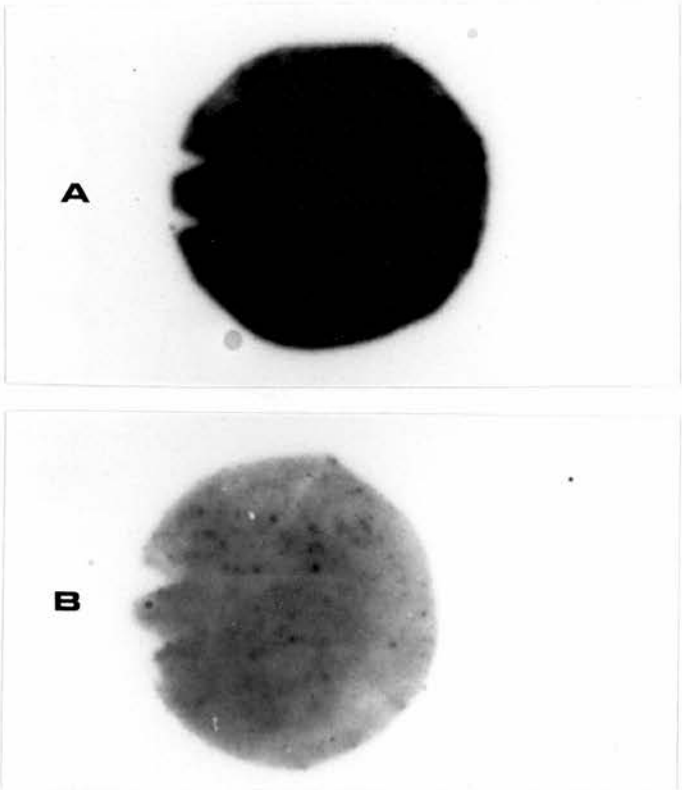


FIG 5.3. AUTORADIOGRAPH OF SCRATCHED CELL MONOLAYERS INCUBATED WITH ^{125}I P14G11.

A - 1:100 dilution in PBS
B - 1:1000 dilution in PBS

non-specific binding with no individual bands visible (i.e. the developed film was completely black at the areas of the nitrocellulose strip.).

Autoradiography of Scratch Damaged Cells.

These experiments were done in exactly the same way as described for antibody D8G8/B11 (5.2.5). A 1:10 and 1:100 dilution of the labelled fraction was tested and controls DAB ELISA stained. The film was exposed this time for 7 days at -70^{o}C. The results (Fig.5.3) show very intense background staining in both dilutions. The scratched areas being visible as unexposed strips. The DAB stained controls showed staining of the cells along the scratch edge only with no high non-specific background staining. The pre-incubation with autologous serum step to block non-specific binding sites had been mistakenly omitted and was thought to be the cause of the high background result.

The experiment was repeated with lower dilutions of antibody (1:1 000 and 1:10 000) and pre-incubation step with neat NBCS for 15 minutes to block non-specific binding sites. The results were completely negative. Either the pre-incubation stage very effectively blocked binding or the antibody dilutions were too low to deliver enough radioactivity to the sites of damage to expose this type of film. Unfortunately no DAB ELISA

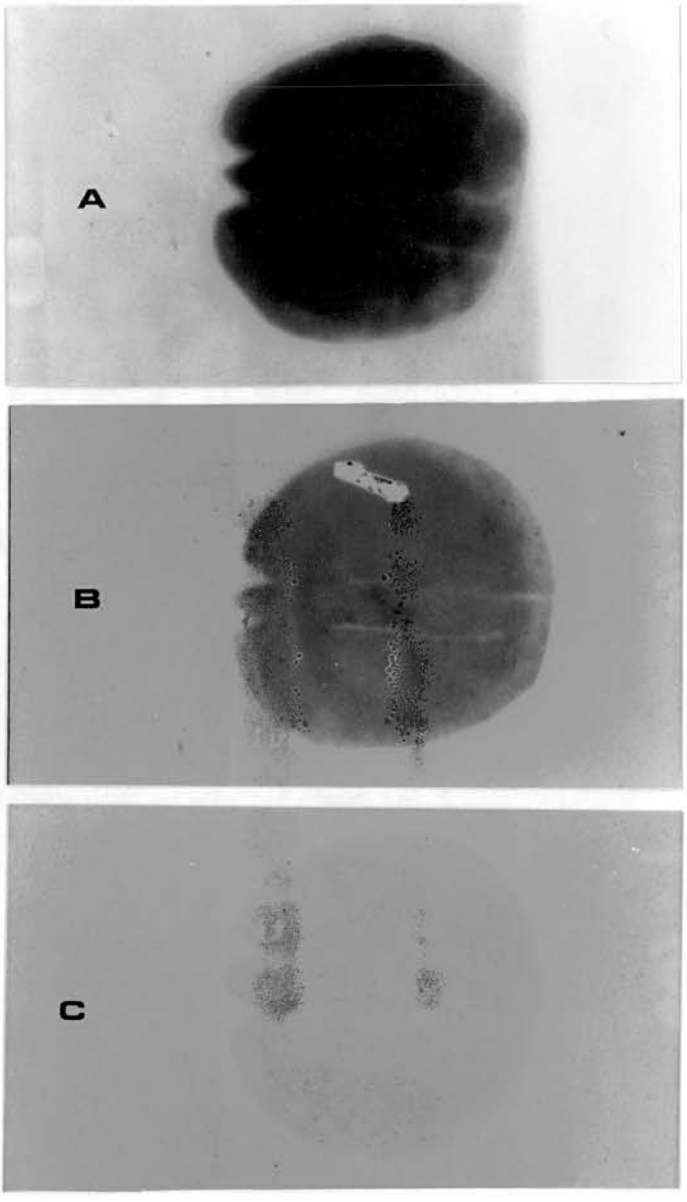


FIG.5.4. AUTORADIOGRAPH OF SCRATCH CELL MONOLAYERS INCUBATED WITH ^{125}I P14G11.

A - 1:2 dilution in PBS
B - 1:10 dilution in PBS
C - 1:100 dilution in PBS

controls were done with these antibody dilutions.

A third trial was done using a new cell line (BAEC). The method was the same as above, this time using 1:2, 1:10 and 1:100 dilutions of the antibody. DAB stained controls of each dilution were also carried out. Exposure was carried out at -70^{o}C in an enhancer cassette for 36 hours. The results (Fig.5.4) show that high background activity is still present with no concentration of exposure along the scratch edge. The DAB ELISA controls show staining of cells at the scratch edge at each dilution.

These high background activity results could be due to interference by other iodinated proteins in the the ascites fluid. For this reason it was decided that purification of the antibody from the ascites was necessary before iodination.

5.2.7. Iodination of Purified P14G11.

P14G11 was purified from ascites by protein A - sepharose affinity chromatography (2.4.3). The ascites (4ml) was clarified by Millipore filtration (0.22um) and diluted with 0.05M Tris / 0.15M NaCl / 0.02% NaN_3 pH8.6 until the pH of the final solution was approximately 8.6 before applying to the column. Elution of ascites and subsequent fractions were all done at room temperature at a constant flow rate of 1ml

/ minute and 5ml fractions were collected. The presence of protein in the fractions was determined by absorbance at 280nm on a Pye Unicam SP8-100 Ultraviolet Spectrophotometer. Protein was detected in the pH5.5 and pH4.3 elution fractions. The fractions were neutralised and tested for antibody content by DAB ELISA on scratch damaged bovine endothelial monolayers.

FRACTION	STAINING OF DAMAGED CELLS
pH8.6 WASH	-
pH5.5 ELUTION	+
pH4.3 ELUTION	++

The negativity of the pH8.6 wash indicates that all the antibody in the sample applied to the column had bound and had been released in the pH5.5 and pH4.3 fractions. The antibody staining pattern of both fractions was identical indicating that both fractions contained the same antibody. Protein A has been reported to have two binding sites of different affinity for IgG's (Lancet et al 1978) and therefore will have different elution properties, in this case pH5.5 and pH4.3. The positive fractions were pooled and concentrated by ultrafiltration and protein content was estimated (Bradford 1979) at 0.3mg/ml (pH4.3) and 2.6mg/ml

(pH5.5).

The pH4.3 fraction was chosen for iodination since it produced the strongly positive DAB ELISA result. 150ul of antibody containing total protein of 45ug was iodinated with 18 500 kBq of $Na^{125}I$ as described. The reaction mixture was gel filtered on Excellulose GF-5 disposable columns. Fractions of 0.5ml were collected. The second fraction from each column was retained as ^{125}I labelled antibody.

Radioactivity in Labelled Fraction	=	323082200 CPM
	=	0.43×10^9 DPM
	=	7.17 MBq
Total Radioactivity Used	=	18.5 MBq
Percent Radioactivity Incorporated	=	39%

DAB ELISA

Bovine cells were grown to confluence on glass coverslips and damaged either by scratching or by air drying for 5 minutes at $37^{o}C$. After fixing in glutaraldehyde and blocking non-specific binding sites with NBCS, the cells were incubated with 50ul of neat and 1:100 dilution of sample. After thorough rinsing they were incubated with peroxidase labelled second antibody and DAB substrate. The results are shown below

TEST	STAINING OF DAMAGED CELLS
NEAT SAMPLE	
AIR DRIED DAMAGE	++
SCRATCH DAMAGE	+
NON-DAMAGED CONTROL	-
1:100 DILUTION	
AIR DRIED DAMAGE	-
SCRATCH DAMAGED	-
NON-DAMAGED CONTROL	-

Autoradiography of Damaged Cells.

The tests in section I were run in duplicate, the second set of coverslips were rinsed and placed on x-ray film and exposed for 48 hours. The 1:100 dilution was negative, whereas the neat sample gave very high backgrounds even with non-damaged cells.

The results of the DAB ELISA show that there was no binding of antibody to non-damaged cells and even in the scratch damaged tests antibody binding was confined to the cells at the scratch edge. Therefore the background activity shown by autoradiography was probably not due to non-specific binding of antibody but suggested that there was still large quantities of free ^{125}I in the preparation.

Protein A-sepharose Affinity Chromatography of Iodinated Fraction.

The labelled antibody fraction was run through a protein A-sepharose column in an attempt to remove the antibody bound ^{125}I from the free ^{125}I. The majority of the radioactivity was eluted in the pH8.6 wash with smaller peaks at pH5.5 and pH4.3 which from previous separations indicates labelled antibody. Fractions corresponding to these peaks were retained and tested for antibody activity by DAB staining on bovine endothelium. The results are shown below.

FRACTION	STAINING OF DAMAGED CELLS
pH8.6	++++
pH5.5	++
pH4.3	-

Positive results from the pH8.6 wash indicate that not all the antibody had bound to the column. This fraction was reapplied to a "clean" column and elution repeated. The strong positive results for the pH5.5 fraction indicates and the corresponding peak of radioactivity indicate labelled antibody. Prior to iodination,

antibody P14G11 eluted in two different pH fractions , 5.5 and 4.3. Purification by the same method after iodination indicates binding of the antibody at the pH 5.5 binding site only. The iodination procedure seems to have changed the antibody affinity for the pH4.3 binding site so that the antibody had either run straight through the column or had bound at the pH5.5 site.

