Measurement of cultured cell adhesion

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MEASUREMENT OF CULTURED CELL ADHESION

Submitted by

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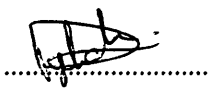

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ABSTRACT

The involvement of cell adhesion in a very wide area from biotechnology to clinical applications made this area very attractive subject of study. To be able to study the underlying mechanism of cell adhesion, it is necessary to measure it. Most studies have been previously carried out qualitatively which will provide information whether cell-substratum compatible. However, a few quantitaive methods have been developed for the measurement of cell adhesion. While these suffer from a limited abilty or need of complex equipment.

In the present work a simple and reproducible cell adhesion measuring device "Microflow chamber" has been developed which produces a wide range of hydrodynamic forces for cell detachment due to existence of convergent channel(s) in it. The reproducibility of this device was shown by determination of adhesion strength of different cell lines. Every cell line has a specific and constant adhesion strength.

The requirement of metabolic energy from initial cell attachment to gaining maximum adhesion strength has been shown. The minimum serum concentration at which CHL cells are able perform adhesion fully was found to be 1%.

The specificity of cell-substrate adhesion has been shown by determining that pre-adsorption of fibronectin and collagen type IV on tissue culture plastic dish strengthened dramatically while modification of these dishes with collagen type I did not make any significant difference on the adhesion of CHL cells. However, HeLa B cells were able to enhance their adhesion strength on collagen type I coated surfaces.

The involvement of endogenous proteins in cell adhesion has been determined by inhibiting protein synthesis or secretion.

CHAPTER 1. INTRODUCTION

1. GENERAL INTRODUCTION

The adhesion of cells embraces a series of phenomena which cover all aspects of biology. For eaxmple, the sticking of the blastomers together after division, the adhesion of sperm to the egg, the invasion of pathogens into an organism, and the metastasis of tumour cells are all phenomena in which adhesion plays an important role. Cells also form adhesions with a wide variety of non-living materials which may be non-organic in nature, such as rocks which marine organisms settle upon, or implanted medical prosthetic devices such as those made of tantalum, or of an organic nature such as the collagen secreted by fibroblast.

It is certain that cell adhesion in animal cells can be considered a complex process, involving proteins of the extracellular matrix, cell surface receptors for these proteins and a complex interplay of physical, biochemical and cytoskeletal events.

An understanding of animal cell adhesion may be important in controlling practical problems such as the control of cell growth on a biocompatible substrate, the proper anchoring of connective tissue on to metal bone prostheses, the prevention of attachment of blood cells to vascular prosteheses. The investigation of all these areas requires the measurement of cell adhesion.

Two approaches for the study of cell adhesion have been characterized. These are; cell to cell adhesions and the adhesion of cells to extracellular substrate. This work is concerned with the latter phenomena which will be discussed below.

1.2.CELL SUBSTRATE ADHESION.

Some cells need to attach to a suitable surface on which they will spread and grow, these cells are called anchorage dependent cells. The cell-substrate interaction is a very complex process involving extra cellular matrix (ECM) proteins, cell surface receptors for these proteins and a complex interplay of extracellular proteins, membrane proteins and cytosolic proteins. However, the mechanism of cell substatum adhesion can be subdivided into a number of steps. These are: first the protein adsorption on the surface, secondly the contact of the cell with the surface bound proteins and finally attachment after which the cell spreads and grows until division where it rounds up and divides (Revel and Wolken 1973; Hughes et al. 1979; Schakenraad and Busscher 1989). The first three of these steps will be discussed briefly.

1.2.1. PROTEIN ADSORPTION ON THE SURFACE

The first reaction that occurs when a virgin surface (e.g. plastic, glass or metal) is immersed in a solution containing proteins is that the latter irreversibly bind and denature onto the surface (Soderquist and Walton 1980; Castillo et al. 1984; Absolom et al. 1987). Such adsorption of proteins to surfaces is largely irreversible and much more rapid than contact of the cell to the surface. It is clear that cells interact with an interface of previously adsorbed proteins rather than the original form of substrate (Revel and Wolken 1973; Horbett and Weathersby 1981; McAuslan et al. 1988; Lee et al. 1991).

The properties of the substrate surface e.g. hydrophilicity, hydrophobicty and surface charge are also known to regulate the amount and activity of the adsorbed proteins (Klebe et al. 1981; Uyen et al. 1990; Fabrizius-Homan and Cooper 1991; Underwood et al. 1993).

For example, fibronectin (an adhesive protein) preferably adsorbs to a hydrophilic substratum rather than hydrophobic (Klebe et al. 1981).

A number of glycoproteins and collagens which are capable of adsorption on the surface and promoting cell adhesion have been identified. The first major glycoprotein of this type to be identified was fibronectin (Vehri and Mosher 1978; Yamada and Olden 1978; Yamada 1983). While later, laminin and vitronectin have joined the list (Wewer et al. 1987; Chi and Hui 1989). Some other adhesive proteins such as thrombospondin (Santoro and Fraizer 1987), entactin (Chakravri et al. 1990) and epilegrin (Carter et al. 1991) have also been discovered. However in the present discussion only fibronectin, collagens, vitronectin and laminin will be discussed .

1.2.1.1. FIBRONECTIN

Fibronectin is the most studied adhesion protein and is present in plasma as extra cellular fibronectin. It is also synthesised by a wide variety of cells to form a cellular fibronectin. This protein is composed of similar polypeptide subunits of 220-250 kD that are linked by disulfide bonds into dimers (Yamada and Olden 1978, Yamada 1983). According to Dufour et al. (1986) both cellular and plasma fibronectins are similar in function and structure, although they are distinguished by certain physical properties such as solubility and mobility on SDS polyacrylamide gels. However, this proposal was rejected by Asaga et al. (1991). They found that collagen gel contraction by human skin fibroblasts requires cellular fibronectin but not plasma fibronectin. This might suggest that these two fibronectins are different functionally.

Fibronectin has many binding domains each of which binds specifically to molecules such as heparin, proteoglycan, collagen,

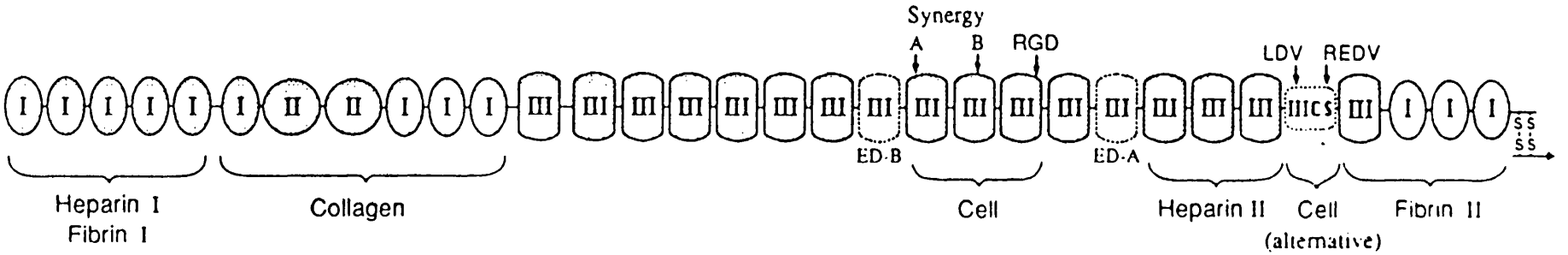


Figure 1.1: The Overal Structure of Fibronectin.

Fibronectin comprises three types of internal repeating units termed types I, II, and III. There are two type III units (labeled ED-A and ED-B) that can be present or absent due to alternative splicing of precursor mRNA. Binding domains are labelled along the bottom, including two domains for binding to heparin, two to fibrin, and one to collagen. The central cell-binding domain contains the RGD site as well as subregions A and B of the synergy region. The IIICS region provides an alternative cell binding domain which contains minimal recognition units with the peptide sequences LDV and REDV. Futher details may be found in the text.

and a cell binding site present in this molecule (Skorstengraat et al. 1986; Akiyama and Yamada 1987; Wolf and Lai 1990) (figure 1.1). It has been reported that at least three distinct types of internal amino acid sequence homology known as type I, type II, and type III exist along the molecule (Hynes 1985; Gutman and Kornblit 1987; Narasimhan and Lai 1989).

It has been shown that fibronectin has a cell binding domain which requires a minimum amino acid sequence of arg-gly-asp (RGD) (Piersbacher and Ruoshlahti 1984; Piersbacher et al. 1985). However, to provide full adhesive activity additional peptide information in the central cell binding domain of fibronectin is required. This second adhesive recognition site (synergistic site) co-operates with the RGD sequence to produce full adhesive activity (Nagai et al. 1991; Kimizuka et al. 1991). However, apart from this minimal adhesive active site, there are other domains in fibronectin that promote cell adhesion. Humphries et al. (1987) identified a domain of fibronectin which has an Arg-Glu-Asp-Val (REDV) amino acid sequence and recently Komoriyo et al. (1991) have identified another fibronectin domain that has an amino acid sequence of leu-asp-val (LDV), both of these domains support cell adhesion.

Although fibronectin is present in serum, in most cell culture conditions (growth medium contains more than 5% serum) the more effective protein in cell adhesion is serum vitronectin (Knox 1984; Steele et al. 1992). This will briefly be discussed below.

1.2.1.2. VITRONECTIN.

Vitronectin is a multifunctional adhesive glycoprotein which is found in the serum and in different tissues (Hayman et al. 1983). Vitronectin is also known as serum spreading factor and has a

molecular weigh of 75 to kD 80 kD (Barnes and Reing 1985). This protein promotes attachment and spreading of a wide variety of cells. Vitronectin, like fibronectin, has binding domains for different molecules including heparin binding, integrin binding domains as well as a cell attachment site (Hayman et al. 1985; Ruoshlahti and Piersbacher 1987; Izumi et al. 1988; Underwood and Bennet 1989; Preissner 1991).

Collagens are another family of proteins that promote cell adhesion which will briefly be discussed below.

1.2.1.3.COLLAGENS

The collagens are a family of highly characteristic proteins found in all multicellular animals. The characteristic feature of collagen molecules is their rigid triple-stranded helical structure (Alberts et al. 1989). So far 14 genetically distinct types of collagens have been identified (Yamagata et al. 1991; Hulmes 1992). Based on their supra molecular structure the collagens can be divided into two main classes; fibril forming collagens (types I, II, III, V and XI) and non fibril forming collagens (types IV, VI, VII, VIII, IX, X, XII, XIII and XIVs) (Vuiro and Crombrugge 1990, Hulmes 1992). Among many other functions collagen promotes cell adhesion and spreading (Klebe 1974; Gulberg al. 1989). The collagens either interact directly with cell surface receptor(s) (Grinnel and Minter 1978; Schor and Court 1979) or via fibronectin (see figure 1.2). Therefore a cell requires extra cellular fibronectin to be able to attach onto a collagen substratum (Kleinman et al. 1979 and 1981). However some cells can synthesise fibronectin, hence these cells could bind to collagen without added fibronectin or fibronectin

containing serum (Scott et al. 1983; Farsi et al. 1985; Herbst et al. 1988; Asaga and Yashirato 1992).

Collagens differ from each other with distinct chemical composition. Therefore certain cells would have a preference for certain types of collagen. For example, some epithelial cells prefer type IV collagen, while chondrocytes prefer type II collagen (Grinnel 1987). Type IV collagen is probably the most studied collagen. This molecule promotes various cell adhesion mechanisms, it also serves to bind other basement membrane components e.g laminin (Tsilibrary 1990; Vandenberg et al. 1991). Although some cells bind to collagen in an RGD dependent manner, for example rat liver cells (Gullberg et al. 1989), others e.g rat fibroblast (Gullberg et al. 1990) and platelets interact with this proteins family in an RGD independent way (Staatz et al. 1991).

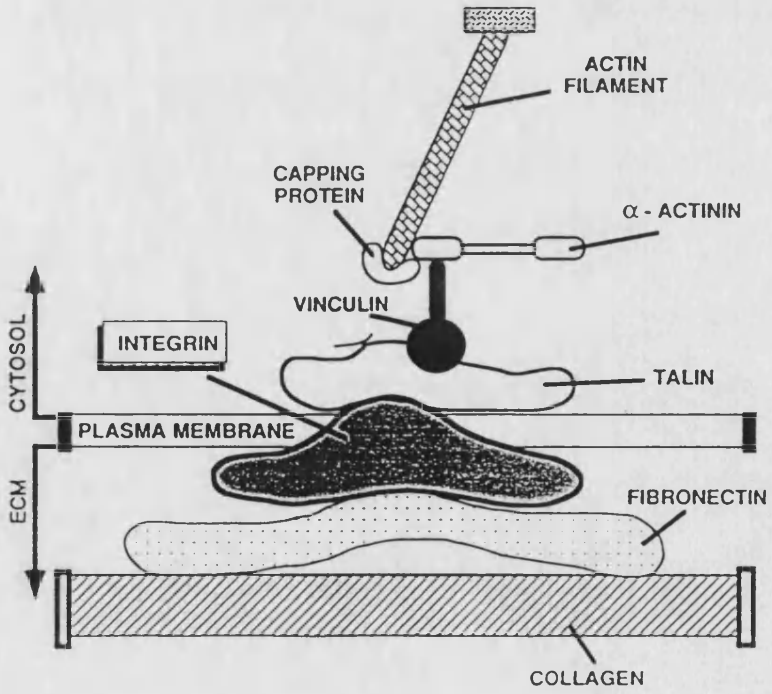


Figure 1.2: The Schematic Binding of Cells to Collagen via Fibronectin.

1.2.1.4. LAMININ

Laminin is the major glycoprotein of the basement membrane and has a molecular weight of 800 kD. This protein is composed of three chains designated A (M_r=400kD), B_1 (M_r=210kD), and B2 (M_r=200kD) which are held together by disulphide bonds (Sasaki 1987) (figure 1.3). All three chains have been cloned and sequenced (Kanemoto et al. 1990). Laminin binds to other basement membrane molecules e.g. collagen type IV and heparin sulphate proteoglycans (Graf et al. 1987). However, it can form a network independent of type IV collagen (Yurchenco et al. 1992).

This protein is able to promote various cellular functions including cell attachment and growth. Although laminin promotes mainly the attachment of epithelial cells, it is also involved in the adhesion of embryonic fibroblasts and endothelial cells (Kleinman et al. 1985; Grant et al. 1989). This adhesion protein contains a sequence of Tyr-Ile-Gly-Ser-Arg (YIGSR) as a cell binding site (Grant et al. 1989). However, recently another adhesive active site in the A chain which has the amino acid sequence of Ile-Lys-Val-Ala-Val (IKVAV) has been identified (Nomizu et al. 1992).

Apart from the specifically adhesive proteins, some other molecules, like polylysine are able to support cell adhesion. The role of polylysine in cell adhesion will briefly be discussed below.

1.2.1.5. CELL ADHESION ON POLYLYSINE

Polylysine is a basic homo polymer and it enhances the adhesion of some cells when it is coated on the culture surface. This molecule affects cell adhesion by forming ionic binding between the positively charged lysine residues and the negatively charged

glycoproteins and phospholipids on the cell surface (Quintin and Philpott 1973; Yavin and Yavin 1974; McKeehan and Ham 1976). In contrast to the other adhesive proteins e.g. fibronectin, collagens, laminin, the adhesion of cells to polylysine is not receptor mediated (Ito et al. 1991; Clapper 1991).

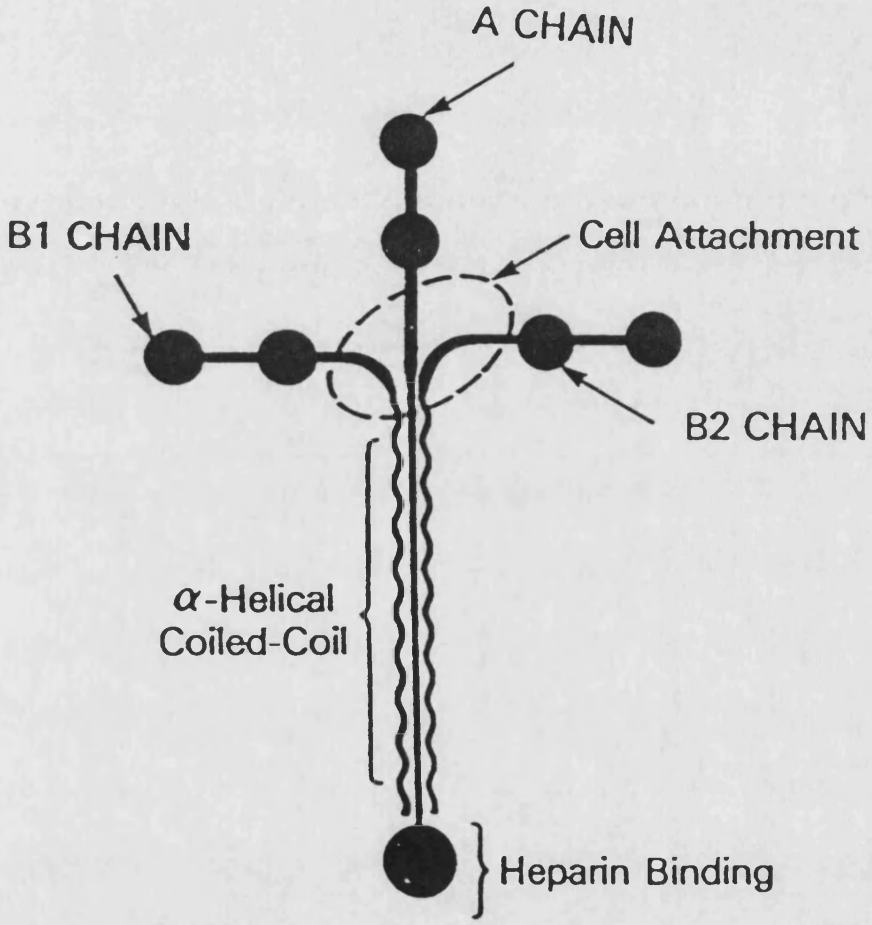


Figure 1.3: A Schematic Drawing of the Laminin, Shows the Arrangements of A, B1 and B2 Chains.

1.2.2. CELL ATTACHMENT.

As a first step towards attachment the cell makes contact with the protein coated substratum (figure 1.4) (Grinnel 1978). Following contact if there are receptors for these adsorbed protein(s) on the cell surface and if the conformation of the adsorbed proteins is not altered by adsorption so as to destroy the high ligand-receptor affinity then cell attachment will take place (Schakenraad et al. 1987; Lydon and Foulger 1988: Anderson et al. 1990).

Most of adhesion receptors are members of the integrin receptor family. This is briefly discussed below.

1.2.2.1.INTEGRINS

The term "integrin" denotes a functional linkage between the extra cellular matrix (ECM) and the cell's interior, thus providing cellular responsiveness to the extra cellular environment. Integrins are a family of adhesion receptors that are heterodimeric transmembrane glycoproteins assembled from dissimilar α and β subunits (Hynes 1987). The α subunits vary in size between 120 and 180 kD whereas β subunits are relatively smaller i.e. 90-110 kD. Each integrin is composed of one α subunit associated with one β subunit. There are 8 known β subunits and 14 known α subunits (see table 1.1) (Hynes 1992). Both the α and β subunits have a relatively large extra cellular domain, a typical transmembrane domain and fairly short intracellular carboxyl terminal domain. However, an exception to this generalisation is the β_4 subunit of the $\alpha_6\beta_4$ integrin. Here the β_4 subunit has a large cytoplasmic domain (Giancotti et al. 1992). The extra cellular domains of the integrins interact with a variety of ligands including the extracelllular matrix glycoproteins, while their intracellular domains interact with cytoskeleton (Hynes 1987;

Humphries 1990). Although integrin receptors mainly mediate cell substrate adhesion, there are a few integrins e.g. $\alpha_4\beta_1$, $\alpha_4\beta_7$, $\alpha_L\beta_2$ and $\alpha_M\beta_2$ which have been shown to mediate cell-cell adhesion.

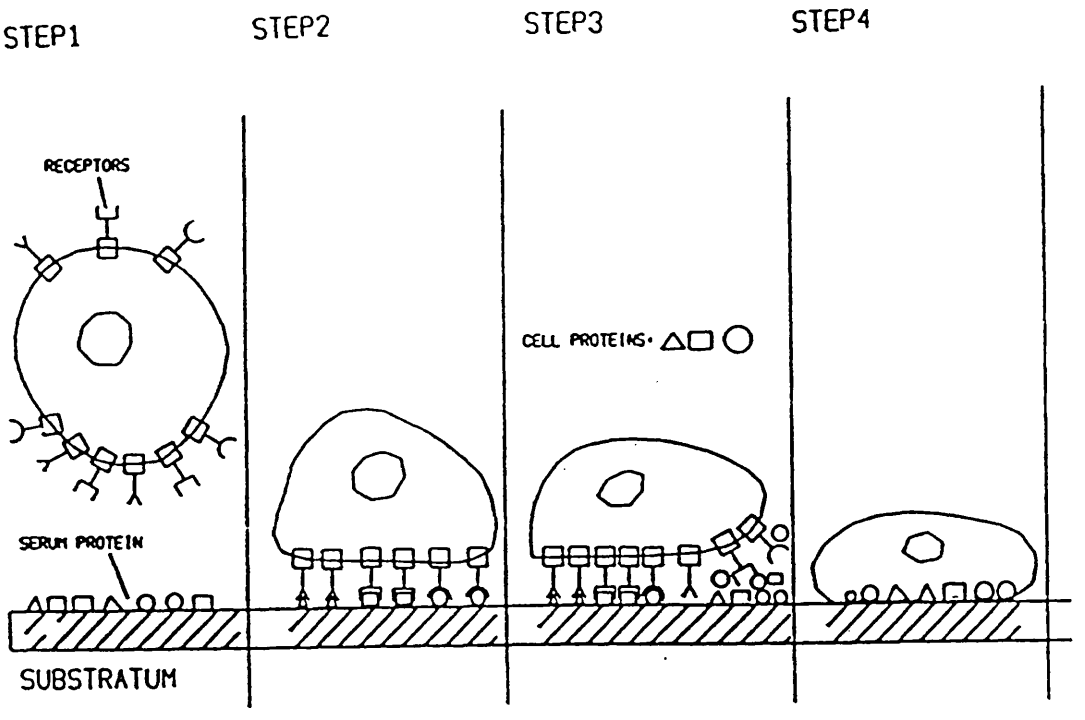


Figure 1.4.: Schematic Illustration of Attachment and Spreading of A Cell In the Presence of Serum Proteins.

Step 1: Suspended cell reaching the adsorbed serum proteins.

Step 2: Initial contact of the cell with the adsorbed proteins and formation of the receptor-protein bonds.

Step 3: Cell-substrate attachment, protein synthesis and secretion.

Step 4: Spreading, formation of focal adhesion and growth.

Table 1.1: The Integrin Receptor Family

β Subunit	α Subunit	Ligands and Counter-receptors
β_1	α_1	Collagens, laminin
	α_2	Collagens, lamini
	α_3	Fibronectin, laminin
	α_4	Fibronectin, VCAM-1
	α_5	Fibronectin
	α_6	Laminin
	α_7	Laminin
	α_8	?
	α_V	Vitronectin, fibronectin
β2	α_L	ICAM-1, ICAM-2
	α_M	C3b component of complement (inactivated),
	α_X	fibrinogen factor X, ICAM-1
β_3	α_{IIb}	Fibrinogen, fibronectin, von Willebrand factor, vitronectin, thrompospondin
	αV	Vitronectin, fibrinogen, , von Willebrand factor, thrompospondin, fibronectin, osteopontin, collagen
β_4	α_6	Laminin
β_5	α_V	Vitronectin
β_6	α_V	Fibronectin
β_7 ($=\beta_p$)	α_4	Fibronectin, VCAM-1
	α_{IEL}	?
β_8	αV	?

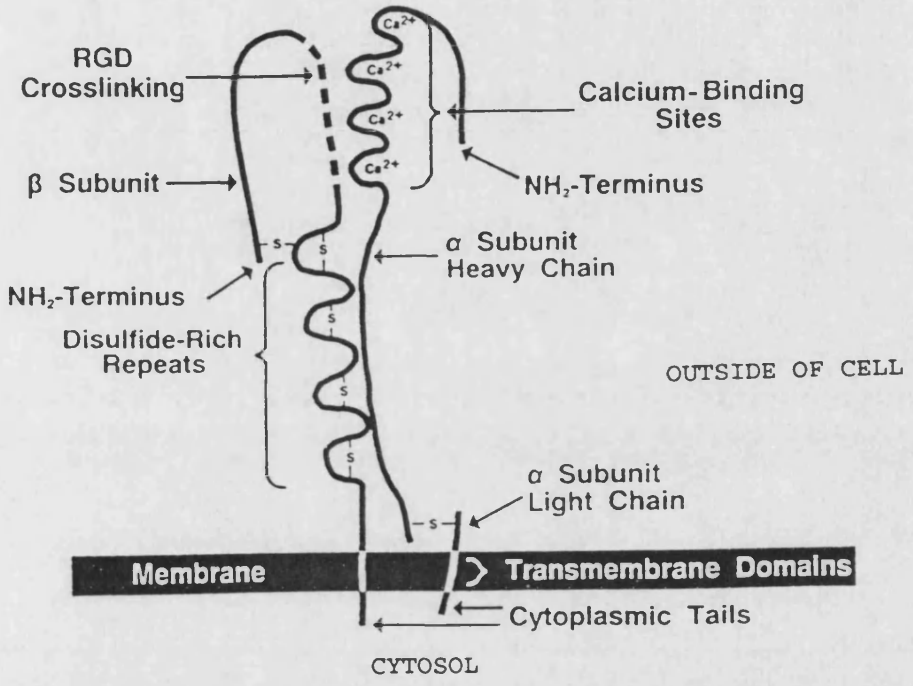


Figure 1.5: Schematic Depiction of A Typical Integrin.

Integrins participate in cell-cell interactions by recognition of integral membrane protein ligands including intracellular adhesion molecules, intracellular adhesion molecule I (ICAM-I), intracellular adhesion molecule II (ICAM-2), and vascular cell adhesion molecule I (VCAM-1) (Loftus et al. 1990; Erle and Pytela 1992).

It is the αβ constitution of the complex that determines the properties of a particular integrin (Solowska et al. 1991). Almost all known integrins are capable of mediating adhesion to at least one matrix protein. Many integrins recognise more than one matrix protein

and many proteins are recognised by more than one integrin. For example fibronectin can be bound at various sites by at least 8 integrins (Erle and Pytela 1992; Hynes 1992). The reason for the former could be that different integrins mediate different functions. For instance, both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ mediate to carcinoma cell adhesion to vitronectin while only $\alpha_v\beta_3$ was found to cluster into focal contacts (Leavesly et al. 1992). The other possibility is that each adhesion protein may have shared a structural feature such as an adhesive recognition sequence, which represents a common binding signal. Therefore one adhesion receptor can interact with more than one ligand (Humphries 1990).

Integrins recognise adhesive proteins that have Arg-Gly-Asp (RGD) amino acid sequence (Ruoshlahti and Piersbacher 1987). However, some integrin receptors can recognize both the RGD sequence or non RGD sequence (D'Souza et al. 1991). For example $\alpha_2\beta_1$ binds Asp-Gly-Glu-Ala (DGEA) in type I collagen, $\alpha_4\beta_1$ binds Glu-Ile-Leu-Asp-Val (EILDV) in fibronectin and $\alpha_x\beta_2$ binds Gly-Pro-Arg-Pro (GPRP) in fibrinogen (Loike et al. 1991).

The characteristic of integrin mediated adhesion is the requirement for divalent cations and temperature (Tuckwell et al. 1992; Makgoba et al. 1992). Each of these factors will be discussed later.

The binding of integrins to a ligand can result in much more than a simple mechanical adhesion of cells. Instead integrins are now seen as components of signaling machines that translate events occuring outside the cell into intracellular messages (Erle and Pytela 1992).Integrins are also recipients of intracellular signals (Cheresh 1992). Now it is commonly believed that integrins can act as true signalling molecules (Curtis et al. 1992; Hynes 1992; Makgoba et al.

1992; Cheresh 1992; Gimond and Aumailley 1993; Juliano and Haskill 1993).

Integrins are a family of adhesion receptors , however here only the fibronectin receptors, collagen receptors and vitronectin receptors will briefly be discussed.

1.2.2.1.1.FIBRONECTIN INTEGRIN RECEPTORS

Fibronectin receptors that interact with the cell binding domain of fibronectin have been isolated from different cells and have been identified. Each of these receptors contains two non covalently bound α and β subunits (Hasegava et al. 1985; Akiyama and Yamada 1987; Akiyama et al. 1990). Some of these receptors are specific for fibronectin i.e $\alpha5\beta1$ while the others are able to bind fibronectin as well as the other adhesive proteins. e g. $\alpha3\beta1$ can interact with fibronectin, laminin and collagen proteins (Elices et al. 1991). The distrubition of receptors can be regulated by availability of ligands. For example, $\alpha5\beta1$ is concentrated in focal contacts in cells spread on a fibronectin substratum, while $\alpha2\beta1$ is a collagen receptor and is concantrated in focal contacts of cells that spread on a collagen substrate (LaFlamme et al. 1992).

By using monoclonal antibodies that recognize the fibroblast fibronectin receptor it has been shown that although fibronectin receptors mediate the initial attachment of the fibroblast , the receptor has no role in the spreading of cells (Akiyama et al. 1989). This finding was rejected by Ingber (1990) who said that fibronectin receptors induce attachment as well as spreading.

Like fibronectin receptors, collagen receptors are members of the integrin receptors family which will be discussed below.

1.2.2.1.2. COLLAGEN RECEPTORS

By using affinity chromatography and by means of antibodies various collagen receptors have been identified. Some of these receptors are specific for collagen while the others can interact with more than one ligand. For example, two classes of collagen receptors have been identified by Wayner and Carter (1987) although the class I receptor is a promiscous receptor i.e. it mediates cell adhesion to collagen and interacts with fibronectin and laminin as well. While class II collagen receptors are specific for collagen Randal and Marks (1989) have identified $\alpha_2\beta_1$ and $\alpha_1\beta_1$ integrin receptors which mediate cell adhesion to collagen type I and collagen type IV and it has been reported that these receptors are specific for collagens since they were unable to bind to fibronectin or laminin (Kramer and Marks 1989). However, according to Staatz et al.(1991) the specificity of collagen recptors is dependent on cell type. That is the $\alpha_2\beta_1$ serves as a specific collagen receptor on platelets and fibroblast, while on other cells, such as endothelial cells or melonama cell lines this receptor may function as both a collagen and a laminin receptor. The reason for the binding of collagen type I by both $\alpha_2\beta_1$ and $\alpha_1\beta_1$ integrins was explained as that collagen type I has separate binding sites for each of these receptors (Gulberg et al. 1992). The binding activity of these receptors to collagen was dependent on the triple helical conformation of collagen (Aumailley and Timpl 1986; Randal and Marks 1989 Vandenberg et al. 1991). However it was later reported that integrins also interact with denatured collagen (Kandenber et al. 1991). Although some of these receptors are RGD dependent some of them are RGD independent. Lu et al.(1989) have purified three RGD dependent collagen receptors from HeLa cells. These were 102, 87 and

38 kD plasma membrane proteins. Whereas Gullberg et al.(1989) identified a collagen receptor from hepatocyte cells which was a 115kD protein and it was found to be functionally independent of the RGD sequence. Like the other integrin receptors metal ions are reqiured for the activity of collagen receptors. It was shown by Beacham and Jacobson (1990) that Mg^{2+} has a crucial role in the collagen receptors interaction with the RGD sequence in collagen.
The other member of the integrin receptor family include vitronectin receptors and these will be discussed below.