5.3. INTERNAL LABELLING OF MONOCLONAL ANTIBODIES D8G8/B11 AND P14G11 WITH TRITIATED LYSINE.

During biosynthesis of monoclonal antibodies, lysine has been shown to be the most efficiently incorporated amino acid. It is therefore the most commonly used means of introducing a radiolabel into the antibody molecule (Cuello et al 1982).

5.3.1. Growth of Hybridoma Cells with ^{3}H Lysine.

Both D8G8/B11 and P14G11 were treated in the same way. Antibody secreting hybridomas were grown to log phase growth in normal RPMI 1640 growth medium supplemented with 5% FCS. They were centrifuged out of this medium and split at a 1:2 ratio into lysine free RPMI 1640 + 5% FCS for 24 to 48 hours or until optimal cell density was reached. The cells were removed from

this medium and resuspended in fresh lysine free RPMI 1640 + 5% FCS containing 0.37MBq/ml of L-[4,5-H3] lysine monohydrochloride. Incubation with the labelled amino acid was carried out for 24 hours, after which the medium was decanted and retained (batch 1). The cells were resuspended in fresh medium (no ^{3}H lysine) and incubated for a further 24 hours. At the end of this second incubation the cells were no longer viable therefore the medium and cells were separated and the medium supernatant stored (batch 2). Both were stored at $4^{o}C$.

5.3.2. Liquid Scintillation Counting

Counting was carried out on an LKB 1217 Rackbeta Liquid Scintillation Counter. Samples were counted over a 20 minute period and counts per minute (CPM) were automatically calculated. Efficiency for tritium was estimated at 20% by an internal standards method.

The medium supernate samples were counted directly in scintillant in a sample size of 10ul. It was found that very little quenching occured with this sample size and still gave a sufficient number of counts. The cell pellet after centrifugation was dissolved in formic acid. The cells from 1ml of suspension in log phase growth ($\sim 10^{5}$ cells) were completely dissolved

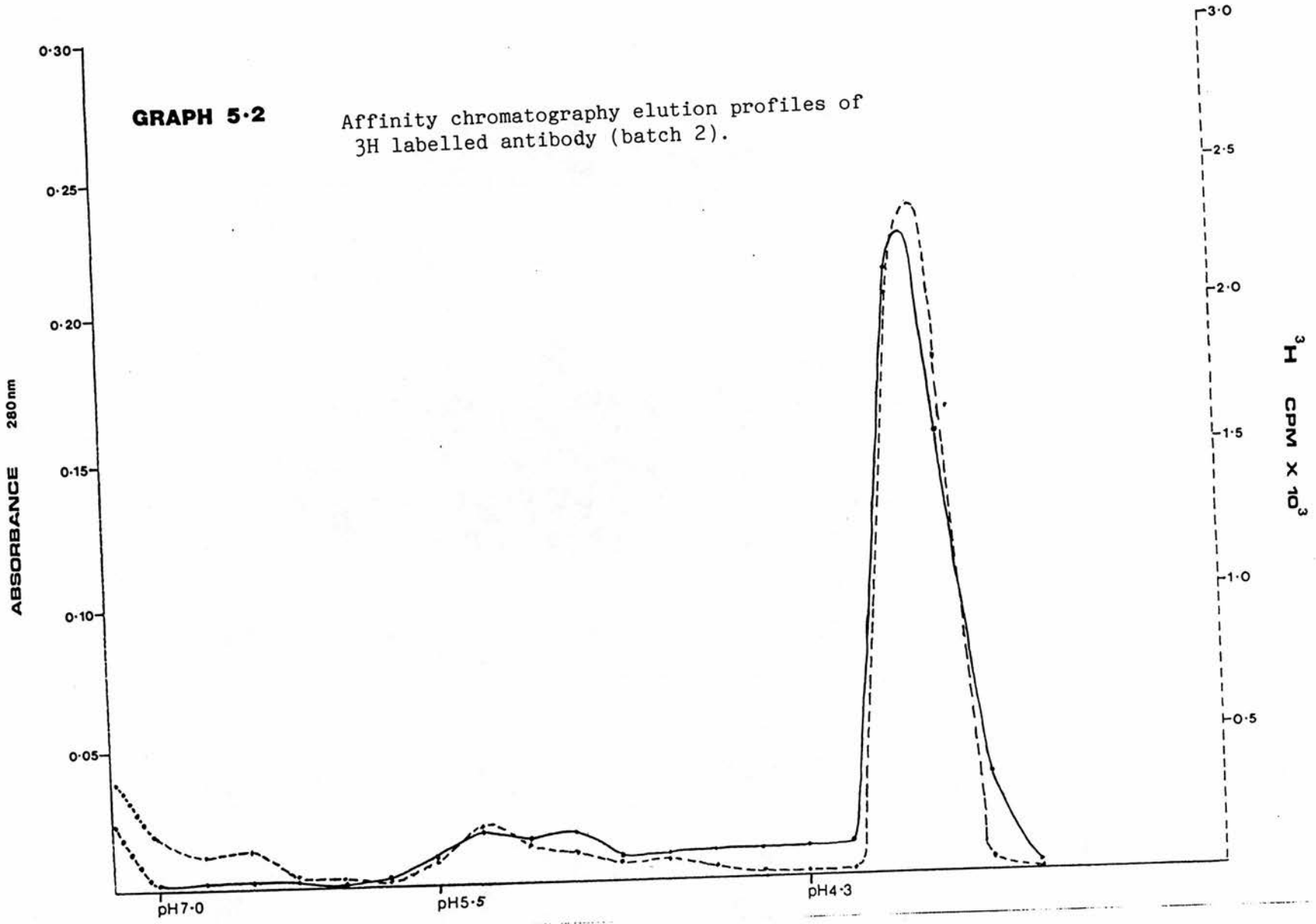
by 100ul of acid. Formic acid, when used in the right cell number to volume ratio, dissolves cellular material completely leaving a clear solution with very little quenching effect. Unlike some other scintillant compatable tissue solvents it can be used in plastic tubes and plates. The control counts with formic acid only show very little difference from the scintillant only controls indicating very little count quenching. The average counts from 10 control samples of 10ul of formic acid in 10ml of scintillant was 12.9 CPM whereas for scintillant alone it was 10.5 CPM. Samples of 5ul of cell preparation were counted.

All samples were counted in 10ml of a commercially prepared toluene based scintillant, Cocktail T 'Scintran' (BDH Ltd). This scintillant was particularly good for complete dispersal of aqueous samples.

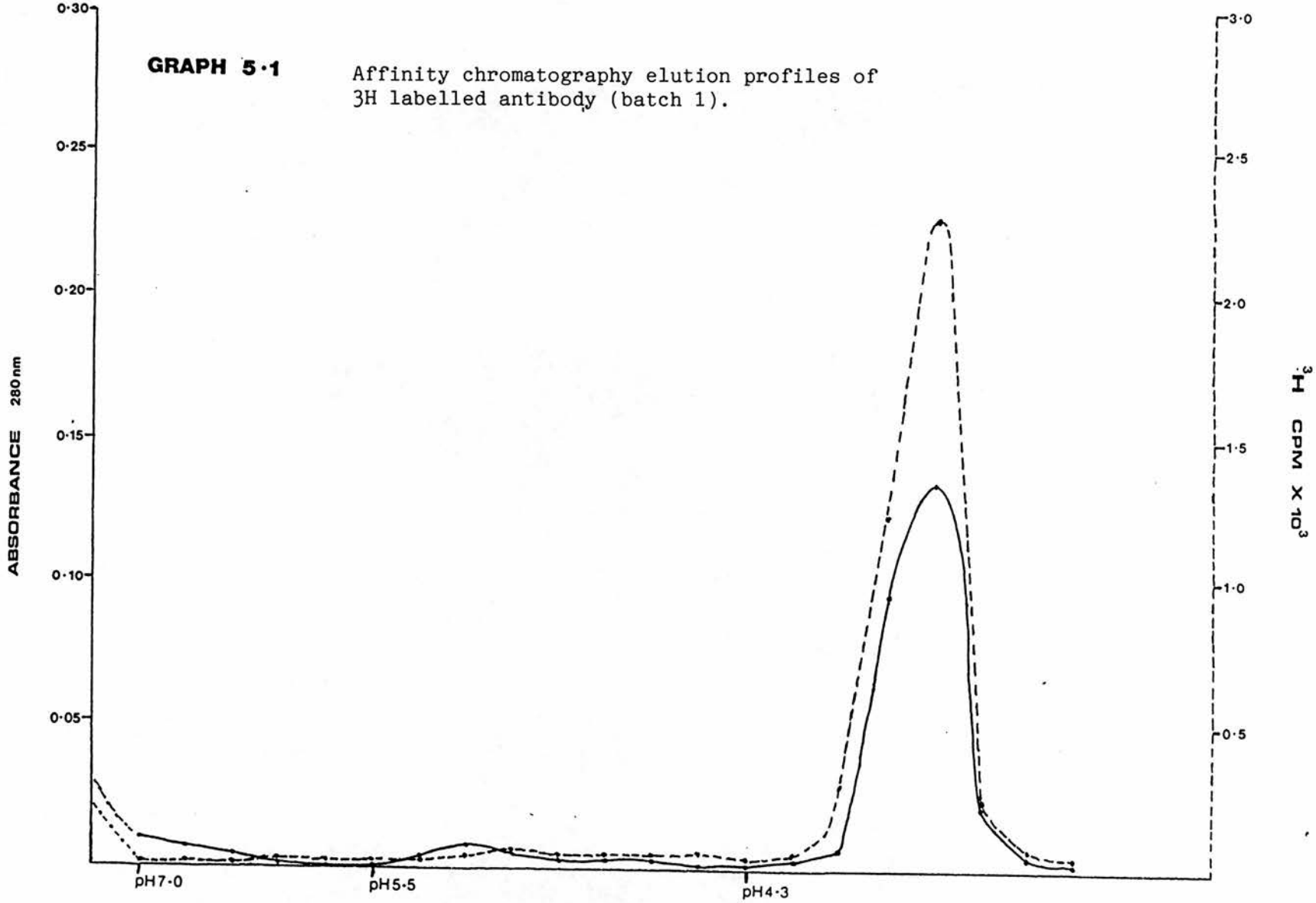
5.3.3. Purification of Labelled Antibody by Protein A-Sepharose Affinity Chromatography.

The pH of the supernate was adjusted to between 8 and 8.5 using 0.15M Tris / 0.05M NaCl pH8.6 buffer. It was applied to the column at a rate of 1ml/min. and 5ml fractions were eluted as described in section 2.4.3. The presence of protein in each fraction was determined by measuring absorbance at 280nm and radioactivity

GRAPH 5·2 Affinity chromatography elution profiles of 3H labelled antibody (batch 2).