1.2.2.1.3. VITRONECTIN RECEPTORS

Vitronectin receptors belong to the β_3 integrin family and they act as promiscous receptors for the RGD containing adhesive proteins e.g. vitronectin, von Willebrand factor and thrombospondin (Kieffer et al. 1991). A Common vitronectin receptor, ($\alpha_v\beta_3$), is composed of a 125 kD α chain and a 115 kD β chain (Pytela 1985). Unlike collagen receptors, vitronectin receptors require calcium not magnesium for their binding activity (Cheresh et al. 1987). Bodery and McLean (1990) have identified $\alpha_v\beta1$ as a vitronectin receptor and it was shown that this receptor binds exclusively to vitronectin in embryonic kidney cells. Whereas it has been reported by Vogel et al. (1990) that $\alpha_v\beta_1$ integrins can interacts with fibronectin as well as witronectin in neuroblastoma cell. Although the regulation of integrin binding is still unclear, it is possible that the binding specificity and activity might be controlled by various means such as RNA splicing, phosphorylation and by the transmembrane lipid environment (Kirchoffer 1991). However, the regulation of integrin functions does vary from cell type to cell type (Cheresh 1992).

1.2.3.CELL SPREADING

A cell begins spreading onto the substratum surface following initial attachment (Grinnel 1974). In order to spread, cells require a suitable stimulus which is usually supplied by the serum present in the growth medium (Knox and Grifths 1980). Although it was suggested by Grinnel and Hays (1978) that cell spreading is a general cellular response of cell substrate interactions and it does not require the binding between a cell-surface receptor and the substratum. However, later it was shown that it is not a general response of cell substrate interaction. Hence Hela cells only spread on a gelatin preadsorbed surface but not on laminin or fibronectin surfaces (Burke et al. 1983; Fairman and Jacobson 1983). Promotion of cell spreading can be carried out by either an adsorbed serum protein (s) (Whateley and Knox 1980; Knox 1984; Neumeier and Reutter 1985) or by secreted cellular proteins which adsorb to the substratum following secretion (Grinnel and Feld 1979; Van Wachem et al. 1987). Recently it has been reported that human keratinocytes are able to secrete a spreading factor which acts directly on the cells not through the modification of the substratum (Malcovati and Tenchini 1991). However, it is commonly believed that spreading can take place through interactions of integrin receptors with adhesion proteins and cytoskeletal proteins (Horwitz et al. 1986, Tamkun et al. 1986; Bidanset et al. 1992; Leavesley et al. 1992; Weitzman et al. 1993) and it requires metabolic energy, as well as metal ions (Bereiter-Hahn et al. 1990). Cellular spreading is preceded by the process of strengthening of cellular adhesion and is also possibly triggered by metabolic events such as formation of focal adhesions which will now be discussed below.

1.2.4. FOCAL ADHESIONS

Focal adhesions can be described as areas of the cell surface at which cells are tightly bound to the substratum. Focal adhesions are also known as focal contacts or adhesion plaques (Burridge et al. 1988; Woods and Couchman 1988).

When many cells, including fibroblasts and epithelial cells, are seeded onto the appropriate substratum they attach, spread and form focal adhesions (Couchman et al. 1982;Kolega et al. 1982). In focal adhesions, extracellular matrix proteins, integrin receptors and cytoskeletal proteins are involved (Laterra et al. 1983, Woods et al. 1986, Kupfer et al. 1986, Singer et al. 1988, Stickel and Wang 1988). That is the β subunit of the integrin receptors links the extracellular matrix to a cytoskeletal protein e.g. α-actinin or talin through plasma membrane (figure 1.6) (Simon et al. 1991; Luna and Hitt 1992). However, recently it has been reported that $\alpha_5\beta_1$ is involved in focal adhesions while the $\alpha_1\beta_1$ integrin is not involved in the focal adhesions. This might suggest that although the β subunit binds to the cytoskeletal proteins the α-subunit may be involved in the formation of focal adhesions (Tawill et al. 1993). This point has been supported by Ylänne et al.(1993) who suggested that the α subunit cytoplasmic domain maintains the fidelity of recruitment of the integrins to focal adhesions and hence regulates the performance of integrins.

Focal adhesions are dynamic structures, thus in cultured cells they assemble, disassamble and then reassemble at specific times during cell growth (Burn et al. 1988). However, this mechanism is attributed to limited proteolysis and phosphorylation of extracellular matrix and cytoskeletal proteins (Anteler et al. 1985; Kamps et al. 1986 and Herman et al. 1986). Changes in protein phosphorylation can

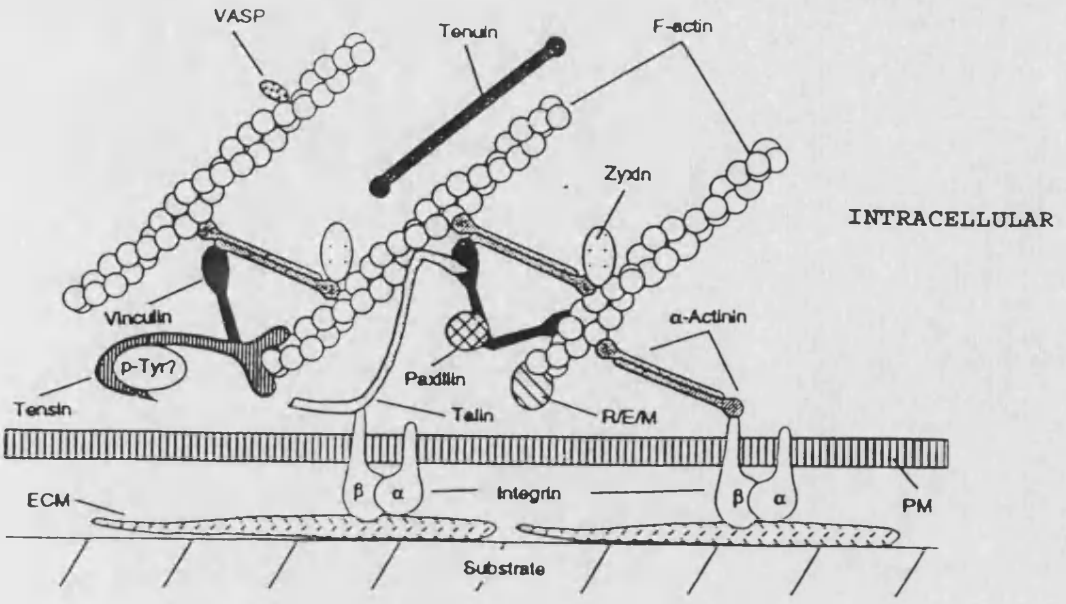


Figure 1.6: A Schematic Model of of Protein-Protein Interaction In Focal Adhesions.

Abreviations are: ECM= extracellular matrix; PM= plasma membrane; R/E/M= member of the radixin/ezrin/moesin family; VASP= vasodilator-stimulated phospho protein.

regulate the structure and function of adhesion, since inhibition of protein phosphorylation also inhibits the formation of focal adhesions (Luna and Hitt 1992).

As described above, The extracellular matrix molecules presumably send some information to the cell interior by acting through their membrane receptors and so can modulate the growth of the cells. This will briefly be discussed below.

1.2.5. CELL GROWTH

For normal anchorage dependent cells, attachment to a substratum and spreading are prerequisites for entry into the growth

cycle and synthesis of macromolecules (Folkman and Moscona 1978; Niven and Aplen 1985). Although this is a phenomena which has been known for a long time, the mechanism of regulation of cell growth upon adhesion is not known in detail. However, some suggestions have been made to explain it. For example, Menko and Boettriger (1987) suggested that binding of extracellular molecules to integrins may activate a second messenger system(s) and initiate a signal that is then transduced to the nucleus to influence cell growth (Menko and Boettiger 1987). The other opinion is that when cells adhere to the substratum, integrin receptors send signals to the interior of cells which would lead to organization of the cytoskeleton, thus regulation of cell shape,induction of gene expression and therefore regulation of cell growth occur (Unemori and Werb 1986; Juliano and Haskill 1993).

Cells in culture are able to divide before attaining confluency. To be able to divide cells must greatly reduce their contact with the substratum and round up. Mitotic cells may temporarily release themselves from the substratum and rebind to the substrate as they flatten following division (Baker and Garrod 1993). The various stages in cell adhesion have been described. It will now be appropriate to discuss some of the factors which play a role in cell adhesion. These factors include:

1-Energy,

2-Protein synthesis ,

3-Metal ions.

Each of which will be discussed briefly.

1.2.6.ENERGY

The facts which are given in above sections and other lines of evidence suggest that cell adhesion is a metabolically active process. However, there are still conflicting reports on this issue while the role of energy in cell adhesion has been debated for more than two decades. A brief review of this will be given below.

Carter (1967) suggested that spreading of mouse fibroblasts on cellulose acetate sheet was passive. The passive spreading means that spreading is not the result of the forces or components which originated from inside the cell, rather it is due to forces acting between the surface of the cell and the surface of the substratum. However, the idea of passive spreading was rejected by Wolpert et al. (1969) who said that if cell spreading was caused by a passive process, it ought not to be significantly affected by lower temperatures, which infact did reduce cell attachment. Michaelis and Dalgarno (1971) were able to show the involvement of metabolic energy in cell adhesion. That is depletion of cellular ATP by metabolic inhibitors resulted in the preventing of cell adhesion and spreading. Later it was reported by Unhjem and Prydz (1973) the attachment of Hela cells in the presence of serum is an energy requiring process while in the absence of serum it is not energy dependent. Unhjem's point was rejected by Grinnel (1974) who found that the attachment of BHK cells in both the presence and absence of serum is an energy dependent phenomena.

Klebe (1975) reinforced the report of Michaelis and Dalgarno (1971) by showing that the blocking of ATP synthesis by metabolic inhibitors inhibited cell attachment. However, the effect of metabolic inhibitors on cell attachment was overcome by adding glucose to the medium. Klebe (1975) was later supported by Juliano and Gagalang (1977). In this report it was indicated (see chapter 4) that the

lowering of temperature (below 10°C) and depletion of cellular ATP diminishes cell adhesion. Contrary to the above reports, Nath and Srere (1977) were able to show that there is no correlation between cellular ATP concentration and the rate of cell adhesion although attachment of cells was inhibited at 4°C.

Bereiter-Hahn et al. (1990) reported that although metabolic energy was not required for cell attachment, spreading of cells required energy. Recently we have reported that initial attachment and gaining of possible maximum adhesion strength is an active process (Yildirim and Whish 1994).

1.2.7. CELLULAR PROTEINS.

As indicated earlier, cell adhesion involves endogenous and exogenous proteins. However, the role of protein synthesis in cell adhesion has been always controversial. For example, Daday and Creaser(1970) have reported that a cellular protein is responsible for the adhesion of retina cells. While Michalis and Delgarno (1971) proposed that protein synthesis is not an effective factor in cell adhesion. Although, to date several contradictory reports have been published. The most important of these will be considered in following paragraphs.

Kolodony (1972) reported that the initial attachment of 3T3 cells to tissue culture dishes was not affected by emetine (80 µg/ml). Whereas under the effect of emetine cells were unable to sustain their adhesiveness. Therefore after 6 hours of incubation most of the cells had come off the culture surface. A contradicton to latter report has been published by Weiss and Chang (1973). These authors reported that the inhibition of protein synthesis of Ehrlich-Lettre hyperdiploid ascites carcinoma (EAT) cells with cycloheximide

increased the rate of cell adhesion. The relevance of protein synthesis in cell adhesion has become a controversial issue.

Pena and Hughes (1978) reported that spreading of BHK cells on fibronectin coated surfaces was independent of protein synthesis. Since the presence of 3 µg/ml cycloheximide did not prevent spreading of cells on this substratum. Grinnel and Feld (1980) found that if the secretion of fibronectin is inhibited, cell spreading is inhibited, unless the surfaces are coated with fibronectin, while Virtanen et al.(1982) reported that inhibition of secretion of fibronectin by monensin did not prevent the spreading of human fibroblast on culture dishes, although this treatment inhibited the formation of focal adhesion. However, later it was reported that although the attachment and spreading of human fibroblasts was not effected by monensin in the presence of serum, monensin treatment prevented cell spreading while it had no effect on initial attachment and spreading in the presence of serum (Pizzey et al. 1983). It was Knox (1984) who showed that the effectiveness of protein synthesis inhibition is influenced by the concentration of serum. That is, below 3% serum cycloheximide did not effect BHK cell spreading. While at 3% or higher serum concentrations cell spreading was completeley inhibited by this agent. However, in the case of fibronectin depleted serum, cycloheximide inhibited cell spreading at both below and above 3% serum concentrations as well as at this concentration.

The attachment of human gingival fibroblasts (HGF) to various collagens e.g. type I and type IV was studied by Farsi et al. (1985). It was found that the depletion of serum fibronectin did not have any effect on HGF cells attachment. The attachment of these cells in the presence of serum was independent of cellular fibronectin since the attachment of cells to collagen was not

prevented by anti fibronectin antibodies. Whereas attachment of this cell type in the absence of serum was found to be dependent on cellular protein synthesis. Brown et al. (1990) noticed that monensin treatment does not significantly affect cell attachment and spreading. These results indicated the importance of fibronectin and endogenous protein synthesis.

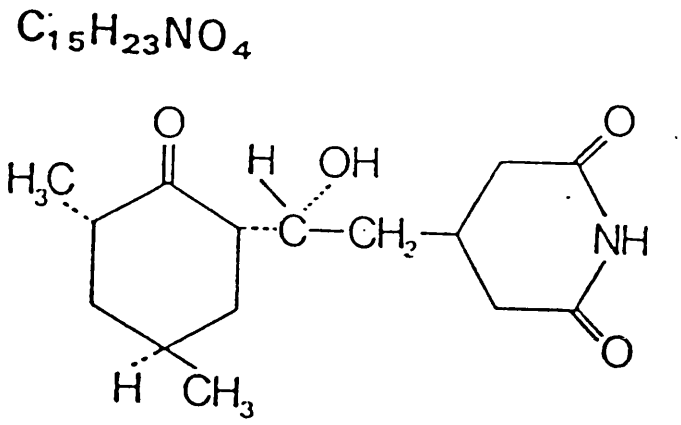
Flickinger and Culp (1990) have reported that prolonged cycloheximide treatment of human fibroblasts prevent the formation of actin stress fibers on a collagen substratum whereas the cells were spreading normally. This treatment did not effect the formation of stress fiber on fibronectin coated substratum.

These studies clearly suggest that cells require endogenous proteins to perform their adhesive function completely.

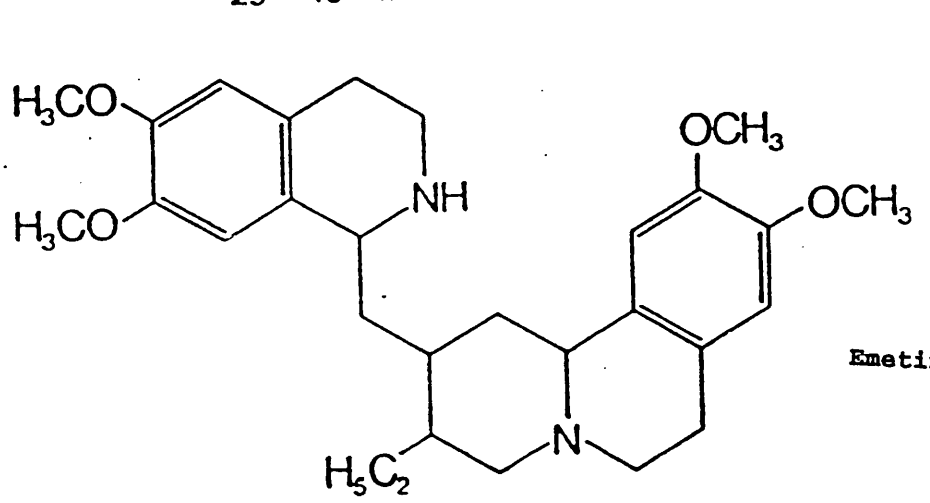
In evaluating the role of protein synthesis in cell adhesion it is necessary to determine whether cell adhesion occurs in the absence of endogenous protein synthesis. However, simply stopping protein synthesis may not be sufficient, since cells can retain adhesion proteins as a large internal pool. The secretion of protein from this pool may mediate adhesion. Perhaps together inhibition of protein synthesis and protein secretion plus the presence of antibodies specific to adhesion protein would be an important tool to evaluate the role of endogenous proteins in cell adhesion.

Perhaps it would be convenient to take a brief look at the inhibitiors of protein synthesis and secretion.

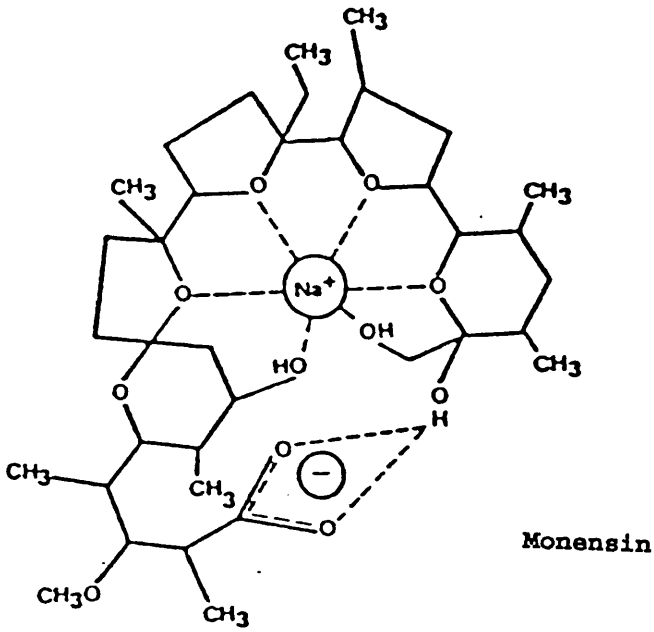
Cycloheximide and emetine are commonly used protein synthesis inhibitors. Cycloheximide (figure 1.7) interacts with the 60s sub unit of eukaryotic ribosome and inhibits translocation of peptidyl-



Cycloheximide



Emetine



Monensin

Figure 1.7 : Structures of The Inhibitors of Protein Synthesis or Secretion.

tRNA from the A site to P site (Pestka 1971; Obrig et al. 1971). Although cycloheximide is only effective for cytosolic ribosomes, emetine is a potent inhibitor of mitochondrial and cytoplasmic protein synthesis. Emetine (figure 1.7) inhibits protein synthesis by preventing the movement of ribosomes along mRNA (Oleinick and Salengo 1976; Oleinick 1977).

The secretion of some adhesion proteins including collagen and fibronectin can be inhibited by monensin (Uchida et al. 1979; Pizzay et al. 1983). Monensin (figure 1.7) is a monovalent ionophore which binds to ions with specificity of Ag>Na>K>Rb>Cs>Li>Ca. The binding specificity of monensin to sodium ions is ten times more than to potassium ions (Mollenhouer et al. 1990). Monensin is able to promote the exchange of protons for univalent ions (particularly Na^+) and hence to increase Na^+ concentrations and to distrupt intracellular proton gradient which would lead to various disarray of the cell function including inhibition of secretion of proteins (Mollenhauer et al. 1990; Decorti et al. 1991).

1.2.8. DIVALENT CATIONS.

Many lines of evidences suggest a role of divalent cations in cell adhesion. Both Mg^{2+} and Ca^{2+} appear to be active at physiological concentrations in many of the systems tested (Takeichi and Okada 1972; Grinnel 1976). Gallit and Ruoshlahti (1988) have reported that Mn^{2+} increased the binding affinity of fibronectin receptors 2-3 fold over their binding in buffers containing Ca^{2+} and Mg^{2+}.

Some cell adhesive molecules express their adhesive function only in the presence of Ca^{2+} and that Ca^{2+} protects extracellular parts of proteins from proteolytic degradation (Ozawa et al. 1990).

According to Chang and Hsu (1990) the presence of multivalent cations in the suspension medium reduces the repulsive force between cell and substratum with which the cell interacts. However the effect of these cations appears to depend on the substratum. For example, the attachment of rat muscle cells to laminin, collagen type I, type IV and fibronectin surfaces was reduced by the absence of divalent cations, whereas, the absence of divalent ions did not effect cell adhesion to polylysine (Clayman et al. 1990). This might suggest that the requirement for divalent cations in attachment is specific for the extracellular matrix derived substrate.

A common characteristic of all integrins is the absolute requirement for divalent cations. All integrin α subunits have three to five putative cation binding sites (see figure 1.5) and presumably the divalent cations exert their effect by binding to these sites and possibly by interacting directly with β subunits as well (Kirchoffer et al. 1991). Divalent cations could regulate the binding affinity of integrins for different substratum. For example, in the presence of Ca^{2+} and Mg^{2+} the fibronectin receptor of placenta recognizes the RGD sequence of fibronectin but not that of vitronectin. While the vitronectin receptor of placenta recognize the RGD sequence of vitronectin but not that of fibronectin. However, in the presence of Mn^{2+} the vitronectin receptor of placenta binds to the cell-binding domain of fibronectin and by replacing divalent cations from Mn^{2+} to Ca^{2+} and Mg^{2+} this receptor demolishes this binding (Yanai et al. 1991). The regulatory effect of divalent cations has been reinforced by Grzesiak (1992). This author has reported that although both $\alpha_v\beta_1$ and $\alpha_v\beta_3$ are RGD dependent integrins, $\alpha_v\beta_3$ binds to vitronectin in either Ca^{2+} or Mg^{2+} and $\alpha_v\beta_1$ binds only in Mg^{2+} and not in Ca^{2+}.

These results also support the possible involvement of the β subunit in cation binding.

In general cell adhesion has been studied qualitatively. However, some quantitative methods have been used to measure cell adhesion. Nevertheless, these methods either have a limited capabilty or suffer from a need of complex equipment. The advantage and disadvantage of these methods will now be reviewed.

1.2.9. MEASUREMENT OF CELL ADHESION

In general, the adhesion of a cell attached to the substratum is defined according to the shear force the cell must resist to avoid being dislodged. It is not necessary that attachment or detachment should be the exact reverse of each other. However both types of measurement have much in common, that is they coexist in any system in which cells are brought to the surface with the possibility of attachment (Bell, 1978).

Much progress has been made in determining the nature of the adhesive interactions at cell surfaces by measuring cell adhesion. That is, the measurement of a cells ability to remain attached when exposed to forces of detachment. Various methods have been developed to perform adhesion assays (Coman 1961; George et al. 1971; Evans and Leung 1984; Frangos et al. 1988; Lotz et al. 1989; Truskey and Pirone 1990). Conveniently these methods can be categorised into the three main classes :

1- Micromanipulation,

2- Centrifugation,

3- Hydrodynamic shear force.

1.2.9.1.MICROMANIPULATION

In these systems the adhesion of a single cell to the substratum or separation of cells can be studied. Coman (1944) originated this technique. In Coman's method, briefly, a flexible fiber, the pulling needle, is inserted into one of adhering cells. The other cell is held stationary against a microscope cover glass using a relatively stiff fiber, the holding needle. The flexible fiber is then moved by the micromanipulator in such a way as to create a tension between the cohering cells. The force exerted on the cells is determined by observing the bending of the flexible fiber through the calibrated reticle of a microscope. In practice the position of the tip of the flexible needle just prior to detachment of the cell was compared to its unstressed position when the cell contact was broken (Coman 1961). The main disadvantage of this method is that since a needle is inserted into the cell this process might damage the cell (Brooks et al. 1967). Moreover it is possible that such damage may release materials from the cytoplasm which could effect adhesiveness (Hubbe 1981). However, this problem has been overcome by the modification of this technique (Evans and Leung 1984). In this method, cells were sucked into micropipettes at known pressures and at the same time the shape of cells in response to suction was observed by light microscopy. Later, Francis et al. (1987) developed a method in which elements of the methods of Evans and Leung (1984) and Coman (1961) are combined.

That is the force is applied to the cells by fine glass micropipette. The micropipette was attached to the adherent cell into a position so that it can be sucked into the micropipette. The force applied to the cell is calculated from the degree of bending of the pipette. In addition the continous direct observation of adhesion zone was carried out by interference reflection microscopy during the

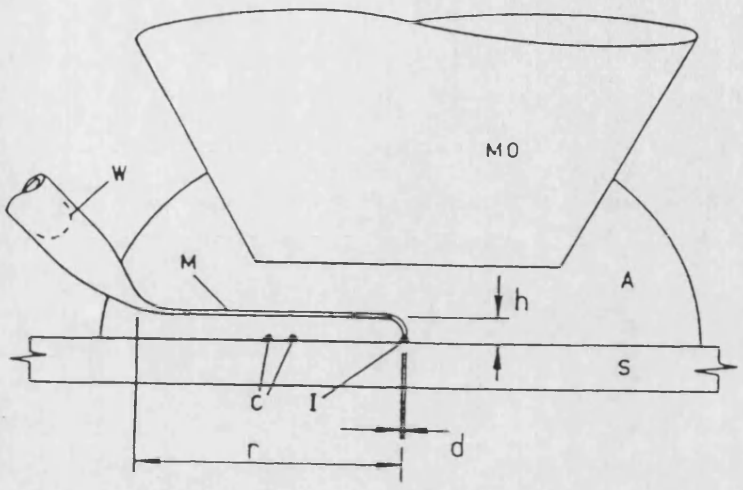


Figure 1.8 = A Cell Adhesion Measuring Device Based On Micromanipulation Methods (Bowers et al. 1989).

Abrevations are: M= micropipette;MO= microscope objective; I= an adherent cell under investigation; C= other cells; S= substrate; A= aqueous medium surrounding the cells; W= the place where the micropipette bore widens; h,r,and d are the micropiptte dimensions.

process (Francis et al. 1987) The developed version of the Francis method has been succeeded by Bowers et al.(1989). In a prototype experimental design the microscope plays a central role, as it supports the cell attached to substratum. A calibrated vertically oscillating micropipette is also positioned between the microscope and the cell bound substrate. The application of the micropipette is manipulated electrically. The pressure within the micropipette is gradually reduced until the suction is sufficient to keep the cell attached to the micropipette tip. Then eventually, a force is reached

which is sufficient to detach the cell from the substrate (figure 1.8) (Bowers et al. 1989).

The main advantage of this technique, perhaps, is that it is possible to distingiush the adhesion behaviour of normal healty and unhealty cells since detachment assays are performed in single cells. However, the main disadvantage of this technique is that it is unable to be used for very small cells or some rounded cells. Moreover only the adhesion of a single cell out of millions of cells can be measured at a time in this complex, expensive and high technology method. Therefore the complexity, time consumption and the fact that relatively small number of cells can be examined in a whole day, contributes to the limitations of this procedure.

Another commonly used method for adhesion studies is Centrifugation which is discussed below.

1.2.9.2. CENRTRIFUGATION

Puncturing and tearing of cells are avoided when detachment takes places in a centrifuge. Easty et al. (1960) have introduced this method. The reported procedures differ in detail but are essentially that described in the following sections. Dispersion of single cells are allowed to sediment by gravity (Easty et al. 1960; George et al. 1971) or by centrifugation (Berwick and Common 1962; McClay et al. 1981). After incubation for a set period the number of attached cells are counted. Together with adherent cells and fluid medium, the cell attached to the substratum is then placed upsidedown in a centrifuge rotor and centrifuged. After cenrtrifugation for a set period at a chosen speed the cells are counted once again under the microscope.

In Easty at al (1960) method the cell suspensions were added into glass tubes of rectangular cross-section. The tubes were placed horizontal and the cells were allowed to settle on the lower glass surface under gravity. The tubes were then centrifuged in the horizontal position.. While Berwick and Common (1962) have spun the cell suspension in a centrifuge for cell attachment. Thereafter with the cell attached side kept uppermost the preparation were centrifuged. In both of above methods the number of attached cells was determined by counting the number of cells per unit area with the microscope before and after centrifugeation.

Lotz et al.(1989) have used radiolabelled cells. Here radioctive labelled cells were added to a substrate coated microtiter well. A second, fluid filled microtiter well was placed over the first and two were sealed. And after first and second cenrtrifugation the detachment was quantified by scintillation counting (see figure 1.9).

The centrifugal method is rather uninformative. Many cells remain adherent in response to the detachment force, these might represent increased adhesion or a change in cell shape which did not allow them to detach. In addition, the centrifugal technique tends to be time consuming and limited in the range of forces which can be applied to detach the fully spread cells. Often the strength of the final adhesion is too large and it exceeds ability of the centrifugal method to measure it. The limitations of the centrifugal and micromanipulation methods could be avoided by using a hydrodynamic shear force on the cells. This will be discussed below.

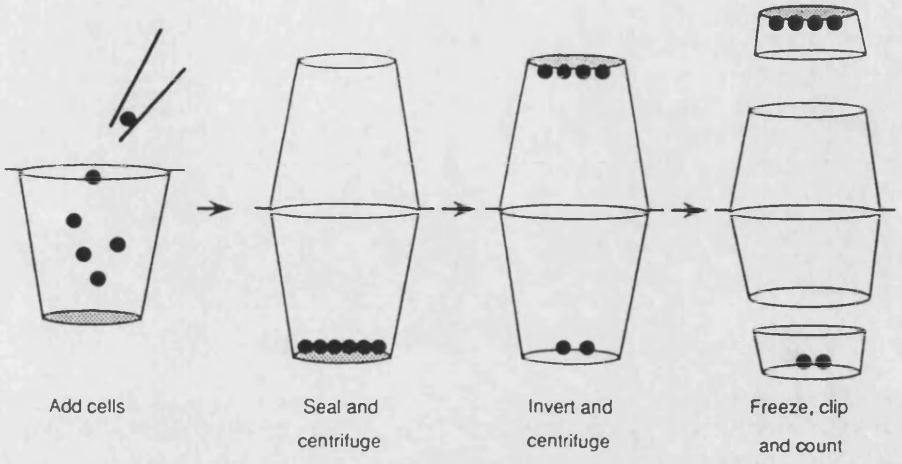


Figure 1.9: A Cell Adhesion Assay In Cenrtrifugation Method (Lotz et al. 1989).

1.2.9.3.HYDRODYNAMIC METHODS.

This type of technique was introduced by Weiss (1961). In this kind of assays, the cells are first allowed to settle onto a substrate and a hydrodynamic force is applied to the surface. In attachment studies, the basic principle is almost the same in all the techniques developed for this purpose (Figure 1.10). A cell containing suspension is passed over the surface and the number of cells which are attached at particular flow rate of suspension is measured after a defined time (Weiss 1961; Mohandas et al. 1974; Forrester and Lackie 1984; Crouch et al. 1985).

For detachment studies, adherent cells are exposed to a known shear field. In these categories the simplest and easiest method is the parallel plate flow chamber. Mohandas et al. (1974) introduced a very basic design and later a number of other workers used this basic design to develop their own parallel plate chambers (Doroszewski et al. 1977; Owens et al. 1987; Truskey and Pirone 1990; Cozen Roberts et al. 1990; Van Kooten et al. 1991; Usami et al. 1993).
As an example, the methodology of Mohandas et al. (1974) is described briefly. In this design the upper portion of a parallel plate flow chamber contains a slide on which cells are growing. The lower portion has a rectangular design (figure 1.10). Fluid flows into and out of the channel through two holes drilled in the glass slides. As fluid passes from the inlet toward the outlet the pressure drops and the number of detached cells as a function of time and applied force are counted. The time and force are used to calculate a value below which essentially no cells will detach but above which all cells will detach, this is called minimum critical shear value.

It was realised that a simple, reproducible and accurate instrument was required to measure mammalian cell adhesion. For this purpose, in the present work a simple but highly reproducible technique called a Microflow Chamber has been developed. This will be described in chapter 3 and the advantage and disadvantage of this techqnique compared to others will be discussed.