GRAPH 5·1 Affinity chromatography elution profiles of 3H labelled antibody (batch 1).



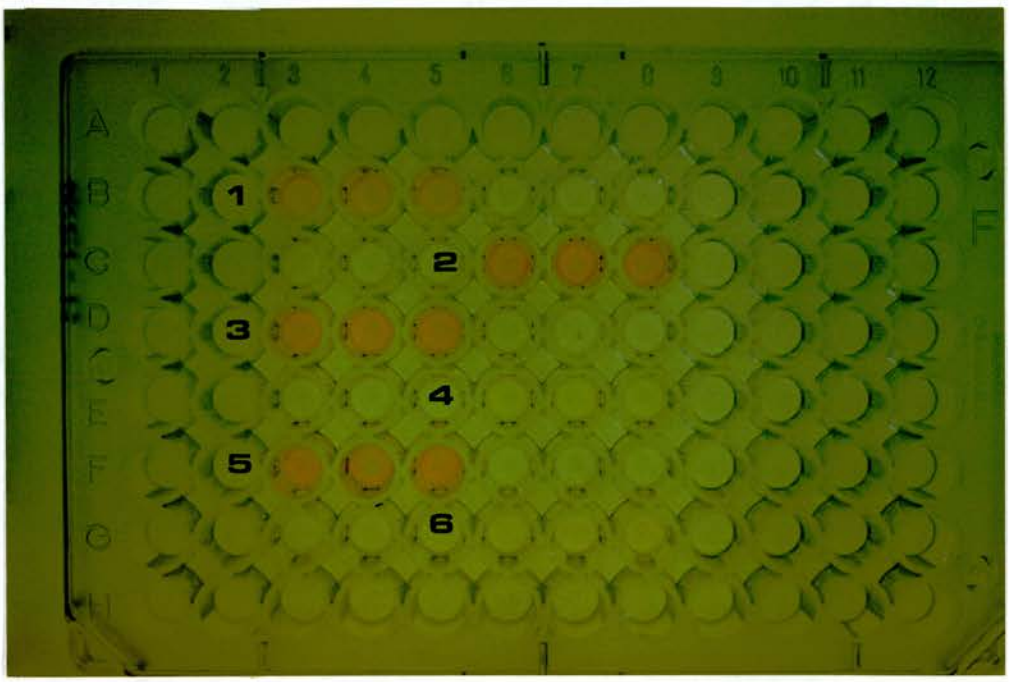


FIG.5.5. o-PD ELISA OF PURIFIED FRACTIONS OF ^{3}H P14G11 ON FIXED PIG AORTIC ENDOTHELIAL CELLS

1 - pH 5.5 fraction (Batch 1)
2 - pH 4.3 fraction (Batch 1)
3 - pH 4.3 fraction (Batch 2)
4 - pH 8.6 wash after sample application (Batch 1+2)
5 - P14G11 unlabelled control
6 - Peroxidase second antibody control

measured. The absorbance profiles (Graphs 5.1 and 5.2) show a peak at the pH 4.3 fraction which is consistent with an increase in CPM, with the peak absorbance and peak radioactivity occuring in the same fraction, suggesting that the radioactivity and the protein are associated. There is a small peak at the pH5.5 fraction of batch 1 (graph 5.1) which in terms of absorbance would be insignificant. Since it is associated with an increase in CPM and therefore probably indicates the presence of a small amount of radiolabelled protein. With batch 2 (graph 5.2), the elution shows very little change in absorbance and no corresponding peak in radioactivity, so it was assumed that radiolabelled protein was present in this fraction.

5.3.4. OPD - Fixed Cell Elisa Screening for Antibody.

The peak protein and radioactivity fractions from the purification procedure were screened for antibody activity by primary fixed cell ELISA (o-PD) (section 2.4.2). The results are shown below and in Fig.5.5.

TEST FRACTION	SCORE
pH 5.5 (batch 1)	++
pH 4.3 (batch 1)	+++
pH 4.3 (batch 2)	++
pH 8.6 wash/sample eluant	-
P14G11 control (SN)	++
Peroxidase only control	-

The negativity of the pH 8.6 sample eluant and wash fraction shows that all the antibody has bound to the column. The samples positive for protein and radioactivity contain active antibody.

5.3.5. Detection of ^{3}[H] Antibody on SDS-PAGE and Transferred Proteins by Scintillation Autography.

A method devised by Bonner and Laskey (1974) shows that efficiency of detection of ^{3}H autoradiographically can be increased by treatment of the gel or blot with scintillant before exposing to film. The light generated by the interaction of the beta particles with the scintillant exposes the film rather than the beta particles directly. A sample containing 3000 DPM of ^{3}H activity can be detected after 24 hours exposure and 500 DPM after one week.

Purified antibody fractions were diluted 1:2 with pH 6.8 gel buffer and after adding glycerol and bromophenol blue tracking dye, 40ul samples were applied to a 7.5% acrylamide resolving gel with a 3.3% stacking gel and electrophoresis carried out as described in section 2.5.2.

The gel was prepared for scintillation autoradiography by the method described by Bonner and Laskey (1974). Fixation and staining of the gel was carried out as described in section 2.5.3. The

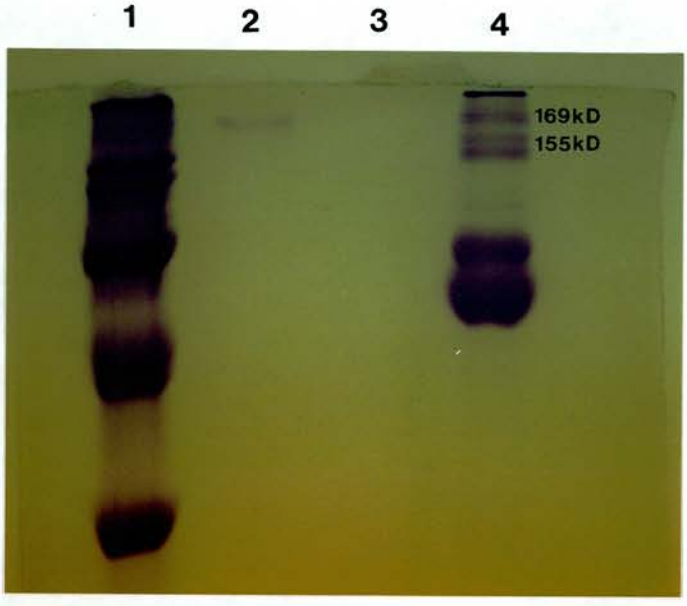


FIG.5.8. COOMASSIE BLUE STAINED CONTROL GEL OF ^{3}H P14G11

Lane 1 - molecular weight standards
Lane 2 - pH4.3 fraction (Batch 1)
Lane 3 - pH4.3 fraction (Batch 2)
Lane 4 - unpurified "cold" P14G11 supernatant control

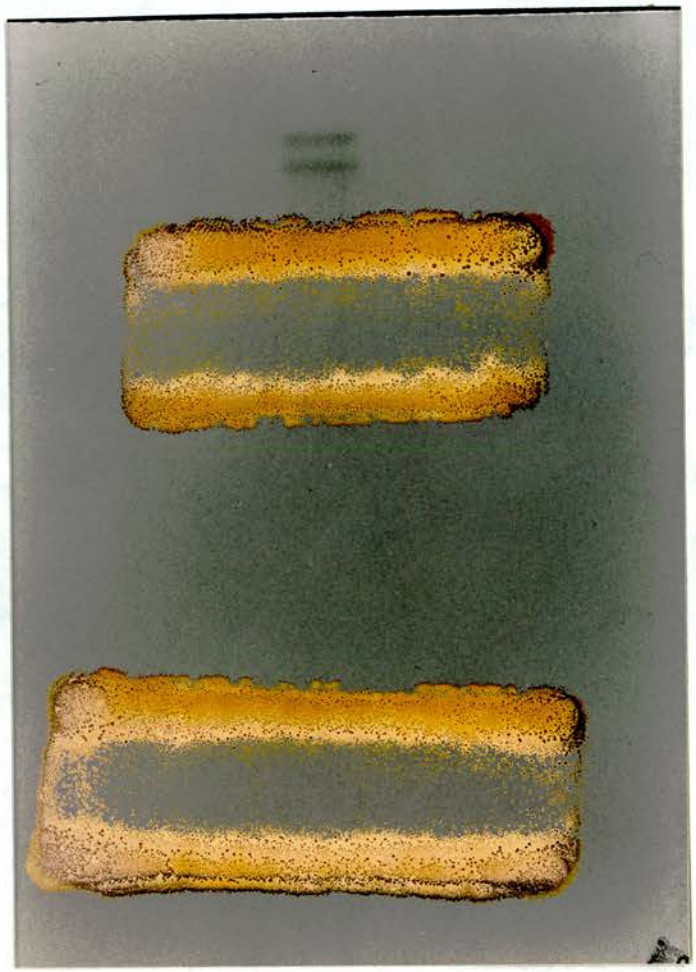


FIG.5.7. AUTORADIOGRAPH OF PURIFIED ^{3}H P14G11 ON A 7.5% ACRYLAMIDE GEL PRE-TREATED WITH SCINTILLANT

20ul samples of batch 1 and batch 2 in the same lane.

FIG.5.6. AUTORADIOGRAPH OF PURIFIED ^{3}H P14G11 ON A 7.5% ACRYLAMIDE GEL PRE-TREATED WITH SCINTILLANT

Lane 1 - pH 4.3 fraction (Batch 1), 20ul
Lane 2 - pH 4.3 fraction (Batch 2), 40ul

destained gel was then soaked in 20 times its volume of dimethyl sulphoxide (DMSO) for 30 minutes. This was repeated in fresh DMSO once. The gel was then transferred to 4 volumes of 22.2% w/v 2,5 - diphenyloxazole (PPO) in DMSO for 3 hours. After washing in 20 volumes of water for 1 hour the gel was dried and placed in contact with Agfa-Gevaert, Curix RP1 x-ray film in a Kodak enhancer cassette at $-70^{o}C$ for 5 days. The results are shown in Figs.5.6 and 5.7. A coomassie blue stained control gel (Fig. 5.8) shows a band corresponding to that of lane 1 Fig.5.6. The coomassie blue was not sensitive enough to pick up the corresponding band of batch 2 (lane 2). When the autoradiograph was superimposed on the control gel the bands corresponded to two bands with estimated molecular weights of 169kD (bands a) and 155kD (bands b). The experiment was repeated with samples from batch 1 and batch 2 run in the same lane (Fig.5.7) and shows two distinct bands with estimated molecular weights of approximately 169kD and 155kD, confirming that the antibody purified from the two different incubation mediums had slightly different molecular weights.