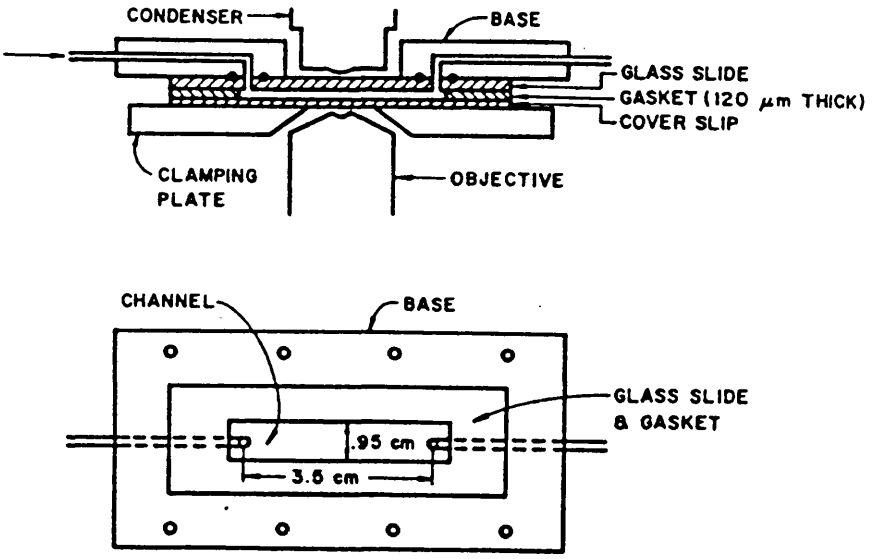


Figure 1.10: A simple Parallel Plate Flow Channel (Mohandas et al. 1974)

In the present work the underlying mechanism of the phenomenon of adhesion strengthening was studied. Therefore, various factors including the effect of energy in cell adhesion was studied. Moreover, the role and origin of serum on cell adhesion strength, the effect of surface modification by adhesion molecules i.e. fibronectin, collagen type I and type IV and polylysine on cell adhesion strength have been studied. Finally, the role of endogenous proteins on cell adhesion strength have been determined. Each of these will be discussed in the following chapters.

CHAPTER 2 MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. GENERAL

All chemicals used in the preparation of solution were from Sigma (London), Poole, Dorset, BDH Chemical LTD., Poole, Dorset, Aldrich Chemical Company, Gillingham, Dorset, Flow laboratories, Irvin, Scotland and Fisons Scientific Apparatus England.
All tissue culture flasks and dishes were from Sterilin LTD. U.K.

2.1.2. CELL LINES

The cell lines tested in present work include; BHK 21 (baby hamster kidney cells), L929 (mouse fibroblast), CHL (Chinese hamster lung fibroblasts), Hela B (human cervical carcinoma epithelial cells) and MDCK (Madine Darby canine kidney epithelial cells). All these cells were obtained from Flow Laboratories.

2.1.3. CELL CULTURE

PBS (phosphate buffered saline), L-Glutamine, penicillin-streptomycin, non essential amino acids, 10x minimum essential medium Eagles (modified with earls salt) (MEM) and trypan blue (0.4% w/v) in 0.85% saline solution were purchased from Flow laboratories. HEPES (N-2-hydroxyethyl piperazine-N-2-ethanesulphonic acid was from BDH. Trypsin and trypsin inhibitor (soybean trypsin inhibitor) was from Sigma.

2.1.4. SERA

Heat inactivated donor horse serum and donor calf serum were obtained from Flow Laboratories. Heat inactivated foetal calf serum was from Globepharm limited Surrey, U.K.

2.1.5. MICROFLOW CHAMBER

The peristaltic pump was from Watson Marlowe LTD. Glass slides were purchased from Chance Proper Limited. Square plastic dishes (144 cm^2) were from Sarsted Ltd. Beaumont Leys, Leicester, U.K. and tissue culture grade round petri dishes were obtained from Sterilin limited U.K.

2.1.6. MODIFICATION OF SURFACES

Human plasma fibronectin was from Flow laboratories. Collagen typeI, collagen type IV and Poly-D-Lysine were purchased from Sigma.

2.1.7. INHIBITORS

Emetine dihydrochloride, cycloheximide (crystalline) , monensin sodium salt, and oligomycin were obtained from Sigma.

2.1.8. RADIOACTIVE COMPOUNDS

[^{125}I]-fibronectin (human plasma) was from Flow Laboratories. [^{35}S]-methionine was purchased from Dupont New England.

2.1.9. RADIOACTIVE COUNTING

Scintillation vials were from Packard Instrument LTD. Germany, GF/C discs were from Whatman, Maidstone, Kent, trichloroacetic acid was obtained from Fisons Scientific Apparatus, England. Optiphase (ethyl substtituted benzene) was used as a scintillation liqiud and was obtained from LKB.

2.2. METHODS

2.2.1. CELL CULTURE

2.2.1.1. REAGENTS AND BUFFERS.

HEPES BUFFER (20mM)

12.58 gram of HEPES was dissolved in double distilled water to give final concentration of 20mM and the pH was adjusted to 7.4 with 0.1 M

NaOH. The buffer was autoclaved (under free steam conditions at 130^{o}C temperature and 15lb/in^{2} pressure for 30 minutes) for sterilization.

PBS

(Phosphate buffered saline) PBS was made according to the instructions of the suppliers i.e. five tablets of PBS were dissolved in 100 ml of double distilled water and autoclaved.

TRYPSIN

1% (w/v) trypsin was prepared by dissolving 10 mg of trypsin lyophilisate in 1 ml of pre cooled PBS. 0.1 ml aliquatos of this solution were dispensed into sterile storage tubes as quickly as possible, as the trypsin will begin digesting itself and stored at -20^{o}C. Each trypsin containing aliquot was thawed immediately before use and diluted in 2ml EDTA (0.02% w/v) solution.

EDTA 0.02% (w/v)

20 mg EDTA was dissolved in 100 ml of PBS and filtered through a 0.2 µm filter for sterilization.

CULTURE MEDIUM

To achieve a 10 fold final dilution of culture medium, MEM (x10 concentration) was diluted in sterile HEPES buffer. To these diluted media other constiuents were supplemented as indicated below;

10% (v/v) foetal calf serum,

200I.U peniccilin, 20 µg streptomycin;

2mM Glutamine and

2% (w/v) non essential amino acids.

SERUM FREE MEDIUM

This was prepared as above except that 10% double distilled water (v/v) was used instead of 10% serum.

OXYGEN FREE MEDIUM

100 ml of HEPES (20mM) was boiled in a conical flask, then it was cooled under nitrogen gas flow. When it cooled to 20-25^{o}C the rest of the ingredients of the medium (see above) apart from serum were added. During the addition of these constituents of the medium and afterwards, a stream of nitrogen was continued for 15 minutes. The pH of medium was adjusted to 7.4 with 4M NaOH. Finally serum was added into this medium and sprinkled gently with nitrogen for 3 minutes.

2.2.2. MAINTENANCE OF CELL LINES IN CELL CULTURE

All the cell lines tested in the present work were used during the logarithmic phase of growth and maintained in cultures according to the suppliers instructions (Flow 1989). The old spent culture medium was decanted and monolayers were washed twice with PBS to remove remaining residues of serum.

To detach the cells from the flask, 0.05% (w/v) trypsin i.e 1% (w/v) stock solution was diluted in EDTA (0.02% w/v) solution was added and incubated at 37^{o}C for 3-5 minutes. While in the case of MDCK cells, 15-20 minutes incubation was necessary to detach the cells. At this point, when the cells had to come off, the trypsin was inactivated with serum containing medium.

Cell viability under the conditions used was always typically 99% as checked by the trypan blue exclusion method (equal volumes of cell suspension and trypan blue were mixed and the cells were observed under the microscope). The viable cells excluded trypan blue. The cell lines were maintained as outlained below.

Hela B, CHL, L929 and MDCK cells were maintained in Eagles minimum essential medium, with Earls salt, supplemented with 20mM HEPES buffer, 10% v/v foetal calf serum, 200I.U penicillin, 20μg streptomycin, 2mM glutamine and 2% (w/v) non essential amino acids.

While BHK 21 cells were grown in RPMI 1640 medium supplemented with donor calf serum with the other constituents as above. Culture medium was added according to the size of the flask used (25 cm^2, 75 cm^2 and 150 cm^2) to obtain a cell density of $1x10^5$ cells/ml. Cultures were incubated in a 5% CO_2/air (v/v) atmosphere and were subcultured twice a week.

2.2.3. MEASUREMENT OF CELL ATTACHMENT IN THE PRESENCE OF OXYGEN OR NITROGEN ATMOSPHERE.

2.2.3.1. MEASUREMENT OF CELL ATTACHMENT IN THE PRESENCE OF OXYGEN OR NITROGEN ATMOSPHERE.

Sub-confluent cells were trypsinized and after trypsinization, trypsin was inhibited by addition of 2ml (for 25 cm^2 flask) of the growth medium. The density of the cells were determined by means of a neubaver hemocytometer. Then a stock cell suspension which contained $5x10^5$ cells/ml were prepared by diluting the above cell suspension with growth medium. Subsequently 2mls of the latter cell suspension were distrubuted onto 35 mm round tissue culture dishes which were incubated at defined temperatures (from 4°C to 37°C). Finally, after the incubation period, the culture medium was transferred into a tube and all unattached cells were removed with twice gentle washing. To wash a monolayer dish serum free medium was used. Any cell not removed by series of these gentle washes were considered to be attached. The number of attached and non- attached cells were counted in an haemocytometer.

2.2.3.2. DETERMINATION OF CELL ATTACHMENT IN A NITROGEN ATMOSPHERE

Cells were grown in normal medium until they reach mid-log phase and about 50% confluency. The cells were then trypsinized from the culture flask under a stream of special oxygen free nitrogen gas.

That is during trypsinization the culture flasks were kept in a polyethylene bag into which passed a continuos stream of special oxygen free nitrogen gas. The EDTA-PBS, and PBS, were also sparged with this nitrogen prior to use. After trypsinization, the cells were placed in oxygen free medium (see 2.2.1.1). For all these experiments, 35 mm diameter tissue culture grade plastic dishes were used and they were also kept in the nitrogen atmosphere for 30 minutes before 2 mls of cell suspension (containing $1x10^6$ cells) were added. Incubation was carried out at 20^oC and rest of the attachment assay procedure was as described at 2.3.1.

2.2.4. MEASUREMENT OF THE CELL ADHESION STRENGTH.

For the measurement of cell adhesion the Microflow chamber which was developed in the present work was used throught this study. The theory, principal and use of this device is illustrated in chapter 3. At this stage it is appropriate to mention that cells are grown on a glass or a plastic substratum for 24 hours and after this time the cell growing substratum is subjected to the hydrodynamic flow in the Microflow chamber for 10 minutes. After this time the Microflow is disassemled and the critical shear stress of detachment was determined by measuring the critical distance and putting its value in a shear stress calculation as described in chapter 3.

2.2.5. SERUM STUDIES.

2.2.5.1. PREPARATION OF MEDIUM WITH DIFFERENT CONCENTRATIONS OF SERUM.

The MEM medium in separate containers was supplemented with different concentrations of serum, namely 10%, 5%, 2.5, 1%, 0.5%, and 0% (v/v). Other constituents of these media were the same as illustrated in section (2.2.1.1).

2.2.5.2. MEASUREMENT OF CELL ADHESION STRENGTH AT DIFFERENT CONCENTRATIONS OF SERUM.

Sub-confluent CHL cells were trypsinized. After trypsinization, trypsin was inhibited by addition of 2 mls (for 25cm^2 flask) of culture medium containing different concentrations of serum (0.5% to 10%). In the case of 0% serum, trypsin was inhibited by the addition of 2 ml of soya bean trypsin inhibitor which was twice as much as required to make sure that trypsin was inhibited. The detachment assay was performed as described in section 2.4.

2.2.5.3. THE EFFECT OF THE ORIGIN OF SERA ON THE STRENGTH OF CELL.

CHL cells were seeded in the medium supplemented with fetal calf serum or new born calf serum or horse serum onto plastic substratum. The critical shear stress of detachment was measured as described in section 2.4.

2.2.6. THE EFFECT OF SURFACE CHEMICAL MODIFICATION ON THE ADHESION STRENGTH OF CHL CELLS.

2.2.6.1. FIBRONECTIN MODIFICATION

2.2.6.1.1.FIBRONECTIN COATING ON PLASTIC SUBSTRATUM.

Lyophilized bovine plasma fibronectin was obtained from Flow laboratories. 1mg of lyophilisate was dissolved in 1 ml of sterilized double distilled water at room temperature in a laminar flow cabinet. The required concentrations of fibronectin were dissolved in 10 ml of sterilized double distilled water. The resulting solution was poured into 100mm diameter tissue culture grade plastic dishes. Fibronectin from this solution was allowed to adsorb on the plastic dishes and water was evaporated overnight. The dried dishes were washed twice with double distilled water and once with PBS immediately before seeding the cells. Control dishes were prepared in an identical manner except that the first incubation was in 10 ml double distilled water without fibronectin (Obrink 1982).

2.2.6.1.2. QUANTIFICATION OF FIBRONECTIN ADSORPTION.

^{125}I-fibronectin (5.3μCi/μg) in the form of a solution was obtained from Flow laboratories and the same day this solution was made up to 10 ml with double distilled water to give final concentration 1μCi/ml. 0.5 ml of this solution was added per well of a 24 well tissue culture grade dish and allowed to adsorb overnight. The water was evaporated and each well was washed twice with 0.5 ml of double distilled water. Both washings were pooled together.

The coated ^{125}I-fibronectin was extracted from the surface by washing twice with 0.5 ml of 1M NaOH. Each extraction lasted for half an hour. Extractions and washings were counted separately for 2-10 minutes on the gamma counter (Curtis and Forrester 1984). Therefore the amount of fibronectin adsorbed onto the dish (%) was determined. Assuming that fibronection was adsorbed as a monomeric uniform layer, the number of molecules adsorbed per cm^2 was calculated. The molecular weight of fibronectin was accepted as 440,000 gm/mole. One mol fibronectin has a numer of molecule equal to the Avagadro number of $6.02x10^{23}$. By using the amount of adsorbed fibronectin, the number of molecules adsorbed per cm^2 was determined.

2.2.6.2. COLLAGEN TYPE IV COATING ON THE PLASTIC

0.75 mg of collagen type IV of mouse sarcoma was dissolved in 2.5 ml of 0.1M acetic acid. The required concentrations of collagen type IV were dissolved in 10 ml of sterilized double distilled water. The resulting solution was poured into 100 mm diameter tissue culture grade plastic dishes. After leaving overnight in a laminar flow cabinet, dried dishes were washed three times with double distilled water and once with PBS immediately before seeding cells (Aumailley and Timpl 1986).

2.2.6.3. COLLAGEN TYPE I COATING ON THE PLASTIC.

Lyophilized calf skin collagen type I was obtained from Sigma. 5mg of the lyopillisate was dissolved in 50 ml of 0.1M acetic acid overnight in a laminar flow cabinet. The required amount of protein was diluted into 10ml of double distilled water. The rest of procedure was the same as described in section 2.6.2 (Kleinman et al 1987).

2.2.6.4. POLY-D-LYSINE COATING ON THE PLASTIC.

Poly-D-Lysine was obtained from Sigma. 5mg of poly-D-Lysine was dissolved in 10 ml of PBS. The required amount was transferred into 10 ml of double distilled water. The rest of the procedure was like that described in section 2.6.2 (Yavin and Yavin 1974).

2.2.7. ROLE OF ENDOGENOUS PROTEINS IN CELL ADHESION

2.2.7.1. PREPARATION OF DRUG SOLUTIONS

2.2.7.1.1. EMETINE OR CYCLOHEXIMIDE

100 mg emetine or cycloheximide was disssolved in 20 ml of complete medium and sterilized by filtering through a 0.2 µm filter. 1 ml aliqouts of this stock solution were dispensed into sterilized eppendorf tubes and stored at -20°C. The frozen drugs were thawed immediately before use and stock soluions were serially diluted to obtain the required drug concentrations (0 to 2 µg/ml).

2.2.7.1.2. MONENSIN

100 mg monensin was dissolved in 20 ml absolute alcohol and kept in the frezer. Immediately before use, the monensin solution was warmed at room temperature for 15 to 20 minutes. 100 µl alcohol containing monensin was added into 100 ml complete medium. From this solution (5µg/ml), a serial dilution was performed to obtain required

final concentrations. No adverse effect of alcohol (1µl/ml) without monensin on the growth or viability of CHL cells were observed and as was also checked by the trypan blue exclusion method.

2.2.7.2. DETERMINATION OF EFFECTIVE DOSE OF DRUGS FOR INHIBITION OF GROWTH OR PROTEIN SYNTHESIS.

To determine the specific dose of monensin, emetine or cycloheximide to work with, the response of CHL cells to these drugs (in terms of their growth or/and protein synthesis) was examined as described below.

2.2.7.2.1. GROWTH

Subconfluent cells were trypsinized and trypsin was inhibited as stated earlier. The resulting cell suspension was inoculated with these drugs. Cell counting was continued with haemocytometer at different intervals for 78 hours. The measurement of population doubling time was used to quantify the response of CHL cells to these drugs.

2.2.7.2.2. PROTEIN SYNTHESIS INHIBITION.

For the protein synthesis inhibition following steps have taken.

2.2.7.2.2.1. DILUTION OF L-[^{35}S] METHIONINE

L-[^{35}S]methionine was obtained from Dupont. The septum of the vial was pierced with a syringe needle and touching the frozen product was avoided. The vial was vented in the fume hood and thawed at room temperature. Any pressure developed could vent through the syringe needle. The needle was removed and thrown in the radioactive waste bag. The contents of the vial was diluted with 10ml of mercaptoethanol (20mM) and aliquoted into 10 eppendorf tubes and stored -80^{o}C. Immediately before use this stock solution was diluted

in complete medium. The final concentration of radioactivity which was added per well of 24 well plate was always 0.5µCi.

2.2.7.2.2.2. DETERMINATION OF PROTEIN SYNTHESIS.

CHL cells were placed in a 24 well plate in the presence or absence of drugs and the cells were allowed to attach to the wells for 2 hours and metabolically labelled by adding 0.5µCi of L-[^{35}S]methionine to each well. The incorporation was followed over a period of 6 hours. At times ranging from 0 to 6 hours, the labelled medium was carefully removed and each well was washed twice with PBS. The cells were then dissolved in 0.5 ml of 0.1M NaOH which was instantly digested the cells. To this mixture 2 to 3 ml of ice cold 10% TCA was added and reaction was left to proceed overnight at 4^{o}C. The precipitated samples were then passed through a GF/C disc (previously washed with 2 ml ice cold 5% TCA and finally with 2 ml of 95% ethanol). The discs were placed in a scintillation vial and dried at 60^{o}C. After drying, 3 ml of scintillant (Optiphase) was added to each vial. The samples were counted in a Packard Tri-carb liquid scintillation counter.

CHAPTER 3 THEORETICAL BACKGROUND OF THE MICROFLOW CHAMBER

3.1. TERMINOLOGY

Before illustrating the theoretical background of the Microflow Chamber, a cell adhesion measuring device, which has been developed in this work, it is appropriate to describe some terminology involved in the design of this device.

3.1.1. INERTIAL FLOW

In this kind of flow, no external forces are exerted on a fluid.

3.1.2. INERTIAL FORCE

The inertial force is that tending to cause flow which is equal to the volumetric flow rate multiplied by the density of the fluid.

3.1.3. LAMINAR FLOW

The particles of fluid are evidently moving entirely in straight lines and parallel to the axis (see figure 3.1), even though the velocity with which particles move along one line is not necessarily the same as that along another line. Thus the fluid may therefore considered as moving in layers or laminae.

3.1.4. TURBULENT FLOW

The path of individual particles of fluids are no longer straight but wavy, intervening and crossing one another in a disorderly manner so that a thorough mixing of the fluid takes place (see figure 3.1). Only laminar flow can be described as steady. In turbulent flow there are continual variations of velocity and pressure at every point.

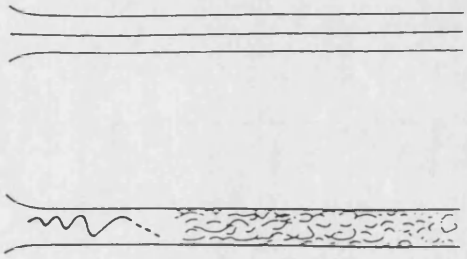


Figure 3.1. A Schematic Profile of Laminar and Turbulent Flow.

3.1.5. REYNOLDS NUMBER

A dimensionless number which is significant in the design of a model of any system in which the effect of viscosity is important in controlling the velocities or the flow pattern of a fluid: equal to the density of a fluid, times its velocity, times a characteristic length, divided by the fluid viscosity. However, Reynolds number is also described as the ratio of inertia force to viscous force . Therefore this number is able to say which one of these forces is dominant. That is, a high magnitude Reynolds number indicates that inertia forces dominate the flow while viscous forces play only a small part, but when the Reynolds numbers is small in value, the viscous forces have the upper hand and inertia forces take second place. Furthermore, the Reynolds number is also able to say whether a fluid is turbulent or laminar in flow characteristics. A low Reynolds number is the indication of laminar flow and high one shows that turbulent flow takes place (Massey 1989).

3.1.6. CONVERGENT CHANNEL

When a change occurs as a decrease in width, relative to the direction of the flow, the transition length is referred to as a convergent channel. Flow through a convergent channel is accelerating.

3.1.7. BOUNDARY LAYER

When a fluid flows over a surface, frictional forces retard the motion of the fluid in a thin layer near to the wall. This layer is called the boundary layer.

3.2. THEORY OF MICROFLOW CHAMBER.

The theory of the Microflow chamber is dependent on the fact that the flow of a viscous incompressible fluid between plane parallel plates, the so called Poiseuille flow, is governed by a parabolic velocity distribution (Millsaps and Pohlhausen 1953). In this flow system, the velocity of flow increases from the boundary surfaces to the centre and at the centre of the parallel plates it reaches a maximum magnitude. (See fig 3.2).

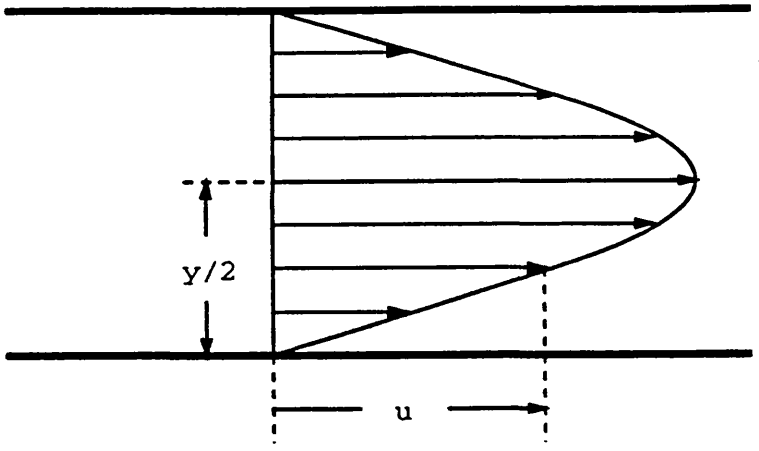


Fig 3.2: The Velocity Profile of Steady Laminar Flow between Parallel Planes. u represents the velocity of flow and y/2 indicate the centre of the parallel plates at where u is maximum.

The dimensions of the Microflow chamber are given in this section (see later) and are designed on the basis of theoretical predictions that in a convergent channel laminarization will take

place. In this system there is acceleration in fluid flow which will lead to laminarization (Tanaka & Yabuki 1986). Laminarization will occur when the acceleration parameter, **K**, defined as

$$K = \frac{\gamma}{u^2} x \frac{du}{dx} \qquad (3.1)$$

,exceeds a value of $2x10^{-6}$ (Launder & Lockwood 1969). In here, γ denotes the kinematic viscosity; u represents the mean velocity; du and dx refer to the difference of velocity and the change of distance respectively. However, laminarization can take place on the convex plate for a smaller value of K than on a flat plate (Launder & Loizou 1993). Thus it is proven that even if there is *turbulence* at the entrance to the channel, it becomes *laminar* very quickly. This is flow re-laminarization and an essential feature of the design of the Microflow chamber.

This *laminar* flow of a fluid is used as a hydrodynamic shear force to detach the cells from the surfaces. It may be argued that the cells growing on a surface is not smooth and so the flow across the cell monolayer could be *turbulent*. The fact is, that if the roughness of the surface is reasonably small, fluid flow will not be affected by this micro roughness (Coulson et al 1990). In addition, if free stream velocity increases rapidly, there is no time for turbulence to develop. Thus the flow remains *laminar*.

It is important to use *laminar* flow in which a difference of pressure is directly proportional to the velocity. In contrast, in *turbulent* flow the pressure difference increases at a greater rate than the velocity. Moreover, in *turbulent* flow there are continual

variations of velocity and pressure at every point whereas there is no such possibility in *laminar* flow (Massey 1989).

General introduction, dimensions, principal and use of the Microflow chamber are given as below.

3.3. INTRODUCTION OF THE MICROFLOW CHAMBER.

The Microflow chamber developed by the author (a cell adhesion measuring device) is shown, diagrammatically in figure 3.3. A convergent channel is accurately developed in this chamber, in which a complete laminar flow is achieved for hydrodynamic detachment of cells from the surfaces. Up to the present time four versions of this device have been designed and developed. The first version is suitable for glass or pre-cut polystyrene slides. The other versions are suitable for glass and plastic petri dishes. Each of these are described below.

3.3.1. FIRST VERSION.

The Microflow chamber is 110 mm long and consists of two parallel plates which are made of machined perspex. Each plate is 20 mm thick (picture. 3.1). The convergent channel is incorporated in the upper part of the chamber. There are two major sections of the Micro flow chamber, a lead in divergent section (30 mm) followed by the convergent channel, the test section, which is 76 mm long (see figure 3.3). The convergent channel starts with a width of 20 mm which reduces to 3 mm at the outlet of the chamber over an overall distance of 76 mm. Thus the fluid in the channel is tapered from the test section inlet to outlet where the depth is kept constant (1mm). Cell growing plastic or glass slides are inserted into the recess which constitutes the lower part of the device. The two halves of the

chamber are assembled and are clamped tight. To ensure tight sealing a silicon gasket is placed around the convergent channel. The Microflow chamber is connected on one side with a reservoir containing running medium and on other side with a peristaltic pump which was obtained from Watson-Marlow. The general arrangement of the Microflow chamber and the attached apparatus is shown in figure 3.4.

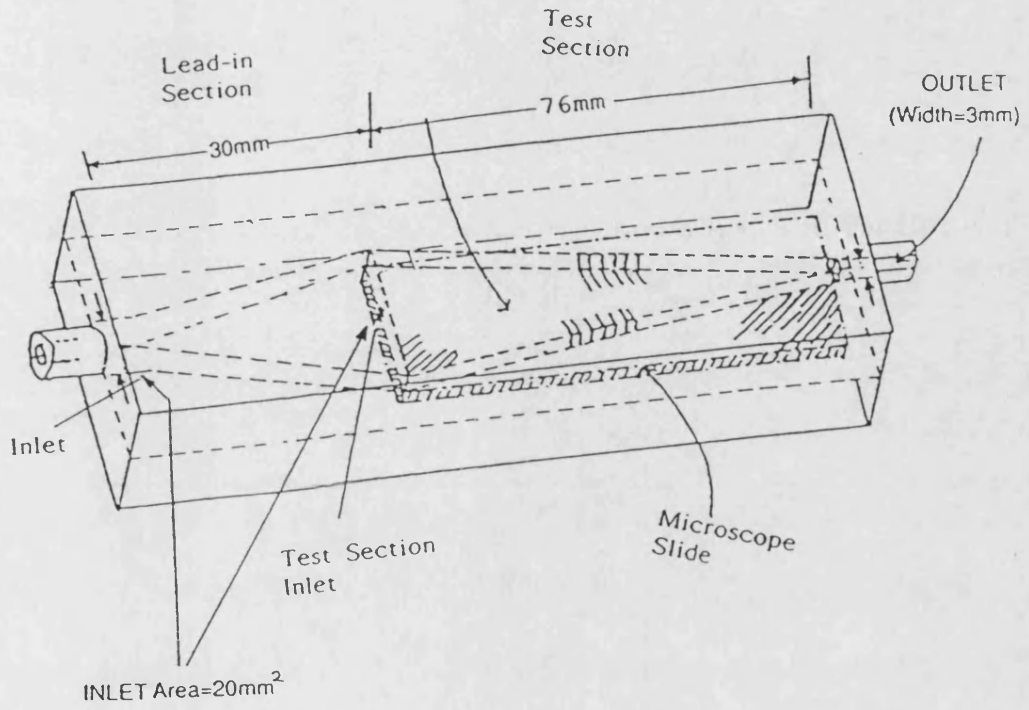


Figure 3.3: Three Dimensional Structure Of First Version Microflow Chamber.

A second version was developed which used standard 100 mm tissue culture dishes as the test surface. Much data was collected but the supplier (Sterilin) changed the dish size to 90 mm diameter. Following this appalling crisis a third chamber was developed using standard 90 mm diameter tissue culture dishes. Finally a fourth chamber was designed to fit into 90 mm square dishes but this one had 3 test channels (see later).