The reason for this variation in molecular weight in what appears to be identical antibody fractions is not immediately obvious. The differences in molecular weight are not enough to explain it in terms of

secretion of two different antibodies which in turn would question the monoclonality of the cell line. A more plausible explanation would be in the amount of glycosylation in the heavy chains of the antibody molecule with the length of time in culture. After the first 24 hour incubation with the radiolabelled lysine the cell numbers dropped and those that were viable looked enlarged and swollen which is an indication of impending cell death. Such cells may alter biosynthesis of antibody for example by reducing the amount of glycosylation of the heavy chains resulting in a "lighter" antibody molecule without altered binding activity. Production and secretion of IgG is not affected by the degree of glycosylation. (Blatt and Haimovich 1981)

5.4. DISCUSSION.

Iodination of two monoclonal antibodies by the Iodogen method described here was not particularly successful. With antibody D8G8/B11 (IgM) a very low percentage incorporation of radiolabel to antibody was attained. Despite this low specific activity (11.1 kBq/mg protein), autoradiography of immunoblots showed that radiolabelled antibody with unchanged activity/specificity had been synthesised. However, for the purposes of imaging individual cells, the specific

activity of the preparation was not great enough. It is possible that the excess of unlabelled antibody in the preparation caused competitive inhibition of binding of labelled antibody to sites of damage and therefore prevented detection by autoradiography. Further work on labelling this antibody to a higher specific activity perhaps by using a different iodination method, may prove useful.

Iodination of antibody P14G11 (IgG2a) by the same method produced a preparation with a much higher percentage incorporation of radioactive isotope. However, subsequent autoradiographic tests showed a high degree of non-specific activity with this labelled antibody. Non-specific antibody binding was ruled out as a possible cause since pretreatment of cells with an autologous serum effectively blocked this in DAB ELISA controls. This pretreatment did not stop high background activity with the same concentrations of antibody on autoradiographs. These results suggested that high quantities of free ^{125}I were present in the antibody preparation. A "clean up" of the preparation on protein A-Sepharose proved this to be right. It did not however improve the results of the autoradiographs and high background activity was still present. This would suggest that the antibody - ^{125}I bond is not stable either as a result of the storage conditions, or

because of the iodination process itself. The high background activity in the autoradiographs is probably due to trapped intracellular free ^{125}I which has entered the cell through small holes in the membrane, caused by fixation.

A different method of iodination should be tried to see if a more stable complex is achieved. The stability of (^{125}I) iodotyrosine compounds is reported to be between two and six weeks at 20^{o}C in 50% aqueous propylene glycol (Evans 1968). The iodinated antibody was stored at 4^{o}C which was perhaps detrimental to the iodotyrosine bond and further work on storage conditions needs to be done.

Internal labelling produces stable radiolabelled molecules where the label is an integral part of the molecule and therefore inherently more stable. However this method is restricted to low energy beta emmitors which are limited in their use for imaging purposes. Detection of tritiated antibody in in vitro preparations is possible by scintillation autography. However, if tritiated antibodies are to be of any use in looking at in vivo or in situ distribution of damaged endothelium, new detection systems must be developed.

CHAPTER 6

TARGETTING UROKINASE (u-PA) TO DAMAGED ENDOTHELIAL CELLS, IN VITRO, WITH MONOCLONAL ANTIBODY P14G11.

6.1. INTRODUCTION

The plasminogen activators t-PA and u-PA are used therapeutically for the dissolution of clots in acute pathological thrombosis. At present the most widely used thrombolytic agent is Streptokinase, a nonenzymic protein produced by strains of beta-haemolytic streptococci. It forms a 1:1 complex with plasminogen resulting in a combination containing an active site which cleaves non-complexed plasminogen molecules to plasmin (Kosow 1975). It does not distinguish between fibrin bound plasminogen and circulating plasminogen, and therefore causes systemic fibrinogen depletion resulting in bleeding complications.

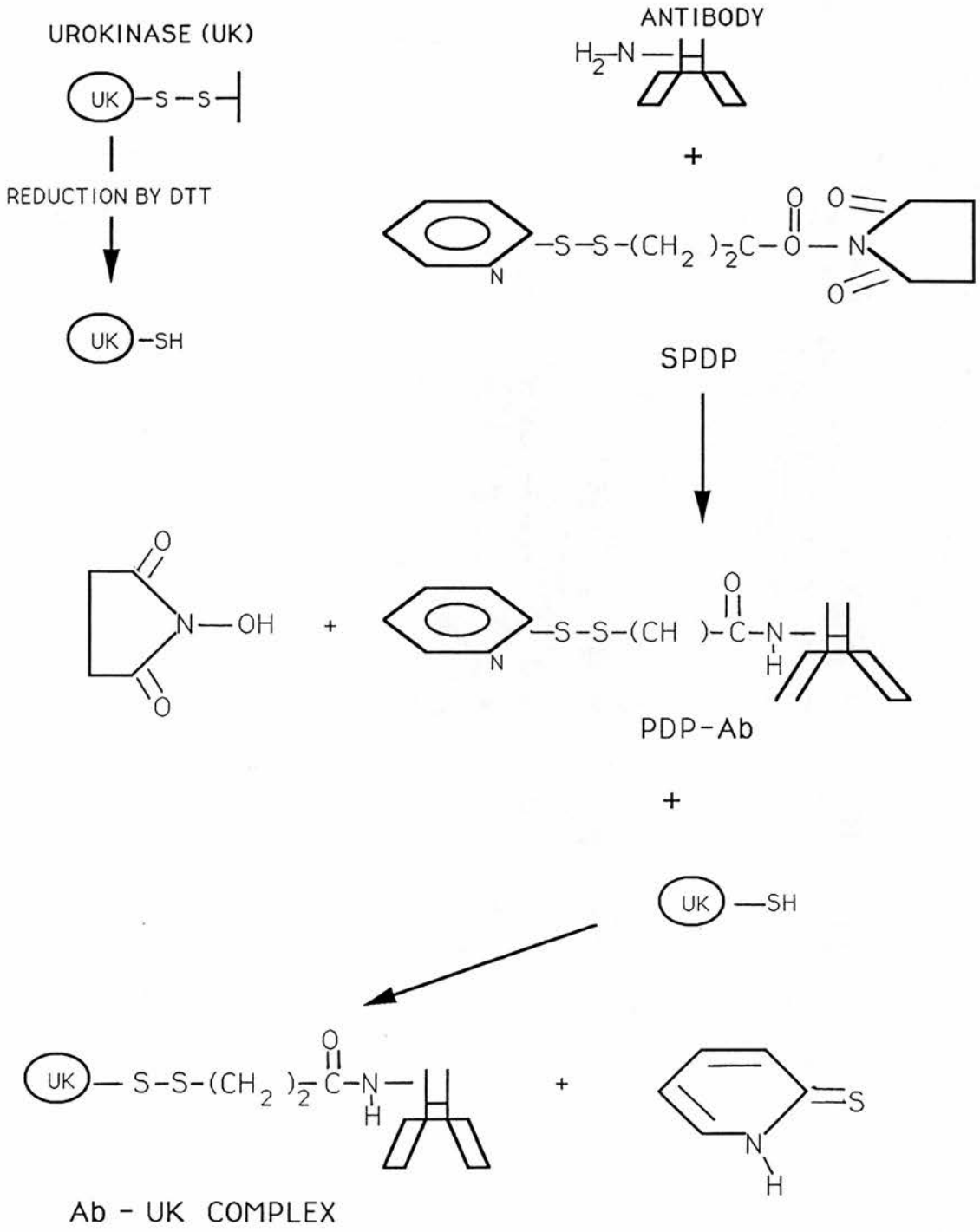
Acylated plasminogen-streptokinase complex (APSAC) has an increased affinity for lysine binding sites on fibrin and an active site inhibited by the acyl group until it reaches its target. On binding to fibrin the acyl group is removed by hydrolysis allowing activation of plasminogen to plasmin . This mechanism reduces systemic fibrinogen depletion and hence iatrogenic bleeding to some extent. Both streptokinase and APSAC cause hypotension and immune reactions as well as bleeding complications, these side effects are well documented (Mander and Sherry 1988, de Bono 1987).

Recombinant human t-PA and scu-PA have a number of advantages over streptokinase and APSAC in terms of

side effects, virtually no hypotension and immune reactions occur with these reagents. t-PA is relatively inactive in the absence of fibrin therefore systemic fibrinogen depletion is avoided to some extent (de Bono 1987). However to obtain rapid coronary reperfusion plasma t-PA levels are raised to approximately 1000 times normal physiological levels, which can result in systemic activation of the fibrinolytic system (Collen et al 1986) .

Single chain u-PA / pro-urokinase has similar advantages over streptokinase and APSAC, and research into its clinical use continues.

Effective thrombolytic agents that do not cause systemic bleeding must distinguish between obstructive thrombi and those preventing haemorrhage. At present no such agents exist. Thrombus formation can occur at areas of the vessel where the endothelial cells have been lost, damaged or altered in response to various events. Such areas do not involve subintimal damage that would result in extravascular bleeding. Monoclonal antibodies that recognise damaged or altered endothelial cells, or exposed basement membrane may prove useful in targetting fibrinolytic agents to such areas. The following experiments demonstrate the conjugation of u-PA with a monoclonal antibody and the restriction of fibrinolysis to areas of endothelial



DIAG. 6.1 SPDP linkage of monoclonal antibody to urokinase

damage.

The monoclonal antibody P14G11 was chosen for the conjugation because it binds to non-fixed damaged endothelium it cross-reacts with human endothelium (section 3.8 and 3.9). It is also an IgG2a subtype which is relatively easily purified by protein A - sepharose chromatography.

The heterobifunctional linking agent n-succinimidyl 3-(2-pyridyldithio)proprionate (SPDP), developed by Carlsson et al 1978 reacts with amino groups on the antibody, thereby introducing a reactive disulphide group to the antibody. Reduction of the disulphide bridge of urokinase leaves a free sulphydril group which interacts with the derivatised antibody resulting in the formation of an intramolecular disulphide link (Bode et al 1985). The reaction is shown in diagram 6.1.

6.2. SPDP LINKAGE OF UROKINASE TO ANTIBODY

Antibody P14G11 was purified from ascites fluid by protein A - sepharose affinity chromatography (section 2.5.3.). Two protein peaks were obtained in pH5.5 and pH4.3 fractions, both contained antibody with identical activity. Protein fractions were pooled, neutralised

and concentrated by ultrafiltration. Protein content was estimated by the method of Bradford (1976).

SPDP was diluted to 2mM in absolute ethanol. P14G11 at 6.5mg in 3.0ml of phosphate buffered saline (PBS) pH7.4 was mixed with 50ul of 20mM SPDP and allowed to react for 30 minutes at room temperature (RT). This was dialysed three times against 1 litre of PBS. Urokinase, from human kidney cells, was dissolved in 0.1M sodium acetate / 0.1M NaCl pH4.5 at 4mg/ml in a 2ml volume. This was reduced by the addition of 0.23ml of 1.0M dithiothreitol (DTT) / 0.1M sodium acetate / 0.1M NaCl pH4.5 for 30 minutes at RT. DTT was removed by desalting on Sephadex G25 (1.5 x 12 cm column) equilibrated with PBS pH4.5. Peak fractions were pooled and concentrated by ultrafiltration to an approximate volume of 5ml. The dialysed PDP - Ab complex and the reduced urokinase solutions were mixed, neutralised and allowed to react overnight at RT. Unreacted molecules were removed by gel filtration on Sepharose - 6B (1.5 x 70 cm column) equilibrated with 0.3M NaCl. Elution was carried out with 0.3M NaCl. The protein fraction was collected in the void volume (20ml) and concentrated by ultrafiltration to 10ml and stored at $4^{o}C$.