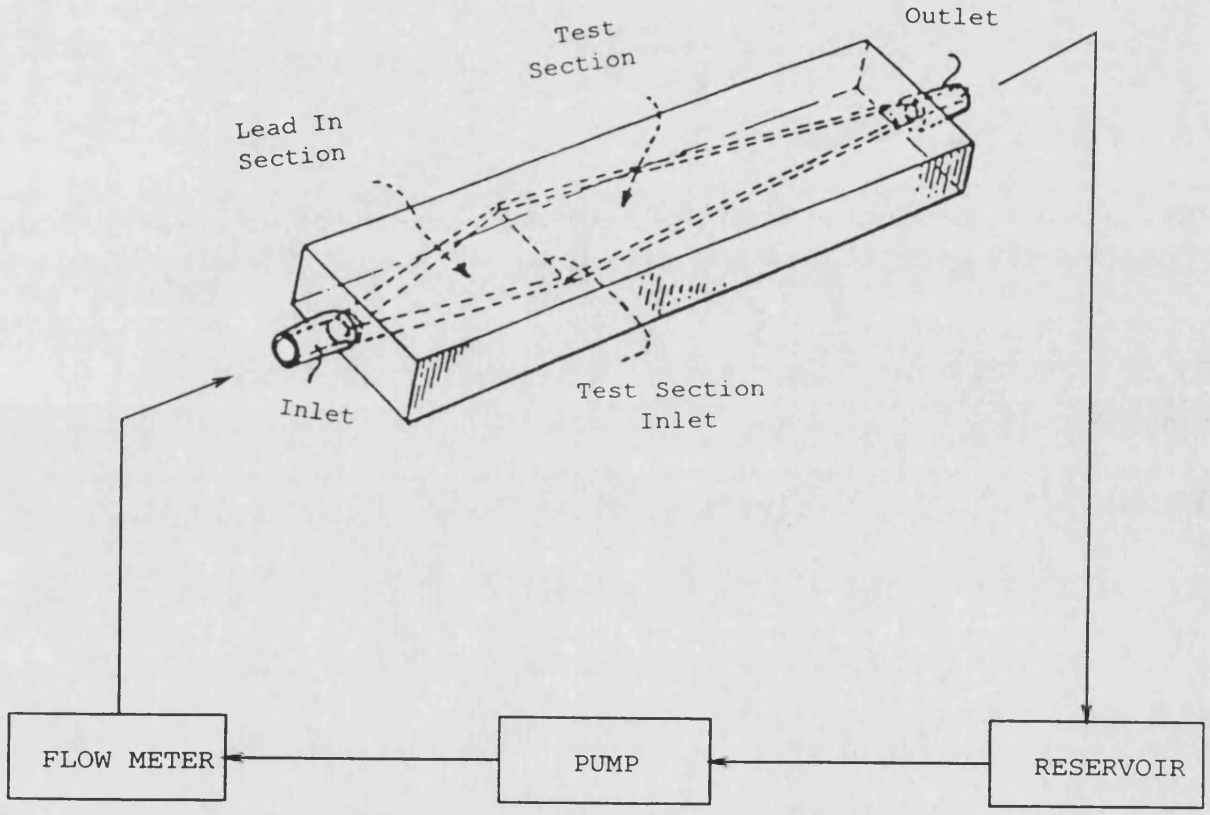
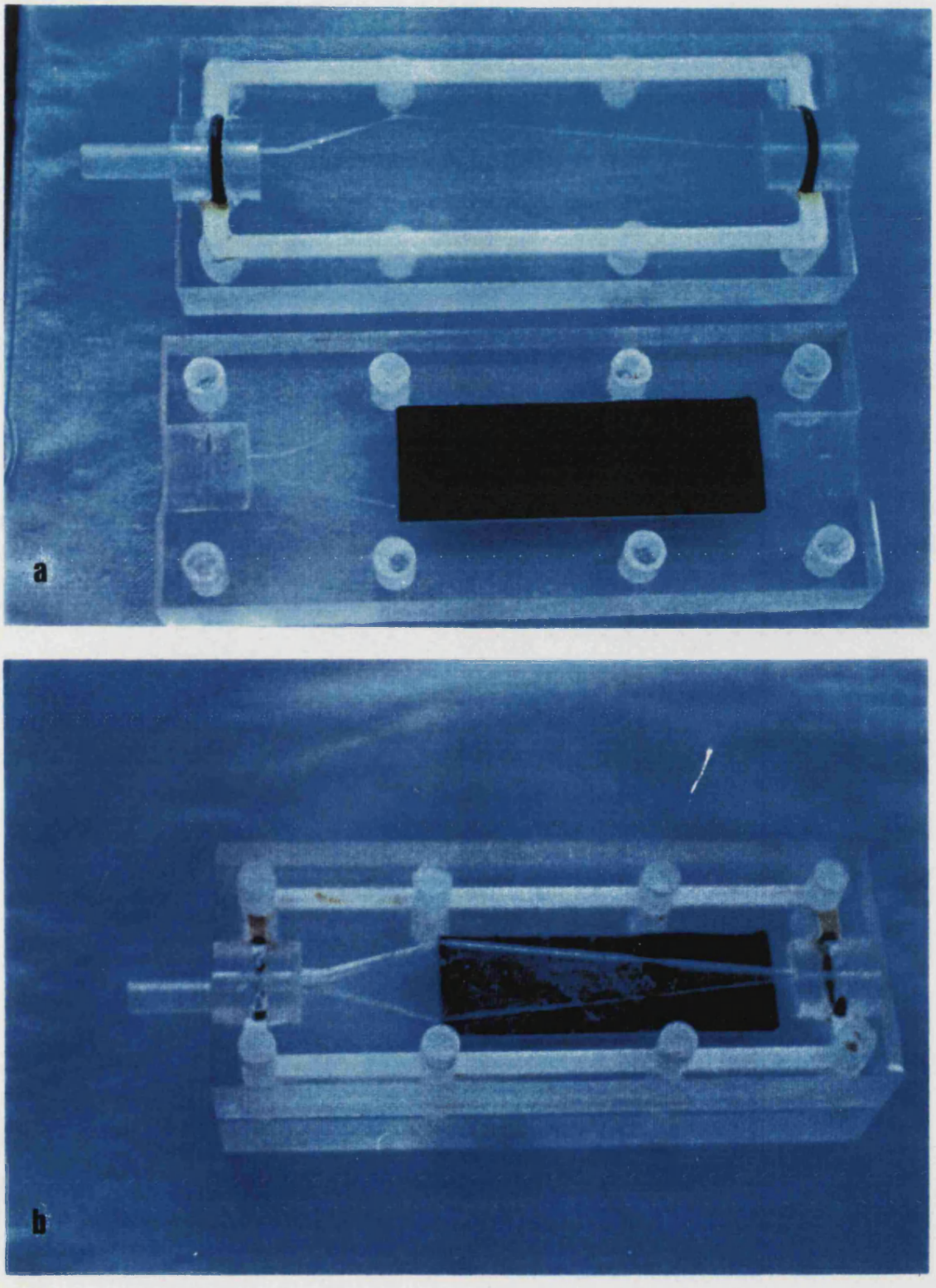


Figure 3.4: General Arrangements of the Microflow Chamber (first version) and the Attached Apparatus.



Picture 3.1: First Version Microflow Chamber

(a= Microflow Chamber Before Assembly; b= Assemled Microflow Chamber)

3.3.2. SECOND VERSION

The geometry of this version is exactly the same as the old version. Here the convergent channel is cast into a tapered, round shaped aluminium casting and coated with nylon (see picture 3.2). The Microflow chamber is 100 mm in diameter and the lead in section is 20 mm wide while the test section is 60 mm long with the narrowest part of test section being 5mm. The depth of the channel in this device is 1.75 mm. The cell culture dish forms the lid of the chamber (see picture 3.2) The whole assembly is clamped with a metal lid. The inlet and outlet of this version are also connected with a peristaltic pump and reservoir containing medium respectively (fig 3.5).

This version of Microflow chamber has been used throughout this work. But unfortunately in the second year of my work the company which supplied culture dishes suddenly stopped production of 100 mm dishes. Therefore it was necessary to spend considerable time redesigning the flow chamber around an available culture dish described below.

3.3.3. THIRD VERSION

Since 90 mm dishes were commercially available it was necessary to design the new version of Microflow chamber according to the size of these dishes. The geometry of this version is exactly the same as the previous versions although the length of the channel and of course the test section are smaller. In this chamber the convergent channel is designed around the base of 90 mm dish. The flow channel being machined out of aluminium alloy. In this Microflow chamber there is a 20 mm long lead in section as in the previous ones while test section is 47 mm long. The width of the convergence at the

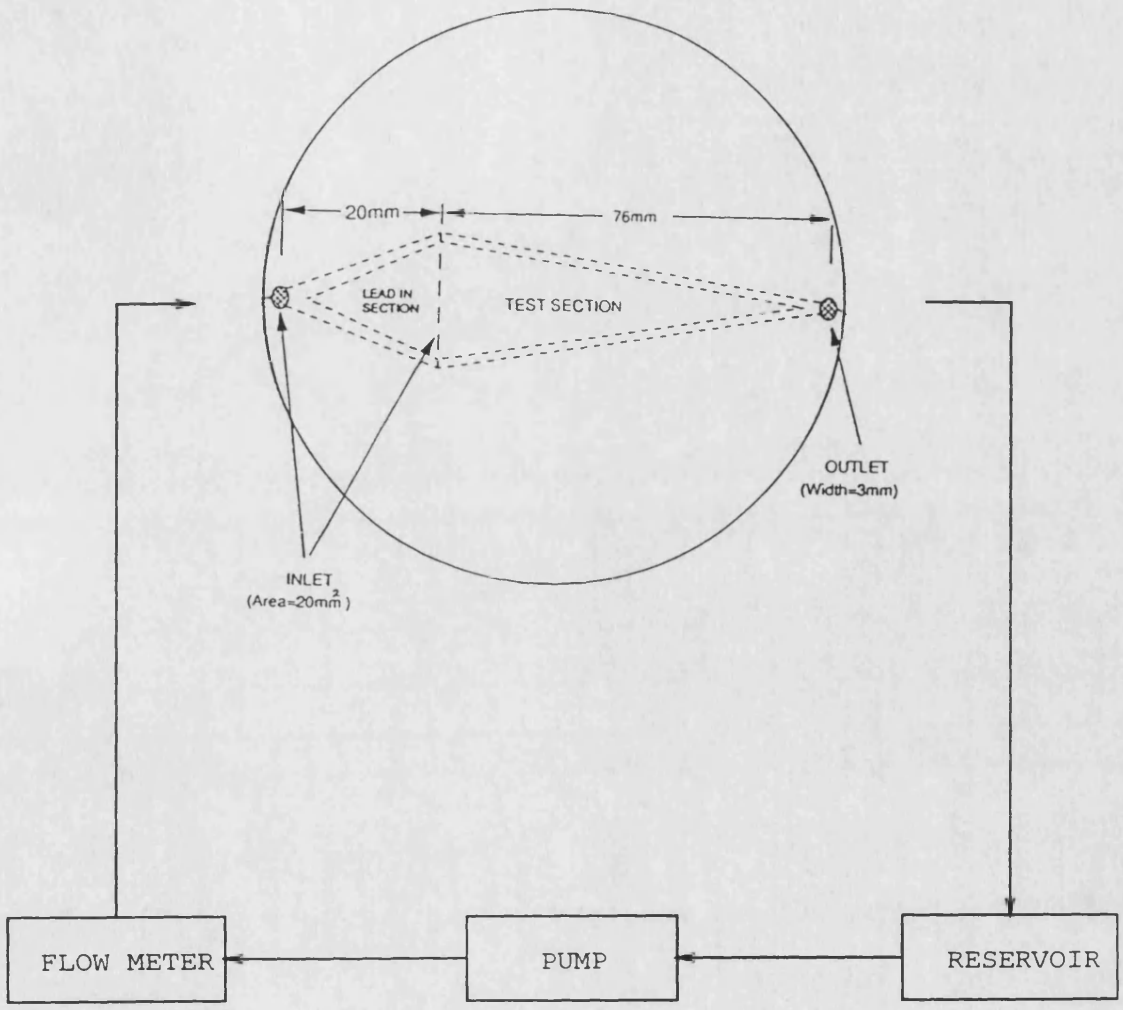
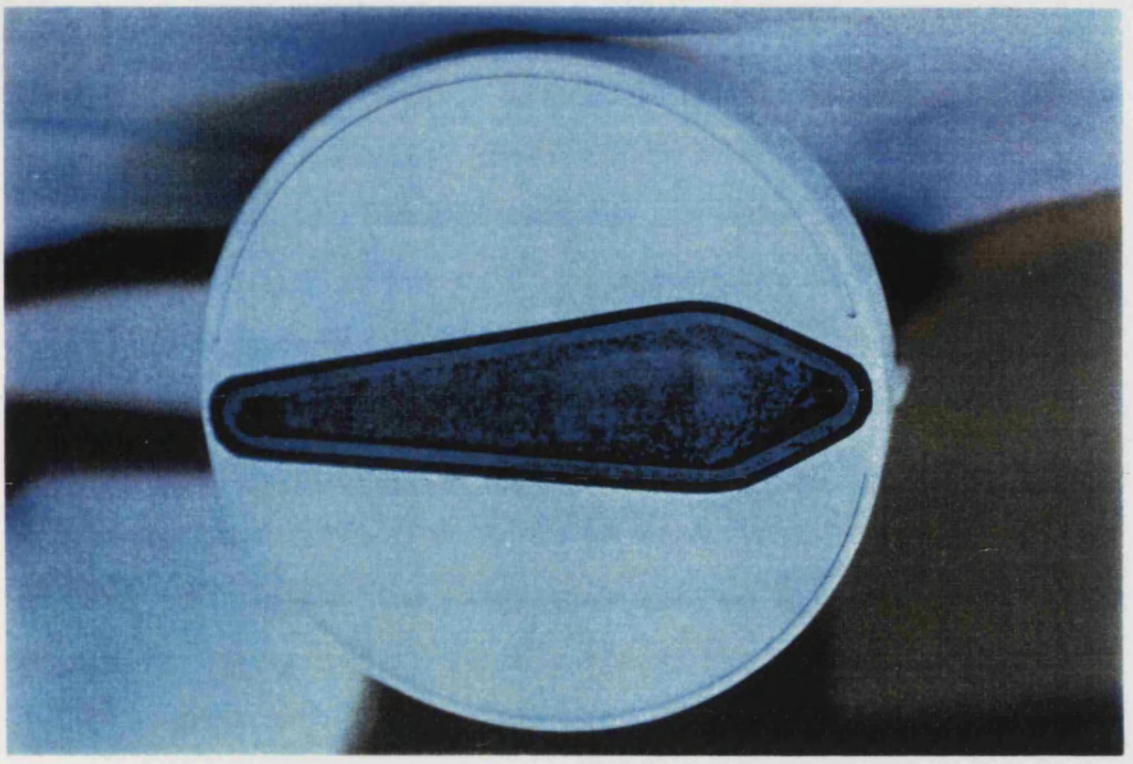
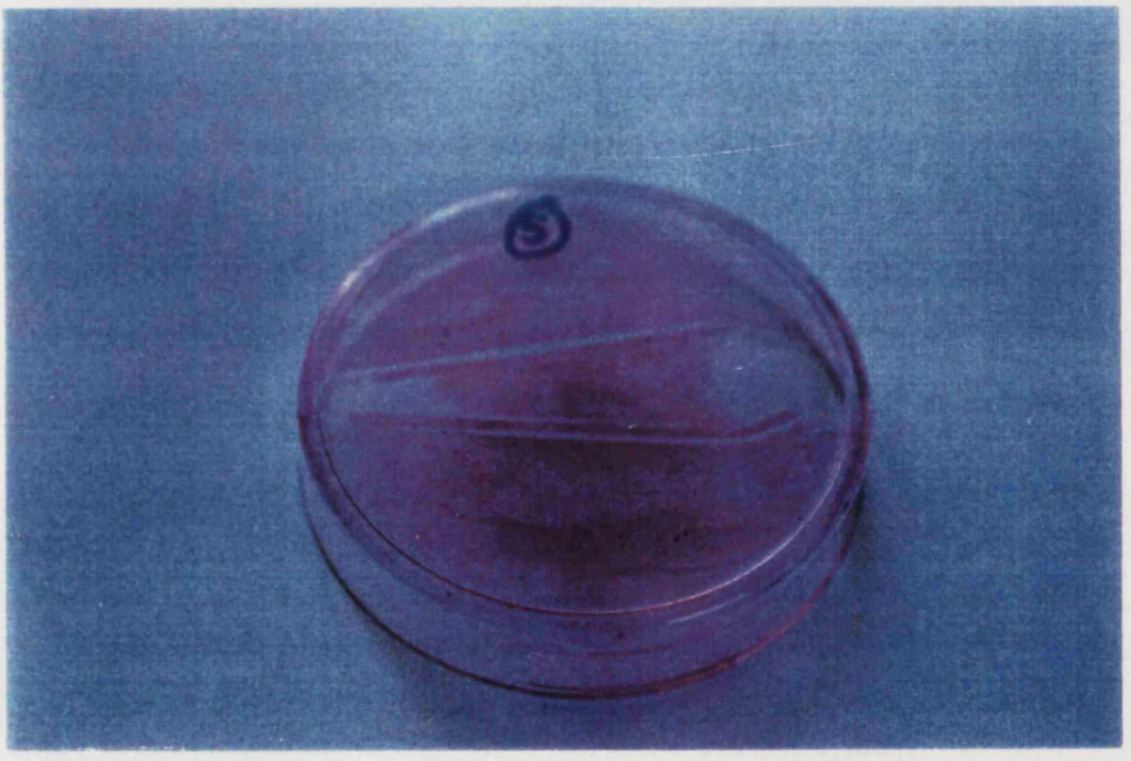


Figure 3.5: General Arrangement of Second Version Microflow Chamber and the Attached Apparatus.



Picture 3.2 a: Second Version Of the Microflow Chamber



Picture 3.2 b: Cell Adhesion Assay of The Cells Growing Petri Dish.

beginning of the test section is 20 mm tapering to 7 mm at the end of the convergence at the end of the test section which is in total 47 mm long. The depth of the channel is 1 mm. The cell culture dish forms the lid of the chamber. The dish and chamber was clamped tight. The general arrangement of the Microflow chamber is like in second version that is given in figure 3.5.