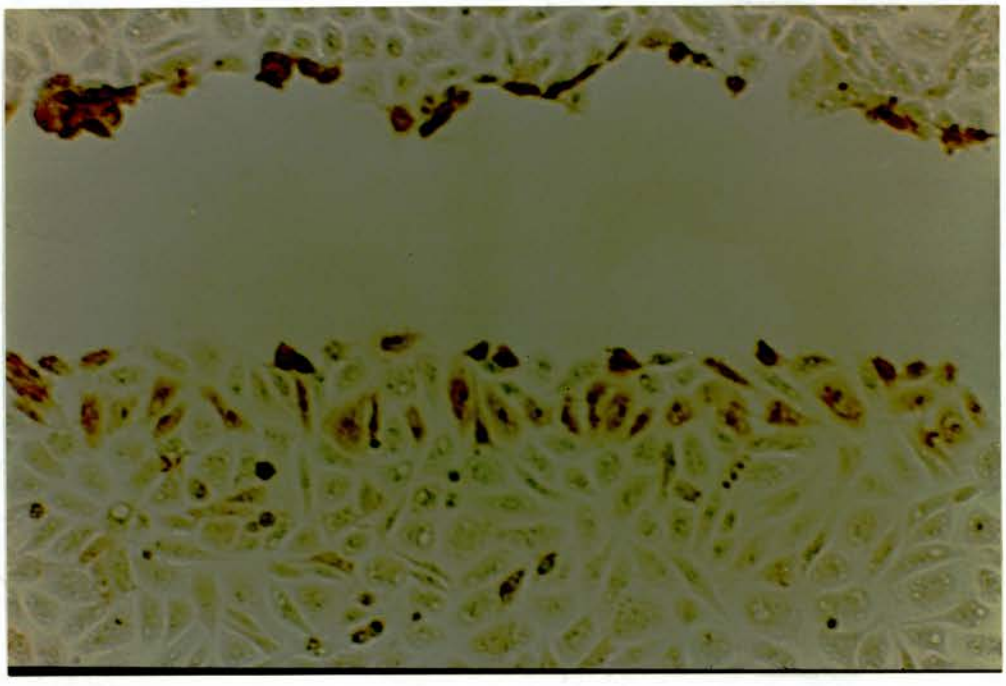


FIG.6.1. Ab-u-PA FRACTION ON SCRATCH DAMAGED CELLS. Cells at scratch edge show antibody binding.

6.3. PREPARATION OF FIBRIN PLATES

Fibrinogen Grade L (Kabi Vitrum) was dissolved in tris buffered saline (TBS) pH7.4 at 1g in 65ml and stored at -20^{o}C in 1ml aliquots. Stock solutions were diluted 1:10 in TBS pH7.4. This grade of fibrinogen contained approximately 3 to 4 micrograms/ml of plasminogen and no additional plasminogen was required for clotting. Thrombin was added to the fibrinogen at 1 unit / ml immediately before the gel was poured. Gels 1 to 2mm thick were made on plastic wells or plates and allowed to set on a level surface for at least 20 minutes.

6.4. ANTIBODY BINDING ACTIVITY OF CONJUGATE

The antibody activity of the urokinase - antibody conjugate (Ab-u-PA) was assessed by antibody / DAB staining of scratch damaged monolayers . Bovine aortic endothelial cells were used because of their abundance and reliable growth. All experiments were carried out on the same cell line at the fourth or fifth subculture.

The Ab-u-PA fraction bound to damaged cells at the scratch edge (Fig. 6.1). If conjugation of the two proteins had indeed occured this result shows that the antibody binding sites had not been destroyed by the conjugation process or masked by the urokinase

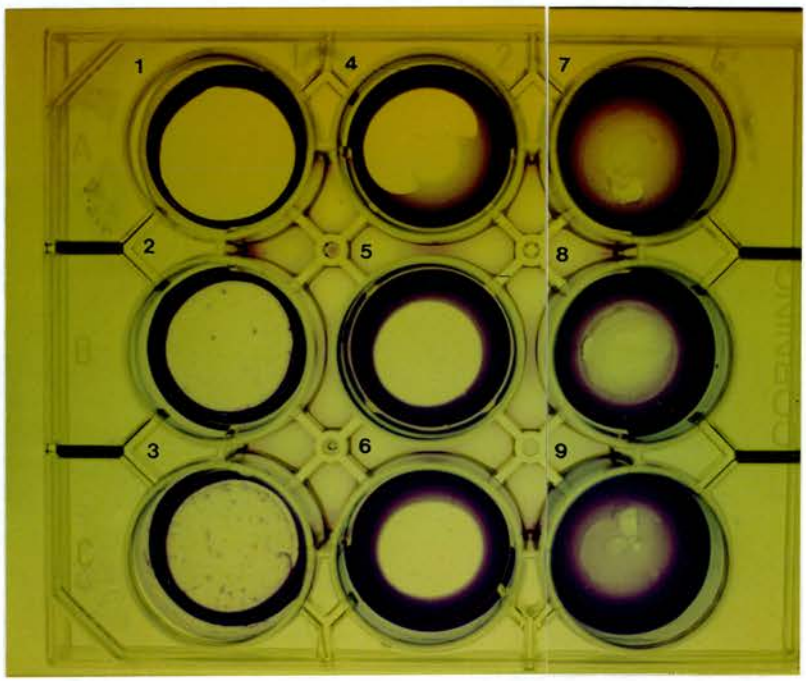


FIG.6.2. LYSIS OF FIBRIN GELS BY Ab-u-PA FRACTION.

1 - urokinase control (1ul of 27unit/ml solution).
2 - undiluted Ab-u-PA
3 - Ab-u-PA 1:2 (in PBS)
4 - Ab-u-PA 1:5 (" ")
5 - Ab-u-PA 1:10 (" ")
6 - Ab-u-PA 1:50 (" ")
7 - Purified P14G11 control
8 - Endothelial cell growth medium
9 - PBS only

molecule, nor had this affected recognition of the antibody by the peroxidase labelled second antibody in the screening process.

6.5. UROKINASE ACTIVITY OF CONJUGATE

Urokinase activity of the Ab-UK fraction was assessed by fibrin gel lysis. Fibrin gels were made in 24mm diameter wells of a 12 well tissue culture plate. Dilutions of the Ab-UK were added as 1ul drops to the centre of each gel. The plate was sealed and incubated overnight at 37^{o}C. The gels were rinsed and fixed in methanol for 15 minutes and stained with 0.25% Coomassie blue / ethanol for 30 minutes. Excess stain was rinsed off with water and the gels were photographed immediately. (Fig.6.2)

Urokinase activity was present in the Ab-u-PA fraction as indicated by lysis of fibrin gels. The control well shows some thining and loss of fibrin in the centre of the well, which probably occured during the fixation and staining process.

6.6. SDS - PAGE ANALYSIS OF CONJUGATE

SDS - polyacrylamide gel electrophoresis (SDS-PAGE) (section 4.2.2) of the Ab-UK complex was carried out under non - reducing conditions . Samples were diluted

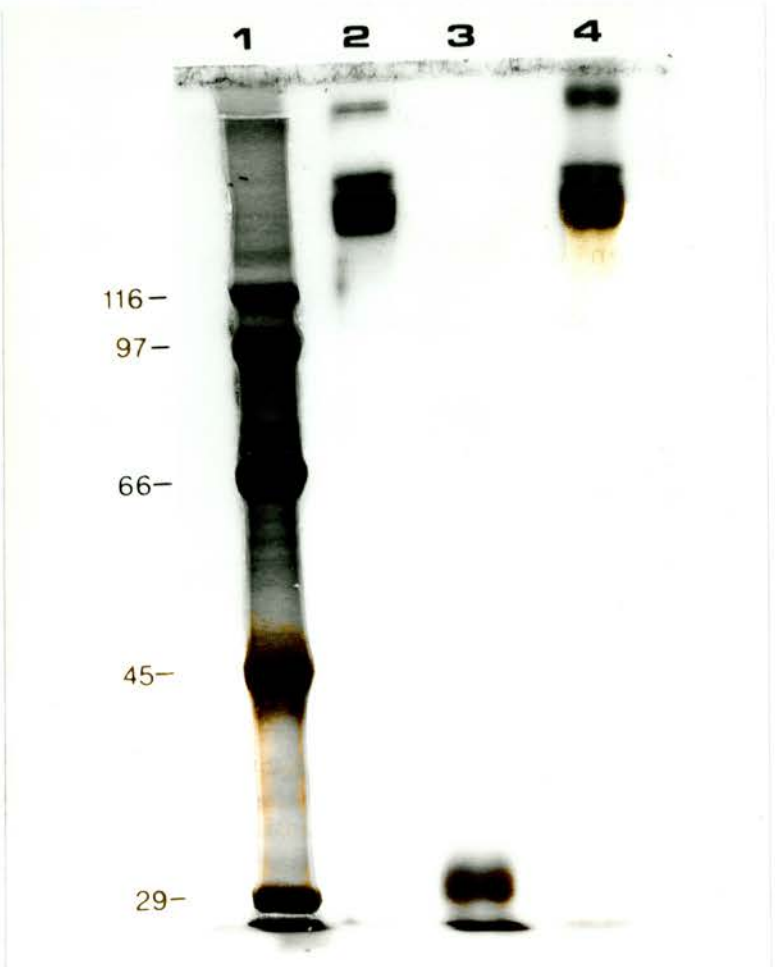


FIG.6.3. SDS - POLYACRYLAMIDE GEL OF Ab-u-PA.

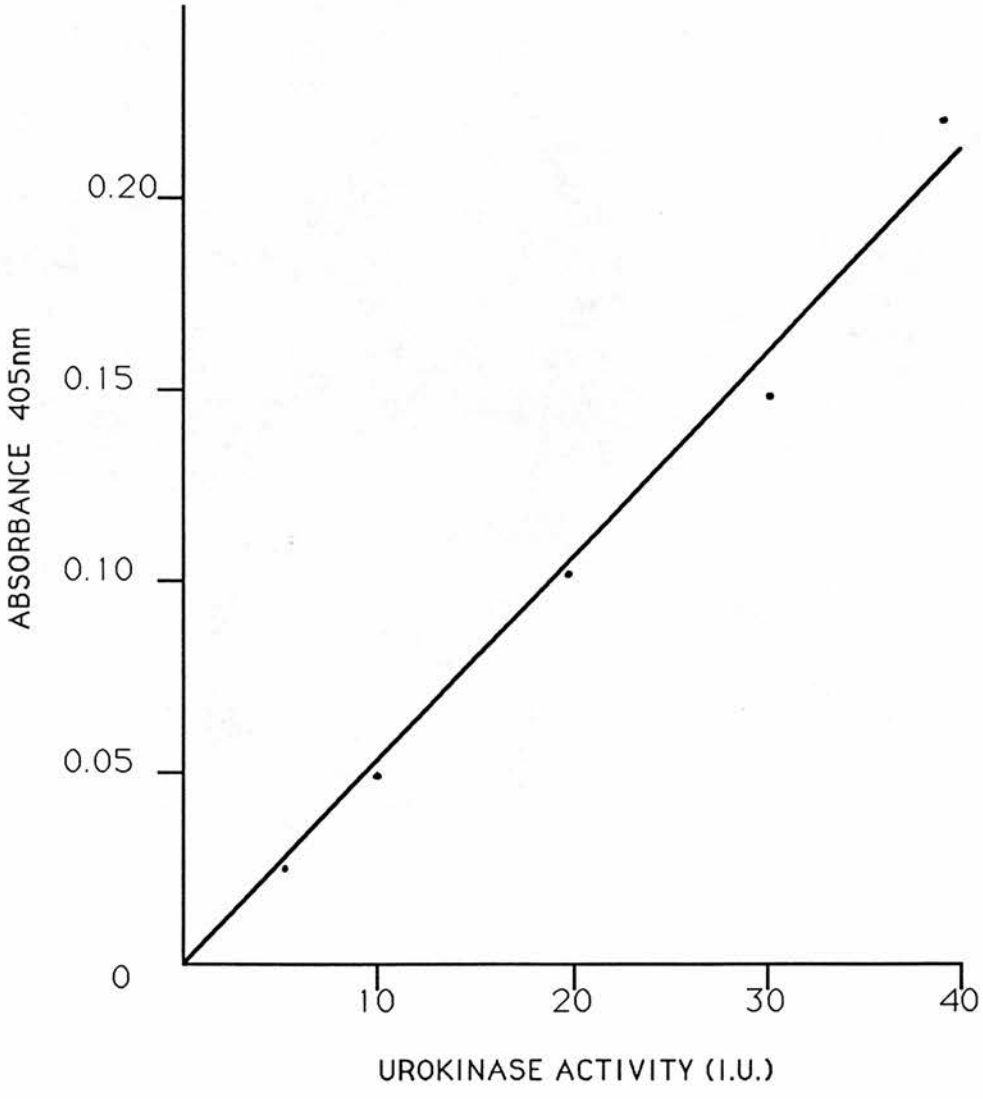
Lane 1 - molecular weight standards
Lane 2 - purified P14G11
Lane 3 - urokinase
Lane 4 - Ab-u-PA

in 0.25M Tris / 0.2% SDS pH6.8 with 5 to 10ug of protein present in 50ul. A drop of glycerol and bromophenol blue tracking dye was added to each 50ul sample, which was then layered onto the appropriate well of a 7.5% resolving gel with a 3.3% stacking gel. Electrophoresis was carried out at 50mA constant current until the buffer front was through the stacking gel and then at 70mA until the front was 2cm from the gel end. The gel was stained in 0.25% Coomassie Blue in ethanol overnight and destained in repeated changes of 1:4 ethanol: 5% acetic acid.

The stained gel (Fig.6.3) showed the purified antibody (lane 2) to run as two bands, a major band (a) which was presumed to be the IgG2a molecule at the 150 - 160kD region and a faint band of higher molecular weight (b). This higher molecular weight band was at first thought to be a contaminating protein from the antibody purification procedure. A control of IgG2a should have been included to confirm this. Lane 3 shows urokinase (molecular weight 33kD). The Ab-u-PA fraction gave the same pattern as the free antibody. In theory IgG and urokinase should have a combined molecular weight of around 180 - 190kD. The high molecular weight band which is much more definite in lane 4 (c) may well be Ab-u-PA and with the majority of the protein in the fraction being unreacted antibody. To test this idea

the two bands were cut out of the SDS gel and placed on a fibrin gel to see which band had fibrinolytic activity. Unfortunately the SDS gel controls also caused fibrinolysis. This was attributed to the incomplete removal of SDS from the gel, which could not be removed by rinsing alone. Attempts to run the samples on native polyacrylamide gels, to overcome the SDS problem, were not successful. Western blotting techniques were applied to the SDS-PAGE samples excluding SDS from the transfer buffers. Transferred conjugate samples (on Immobilon P membrane) were applied to fibrin gels for up to 48 hours. No lysis was noted after this time and it was assumed that conditions of transfer had inactivated the enzymatic properties of the u-PA molecule.

The only statement that can be made from the results of the SDS-PAGE analysis was that the Ab-u-PA fraction did not contain free urokinase. Since this fraction did have fibrinolytic activity (6.4) it was assumed to be due to the presence of urokinase which was antibody associated.



GRAPH 6.1 Standard curve of urokinase activity
(Ukidan 5000, Serono Laboratories UK.)

6.7. QUANTITATIVE ASSAY OF UROKINASE WITH CHROMOGENIC SUBSTRATE S-2444.

Chromogenic substrate S-2444 (Kabi Diagnostics) works on the principle of the rate at which p-nitroaniline (pNA) is released in the reaction :

pyro-glu-gly-arg-pNA + H_2O

u-PA

pyro-glu-gly-arg-OH + pNA

pNA release is measured photometrically at 405nm with the correlation between absorbance and u-PA activity linear in the range 5 - 40 Ploug (1.5 I.U) or CTA units (1.0 I.U).

The standard curve (Graph 6.1) was prepared using a urokinase preparation from Serono Laboratories UK (Ukidan 5000) which had an activity of 5000 I.U / ml. The urokinase used in the antibody conjugation procedure was obtained from Sigma. Enzyme activity of this preparation was given in Sigma Units, each Sigma unit being equivalent to 5000 - 10000 Ploug units, making the preparation of a standard curve from this urokinase extremely difficult. Absorbance and activities are given below.

UROKINASE ACTIVITY (I.U)	ABSORBANCE 405nm 1	2	$\bar{X}$
Standards			
5	0.022	0.022	0.022
10	0.048	0.048	0.048
20	0.099	0.092	0.096
30	0.151	0.124	0.138
40	0.172	0.230	0.201
Sample	0.104	0.103	0.104

From the standard curve the activity of urokinase in the Ab-u-PA sample is approximately 20 I.U. in 100 microlitres = 200 I.U / ml. Protein concentration of the conjugate fraction was estimated at 9 micrograms / ml (Bradford 1976).

6.8. UROKINASE ACTIVITY OF CONJUGATE WHILE BOUND TO DAMAGED ENDOTHELIAL CELLS

Bovine cells were grown to confluency on 16mm diameter glass coverslips. The endogenous fibrinolytic activity of the cells (6.1) was inactivated by incubation with a 1:100 dilution of guanidine activated plasminogen activator inhibitor - 1 (PAI-1) from human umbilical vein endothelium (HUVEC) conditioned growth medium (a gift from Dr Ian McGregor of Scottish

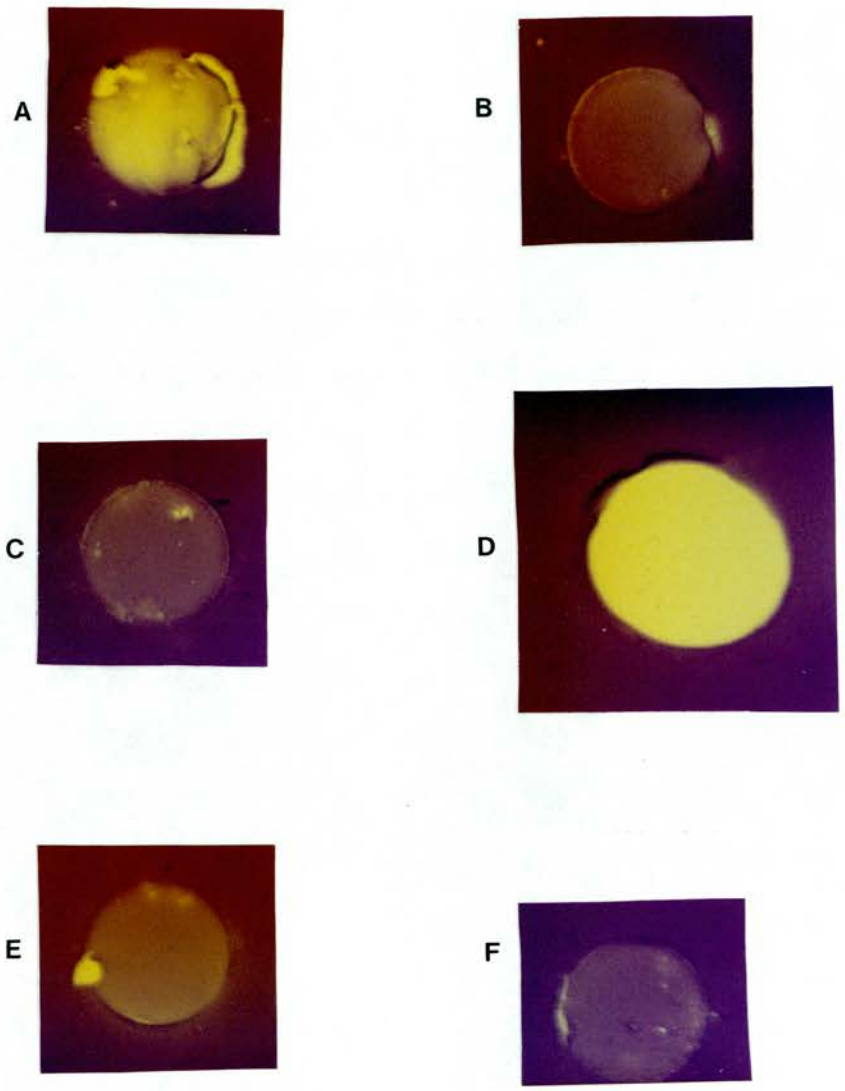


FIG.6.4. FIBRINOLYTIC ACTIVITY OF Ab-u-PA WHILE BOUND TO CELLS DAMAGED BY AIR DRYING.

A - undamaged cells control (endogenous fibrinolysis)
B - undamaged cells + PAI-1
C - damaged cells only (no endogenous fibrinolysis)
D - damaged cells + Ab-u-PA
E - undamaged cells + Ab-u-PA
F - glass coverslip control

National Blood Transfusion Service). The cells were rinsed well with PBS to remove all traces of inhibitor and then damaged either by scratching or air drying for 20 minutes at 37°C. After fixing for 10 minutes in 0.1% gluteraldehyde, the cells were incubated with Ab-UK at 1:10 in PBS for 1 hour at 37°C. Each coverslip was rinsed 5 times in PBS and carefully lowered cell side down onto a fibrin plate, prepared as above, with appropriate controls. Plates were sealed in a humidified container and incubated at 37°C for 24 hours. The plates were then flooded with methanol and the coverslips gently removed. After fixing gels were stained with Coomassie blue. Each test was done in duplicate with one set of coverslips being stained for antibody.

Endogenous fibrinolytic activity of undamaged endothelial cells (Fig.6.4A) was inhibited by PAI-1 (Fig.6.4B). Damage to the cells by air drying switches off endogenous fibrinolytic activity (Fig.6.4C). Air dried damaged cells incubated with the Ab-u-PA fraction showed complete lysis of the fibrin which had been in contact with the cells (Fig.6.4D). Undamaged cells treated with Ab-u-PA did not show fibrinolytic activity (Fig.6.4E). Ab-u-PA DAB stained controls of D showed heavy staining of damaged cells with antibody and with E, no staining of undamaged cells. Fig.6.4F is a plain

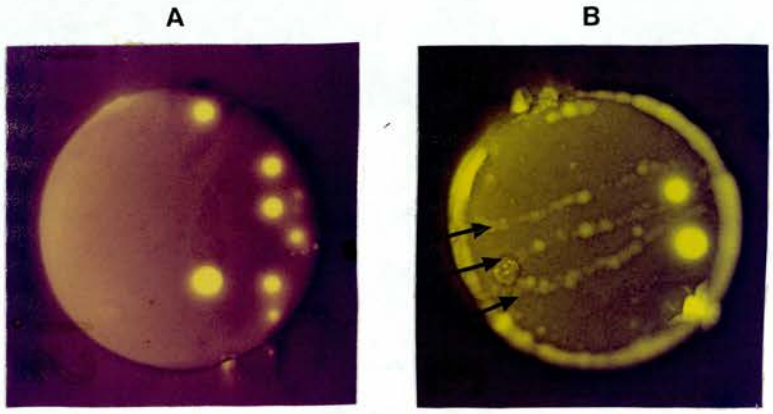


FIG.6.5. FIBRINOLYTIC ACTIVITY OF Ab-u-PA ON SCRATCH DAMAGED CELLS.

A - scratch damaged cells + PAI-1 only

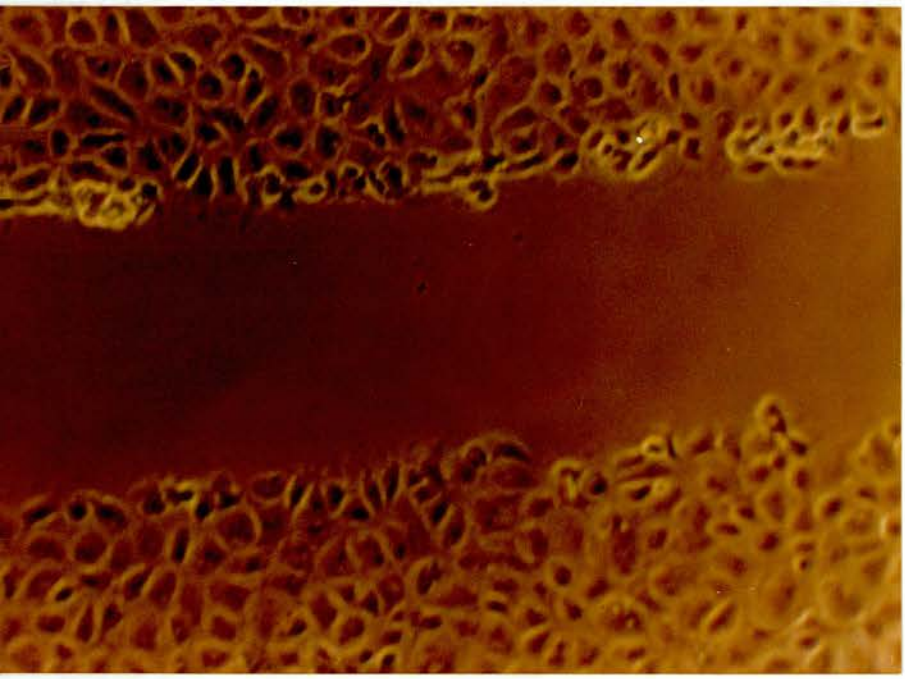
B - scratch damaged cells + PAI-1 + Ab-u-PA

glass coverslip control.

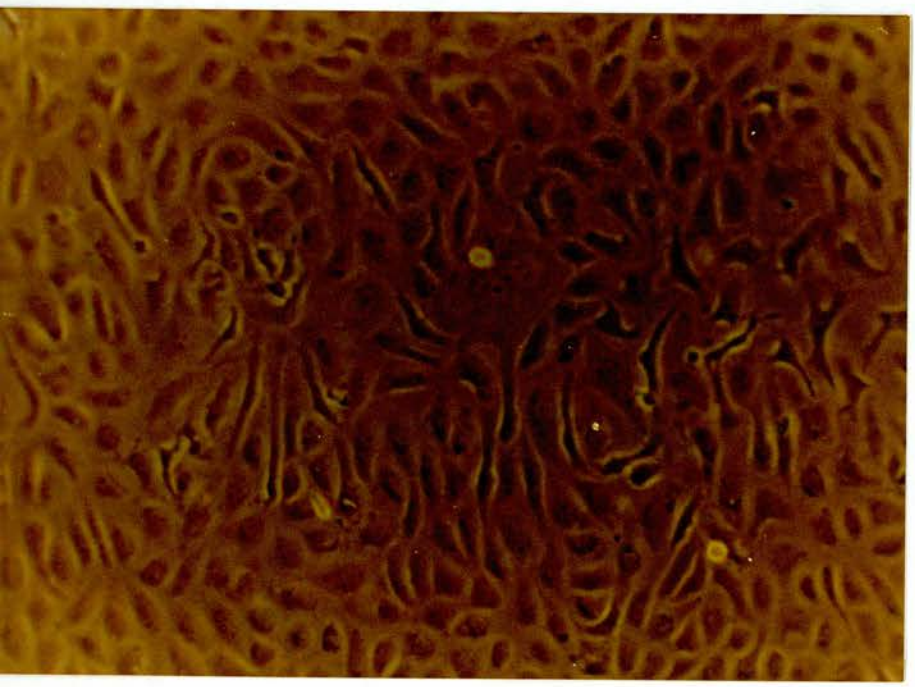
Scratched damaged cells have perhaps more physiological significance. The results of the air dried damage model showed this type of damage to inactivate u-PA and t-P activity of the cells. With scratch damaged cells only a very small proportion of the cells on the coverslip are damaged and it is not known if this type of damage inactivates endogenous PA activity. Fig.6.5A shows a fibrin gel incubated with scratch damaged cells (3 scratches through the centre of the coverslip) and treated with PAI-1 only. Small areas of fibrinolysis were present but these were not associated with the scratches. Fig. 6.5B shows a gel incubated with scratch damaged cells, treated with PAI-1 and incubated with Ab-u-PA after thorough rinsing. Lysis of the gel at the areas of scratch damage are clearly visible. DAB stained controls showed heavy staining of cells along the scratch edge after incubation with Ab-u-PA. Photographs of the DAB stained controls are not shown (see Fig.6.1 for example).

6.9. REGENERATION OF CELLS AT SITES OF DAMAGE TREATED WITH Ab-u-PA CONJUGATE

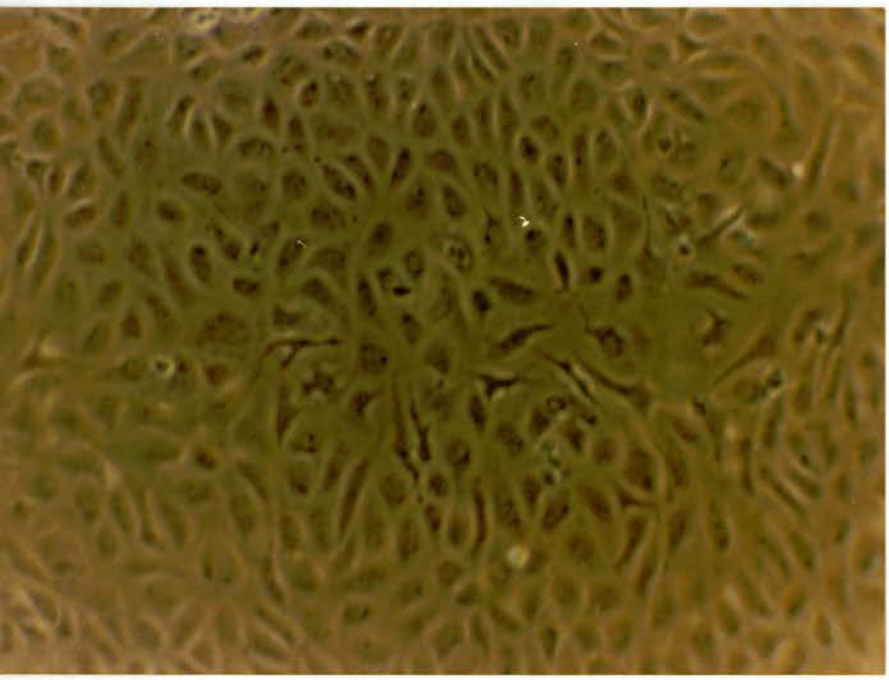
Endothelium denuded areas are re-covered by spreading and multiplication of the cells along the



5. DAMAGED MONOLAYER 2 HOURS AFTER SCRATCHING.



7. DAMAGED MONOLAYER 12 HOURS AFTER SCRATCHING.
luded area has been completely recovered. Cells
site of damage are elongated and less dense than
in undamaged areas.



3. DAMAGED CELLS 24 HOURS AFTER SCRATCHING.
ratched area has completey regrown.

scratch edge. Under optimal culture conditions, a small denuded area (3 to 4 cells wide) was usually re-covered by cell spreading in 4 to 6 hours and complete regrowth of the cells occurred within 12 to 24 hours, depending on the width of the scratch. The effect on regrowth of a scratched area of bound Ab-UK was investigated. Bovine cells of the same cell line as used for the other experiments were grown to confluence in 24 well plates and scratched as before. Ab-UK was added at a 1:10 dilution in complete growth medium and the cells were replaced in the incubator for 24 hours. Observations were made at 2 hourly intervals and photographs were taken (Figs.6.6, 6.7 and 6.8). Unconjugated P14G11 (1:10) and urokinase at 0.025 units / ml were included in the controls. There was no difference in speed of regrowth of the cells treated with Ab-UK from any of the controls. These cells were then fixed and stained for antibody by DAB substrate reaction with negative results.

6.10. DISCUSSION.

The results of these experiments have shown that monoclonal antibody P14G11 to damaged endothelium in vitro can be conjugated to urokinase without loss of antibody specificity or u-PA fibrinolytic activity. The

u-PA activity was preserved while the conjugate was cell bound and fibrinolytic activity appeared to be restricted to areas of damage. Further work needs to be done to quantify binding of urokinase to the antibody molecule and to quantify it.

Bovine aortic endothelial cells were chosen for these initial experiments because of their availability and ease of growth and they produce less tissue - plasminogen activator (t-PA) than human cells although urokinase type - plasminogen activator (u-PA) production does increase with length of time in culture (Levine et al 1979). Endogenous production of plasminogen activators was inactivated with PAI-1 to prevent interference with the urokinase activity of the test substance. The PAI-1 used was from a human source, but was known to have inhibitory action on bovine t-PA and u-PA (personal communication from Dr McGregor). Damage to bovine endothelial cells by air drying was found to inactivate endogenous fibrinolysis.

The conjugate also binds to unfixed damaged cells and does not inhibit or restrict regrowth of cells over denuded areas (6.9). The presence of active urokinase after or even during cell regrowth is highly unlikely due to the presence of inhibitors in the growth medium from the endothelial cells. After cell regrowth the monolayer was tested for the presence of antibody and

very little staining was found. The Ab-u-PA had probably been internalised and degraded by regenerating cells.

The potential use of thrombolytic agents targeted by coupling to monoclonal antibodies which recognise damaged endothelium must remain speculative until they can be tested further in and in vivo. It is unlikely that they would be useful in treatment of coronary thrombosis where established fibrin thrombus is greater in proportion to damaged endothelium. Anti-fibrin antibodies are already in existenceand would prove much more effective in the dissolution of established fibrin clots (Hui et al 1983).

Ab-u-PA preparations might have an important role in preventing thrombus formation at the site of local endothelial damage until the endothelium had time to heal. This could have applications in vascular, and particularly in microvascular, surgery, where prevention of local thrombosis is desirable, but systemic anticoagulation potentially hazardous. Local application of a targetted thrombolytic drug at the time of surgery would provide a means of ensuring such a local antithrombotic effect.

CHAPTER 7

SUMMARY AND CONCLUSIONS

The aims of the work described in this thesis were to :

1. Develop monoclonal antibodies specific to damaged or regenerating endothelial cells .

2. To partially characterise the antigens recognised by these antibodies.

3. To use such antibodies for the detection of damaged / regenerating endothelial cells in vascular disease and assess their suitability for diagnostic uses.

4. To conjugate these antibodies with thrombolytic agents and target such activity specifically to areas of damage.

Antibodies raised against bovine, porcine and human endothelial cells produced a number of immunohistochemical staining patterns on in vitro damaged cell models. The majority of antibodies staining damaged cells were raised against bovine and porcine aortic endothelium. Human umbilical vein endothelial cell immunisation group produced fewer stable antibodies against damaged cells. No anti-damaged endothelial cell antibodies were found with the adult human saphenous vein immunised group. This group did however produce antibodies directed against intracellular granules of giant cells in senescing cultures. Further investigation into the behaviour and

characterisation of these antibodies may be justified.

Most of the anti-damaged cell antibodies raised were IgM subtypes which bound to fixed damaged cells but not to unfixed damaged cells. Antibody P14G11, an IgG2a subtype was an exception and bound with equal affinity to both fixed and unfixed cells. This effect might be due to the differences in size and stereochemistry of the IgM and IgG2a molecules in attaining access to intracellular proteins through damaged cell membrane.

Antibody cross-reactivity between bovine, porcine and human endothelium existed. However, these antibodies were not tested on cultured endothelium from sources other than aortic, saphenous vein or umbilical vein. Antibody reactivity with microvascular or lymphatic endothelium is not known. Cross-reactivity with fibroblasts and smooth muscle cells exists with some of the anti-damaged bovine endothelial cell antibodies. However these findings were not consistent and more work must be done on defining specificity and cross-reactivity of these antibodies, not only on vascular cells but also with other cell types.

Cells damaged by other methods; air drying, heat, hyperoxia, exposure to thrombin and to hydrogen peroxide could also be detected by antibodies to scratch damaged cells. Endotoxin damage could not be

detected by these antibodies.

Cell monolayers grown on 24 well and 96 well culture plates under normal conditions were shown to have defined areas of non-deliberate damage. This appeared to be sublethal injury under normal conditions but under conditions of induced injury, such as treatment with thrombin and hydrogen peroxide, these cells appeared to have a predisposition for lethal injury.

The causes of this "spontaneously" occuring injury have not been defined but may have relevance in the interpretation of the in vitro behaviour of "normal" endothelial cells and may therefore warrant further investigation.

A limited number of antibodies were tested on frozen sections of lung, lymph node and thyroid gland tissues. Only antibody D6F1 gave a positive staining of endothelial cells in lung sections. The others either gave negative results or high background non-specific staining. Further work should be done using tissue sections, with improvements and changes to the staining techniques and tissue fixation procedures.

In recent years many endothelial cell antigenic components have been identified and characterised , highlighting the complexity of the structure and function of these cells. Adhesion molecules such as

ELAM-1 (Bevilacqua et al 1989), ICAM-1 (Simmons et al 1988), VCAM-1 (Osborn et al 1989), PECAM-1 (Newman et al 1990) and the co-expression of haemopoietic CD antigens on vascular endothelial cells (Favaloro et al 1990) have added to the understanding of the processes of inflammation. Other cell surface and cell membrane proteins have been characterised and putative functions ascribed to them (Darnule et al 1983, Dickinson et al 1986, Arvieux et al 1986, Kaplan et al 1983, Sage et al 1986).

Attempts were made to determine the molecular weights of the antigens recognised by the monoclonal antibodies by SDS PAGE analysis of endothelial cell proteins. Using differential staining techniques, subtle differences were found in total protein and lipoprotein/sialoglycoprotein band patterns between bovine and porcine aortic and human umbilical and saphenous vein endothelium.

Changes in band pattern were also seen in cells of increasing culture age. This finding determined the number of subcultures at which cells were used for immunoblot analysis.

Molecular weight estimations were determined using standard procedures. Variations in the results occurred from gel to gel even if experimental conditions were repeated as accurately as possible. To overcome such

variability the estimated molecular weight of a particular protein band was taken as the average from three gels. The most probable source of errors would appear to be in the length of time the gel was run and also in the timing of the staining and destaining procedure. This ultimately affected the relative mobilities of the proteins through variations in the size of the gel and the distance travelled by individual protein bands. It seems likely that more stringent experimental technique would solve the problems incurred by such inaccuracies.

Separated endothelial cell proteins were transferred electrophoretically to nitrocellulose or polyvinylidenedifluoride (Immobilon P) membranes for antigen determination with monoclonal antibody. Three molecular weight regions were detected by antibodies to damaged cells; 75kD, 47-55kD and 15-18kD. Non-specific IgG staining was characterised by staining of bands at 18kD and 30-33kD regions. These findings contradict previous studies which have shown non-specific IgG binding to the vimentin component of intermediate filaments (Hansson et al 1984). Vimentin is a single chain molecule of approximately 55kD. The results of the experiments shown in this work would suggest that vimentin may not be the only non-specific binding site for IgG. Antibody D8G8 reacts with three proteins in

this molecular weight range, 15,17 and 18kD. Pre-blocking of non-specific binding sites with IgG does not inhibit D8G8 binding, showing that it is not a non-specific effect and that binding of this antibody occurs at a different epitope on the same protein molecule.

The SDS-PAGE experiments have shown that at this level of protein separation there is a great deal of structural homogeneity between endothelial cells from different species. It has also shown changes in cellular protein content associated with in vitro ageing. This may have relevance in the interpretation of results of work involving highly passaged cells.

The conclusions to be drawn from this work are limited by the inadequacies of the separation technique. Many different proteins have the same relative mobilities, molecular weights but differ in structure and function. From the immunoblot experiments antibodies that stained identical bands may in fact be binding to functionally different proteins. Two dimensional electrophoresis, with separation by isoelectric focussing in one dimension and by conventional reductive electrophoresis in the second dimension would enhance this work and give a more detailed analysis of endothelial protein content and antigenicity. Purification of the antigens could be

achieved by immunoprecipitation giving a more detailed picture of structure and function. However this was beyond the scope of this thesis and is part of the work in progress.

Attempts to label two of the anti-damaged cell monoclonal antibodies with radionuclides for use in an in vivo model system were met with limited success.

Radioactive iodine labelling by the Iodogen method was partially successful with antibody D8G8. The labelled antibody - antigen complex could be detected by autoradiography on Western blots. However autoradiographs of scratch damaged cell bound antibody did not work. This may have been due to the presence of unlabelled antibody in the preparation which had greater affinity for the antigenic binding sites than the radiolabelled antibody. Competition binding assays between labelled and unlabelled antibody should have been performed to confirm this. On the other hand there simply may not have been enough radioactivity present in this damaged cell model to be detected by autoradiography.

Radioactive iodine labelling of antibody P14G11 was not successful. Autoradiographic analysis of Western blots, scratch damaged and air dried damaged cells gave high background and non-specific activity after repeated purification procedures. This was thought to

be caused by the dissociation of iodine label from the antibody. The instability of the complex may have been due to the coupling conditions and perhaps P14G11 being an IgG molecule could have withstood the slightly harsher conditions of lactoperoxidase or Chloramine T coupling.

It did however prove feasible to produce tritium labelled D8G8 and P14G11 by internal labelling with tritiated lysine. Both tritiated antibodies were stable for a considerable length of time. However, the difficulties in detecting low energy beta emmision by direct visualisation procedures makes the use of tritiated antibody in in vivo studies impractical.

Covalent linkage of antibody P14G11 with urokinase proved more successful in terms of conjugate stability and specificity of antibody binding. In the in vivo damaged cell model, fibrinolytic activity was confined to areas of damage. This activity was attributed to antibody bound urokinase. Binding of the antibody - urokinase conjugate did not inhibit cell regeneration. It is unlikely that such a conjugate would be useful in the treatment of coronary thrombosis where thrombus is already well established. However, local application of such a thrombolytic agent may prove useful in vascular surgery where local fibrinolysis is desirable but systemic anticoagulation potentially hazardous.

APPENDIX : LIST OF SUPPLIERS.

All general laboratory chemicals, unless otherwise stated were obtained from :

Sigma Chemical Company Ltd.
Fancy Road
Poole
Dorset BH17 7TG.

and

BDH Ltd
Broom Road
Poole
Dorset BH12 4NN.

All electrophoresis chemicals were "Electran" grade from BDH.

Electrophoresis and protein blotting equipment was from

Pharmacia LKB Biotechnology
351 Midsummer Boulevard
Milton Keynes MK9 3HP

All tissue culture culture mediums, serums and plastics were obtained from :

Flow Laboratories Ltd
Woodcock Hill
Harefield Road
Rickmansworth
Herts. WD3 1PQ.

Collagenase was obtained from :

Boehringer Mannheim UK Ltd
Bell Lane
Lewes
East Sussex BN7 1LG.

All antisera used in the antibody detection systems were purchased from :

Dako Ltd
16 Manor Courtyard
Hughendon Avenue
High Wycombe
Bucks HP13 5RE.

Radiochemicals were purchased from :

Amersham International plc
Lincoln Place
Aylesbury
Bucks. HP20 2TP.

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