In the first three version of the Microflow chamber there is only one channel with the result that only one data is obtained for each dish. To gain better statistical results a fourth version of the Microflow chamber has been developed which will be described below.

3.3.4. FOURTH VERSION OF MICROFLOW CHAMBER.

In this chamber there are three channels and each of these channels are identical. Thus from one culture dish three determinations can be obtained. The geometry of each of these channels is the same as previously described (see also figure 3.6) ones and the lead in section is curved. The channels are machined from a perspex. The cells are grown in 100 mm square plastic dish which forms the lid of the chamber. The whole assembly is clamped with a metal lid. Like the other chambers the inlet of first channel is connected to a peristaltic pump which pumps the running medium, however the outlet is connected to the inlet of the second channel. Whose outlet of the second channel is connected to the inlet of the third channel. Finally the outlet of this channel is connected to the reservoir containing the medium. Therefore the three channels actually run in series running medium was cycled in thisthree channels device. The length of test section in each of these channel is 60mm (see picture 3.3).

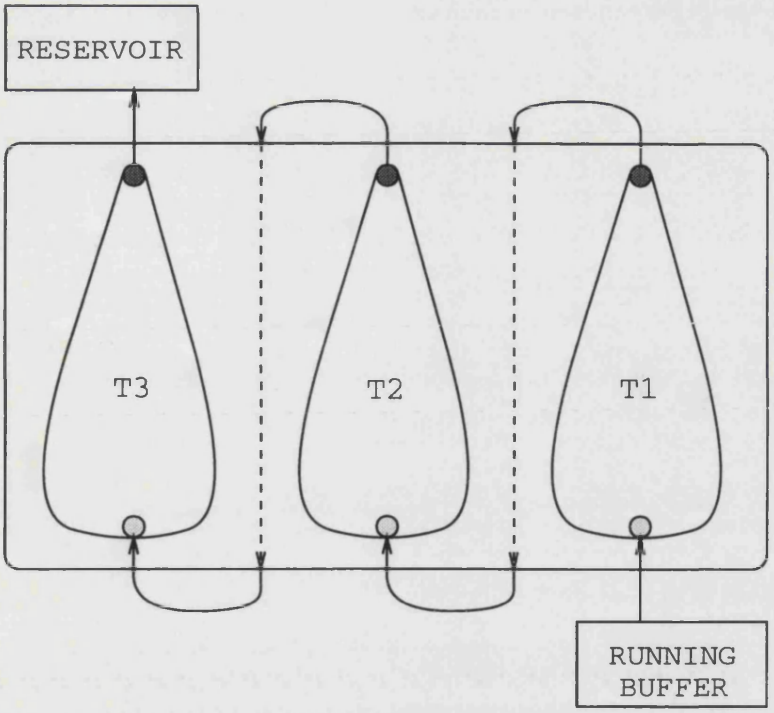
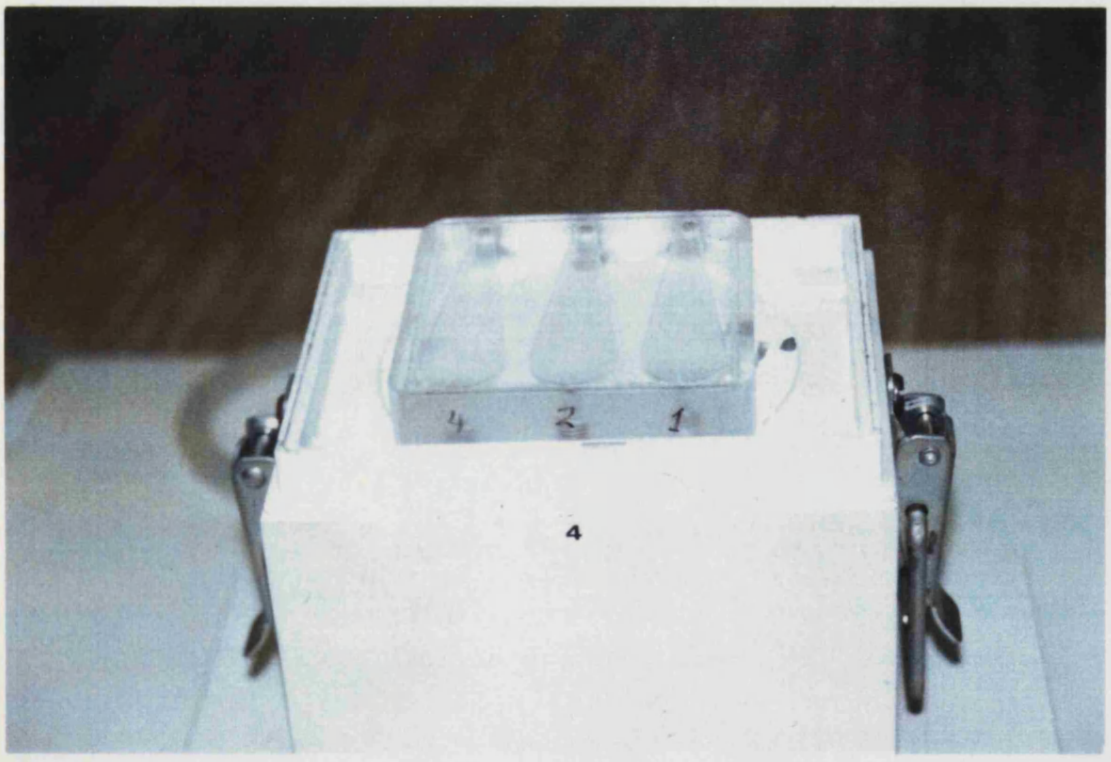


Figure 3.6: Simplified Illustration of Fourth (Three Channels) Version Microflow Chamber.

(○ - Inlet, ● - Outlet, T1 - Test Section 1, T2 - Test Section 2, T3 - Test Section 3).



Picture 3.3: Fourth Version Of Microflow Chamber

3.4. DETACHMENT ASSAY.

3.4.1. PREPARATION OF SAMPLES.

A Sub confluent monolayer of cells was trypsinized with 0.05% (v/v) trypsin in EDTA-PBS buffer and the action of this proteolytic enzyme was stopped by 1.5 ml's of serum (10% V/V) containing culture medium. The resulting cell suspension, at the concentration of $2x10^5$ cells/ml, was inoculated into complete growth medium. This cell suspension was poured into $144cm^2$ plastic dishes already containing five sterilised microscope slides or in the relevant tissue culture grade plastic dishes. The cells were allowed to grow for 24 hours in 5% (V/V) CO_2/air atmosphere at 37^oC. Finally, the adhesion strength of these cells were measured by inserting this cell growing substratum on the Microflow chamber and after passing the running medium over test substratum in a defined flow rates. The general procedure of detachment assay is outlined briefly in figure (3.7). After 10 minutes of running; the distance from the beginning of the test section to a point at which cells start to detach (critical distance) is measured by a ruler. The critical distance is a sharp boundary line and one side of this point cells remained attached while the other side all cells detached (see picture 3.2). By putting this magnitude of the measured critical distance in the relevant equation, for example for the first version of the Microflow chamber equation 3.10 or for the Third version of chamber in equation 3.11 (see later), the adhesion strength of the cell can be determined as Nm^{-2}.

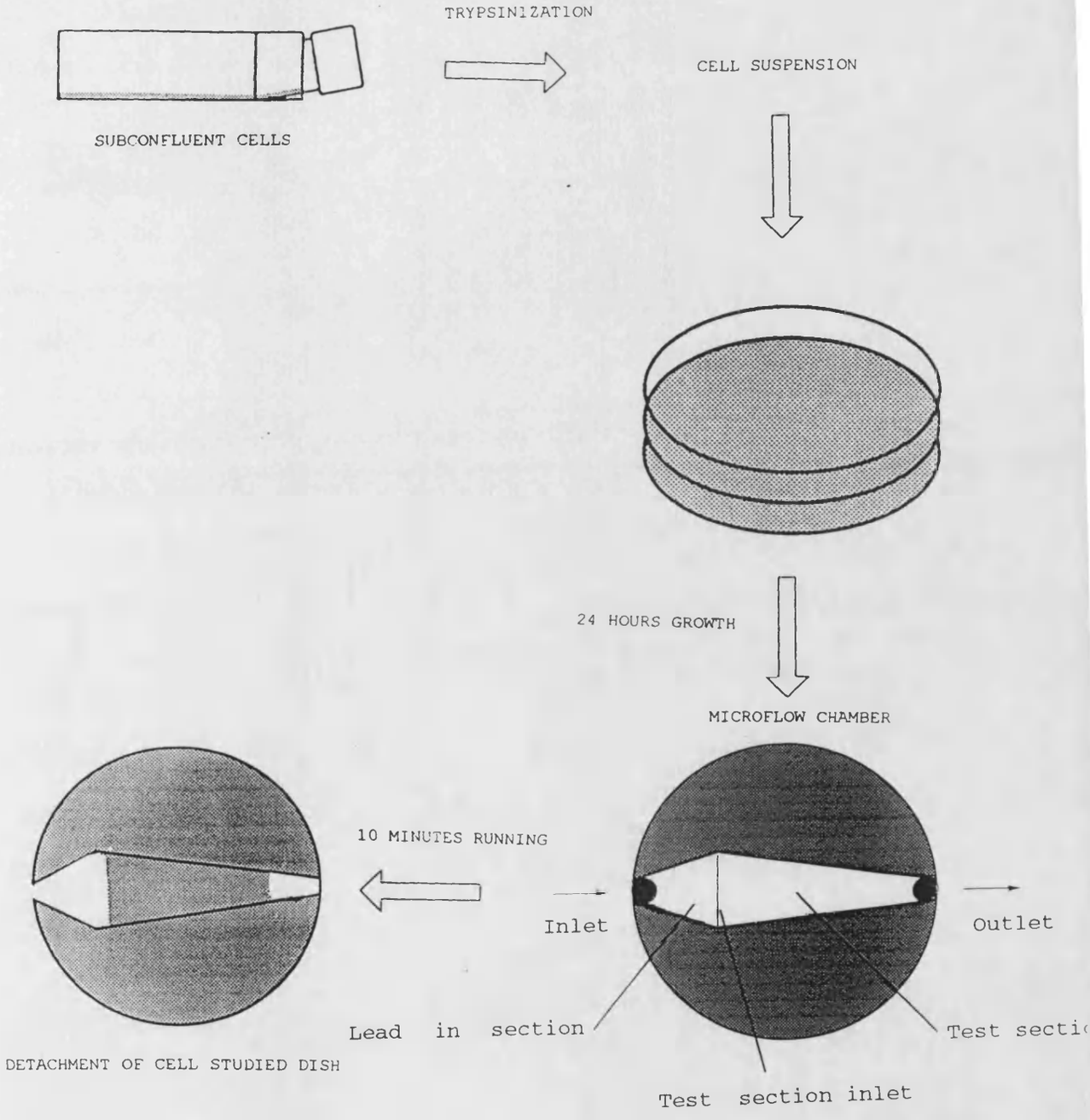


Figure 3.7: General Outline of The Cell Detachment Studies

3.4.2. RUNNING MEDIUM

MEM or RPMI 1640 growth medium was diluted in 20 mm HEPES buffer to give a final concentration of growth medium of 10% (v/v) in this buffer. The pH was adjusted to 7.4 with 0.1M NaOH.

3.4.3. PRINCIPLE AND EXPERIMENTAL PROCEDURE OF THE DETACHMENT ASSAY.

In the first version, the slide with the cells growing on it is inserted into the recess which is constituents the lower part of the chamber and in the rest of versions, the cell growing dish is assembled as the upper part of the chamber. After the assembly of the chamber the running medium from the reservoir is pumped through it at a predermined flow rate for 10 minutes. The flow rate can be controlled by simply varying the rotation per minute (RPM)of the peristaltic pump (figure 3.4,).

As soon as medium enters the chamber, the lead in section reduces the turbulence and stabilises the flow, it then enters the test section and accelerates as, it travels down the tapering width but constant depth, it also constantly relaminarises as any turbulence develops. The increasing fluid velocity results in an increase in the hydrodynamic shear stress along the cell growing surface (figure 3.10). At a certain critical point the surface shear stress becomes sufficiently large to cause the detachment of the cells. The critical distance from the inlet to this detachment boundary is used as a direct measure of the critical shear stress (c.s.s.) in term of Nm^{-2}. The flow rate was calculated by measuring the volume of liquid pumped per second at a specific speed of the peristaltic pump. The calculation will be given below.

3.4.4. CALCULATION OF THE CRITICAL SHEAR STRESS.

The distance from inlet to a point at which cells start to come off is described as the "*critical distance*" and the shear stress, which is applied by the fluid flow, at that point is referred to as the "*critical shear stress*". This shear stress in flow channel can be calculated by using following equation:

$$\tau = \mu x \frac{du}{dy} \qquad \textbf{(3.2)}$$ (Massey 1989; Coulson et al 1990)

In where:

τ= shear stress;

μ= viscosity of fluid;

du= the velocity of fluid

dy= the depth of the channel.

As mentioned earlier, serum free medium was used as the running buffer. Surprisingly the addition of 10% serum to the medium did not change the viscosity and there was no change in the viscosity of the running medium between 20°C and 37°C (WJD Whish personal communication) therefore in our calculations μ is accepted as 1.2 centi poise.

$$1Poise = 0.1\left(\frac{Newton.\sec}{m^2}\right)$$

As it has been described before, the depth of the Microflow chambers are different. i.e. the first, third and fourth version of chambers have 1 mm channel depth while it is 1.75 mm in the second version. However in here as an illustration we will give an example calculation for the first version of Microflow chamber in which depth is 1mm.

Dividing flow rate by the cross section area gives mean velocity (Massey 1989).

$$u=\frac{V}{A} \qquad (3.3)$$

In where;

u= mean velocity;

V= flow rate and

A= cross section area.

Crossection area in any point of channel is:

$$A= DxW \qquad (3.4)$$

in where;

D= depth of channel,

W= width of channel.

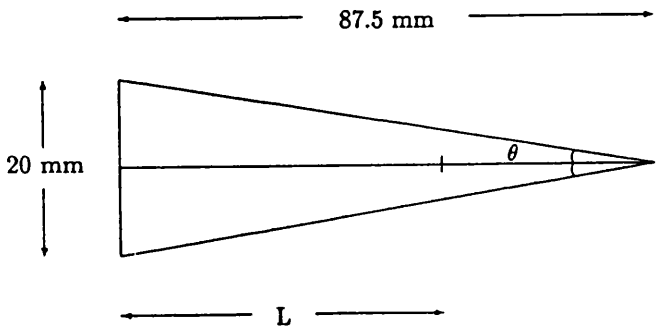


Fig 3.8: Schematic Representation of Convergence in The Channel of The First and Second Version The Microflow Chamber.

Since the depth is constant (1mm in the first version channel) it is only necessary to find out the width of channel at any point. The shape of the channel is a triangle i.e. the convergence reaches zero at a length of 87.5 mm (figure 3.8) in the first and second version of the Microflow chamber while in the third version convergence reaches zero at a 73 mm distance (see figure 3.9). Therefore, by

using basic geometrical calculations the width of the channel at any distance from inlet can be easily determined. The calculation of

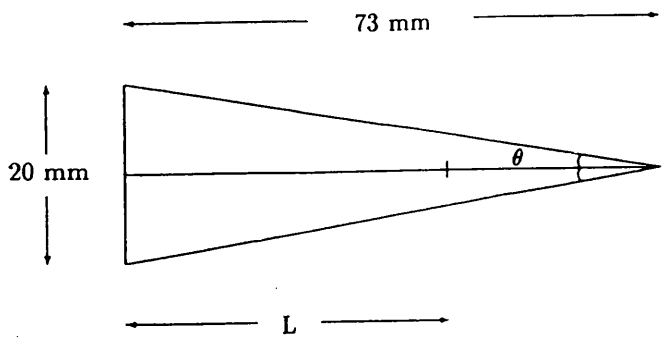


Fig 3.9: Schematic Representation of Convergence in The Channel of The Third Version The Microflow Chamber.

critical shear stress for the first version of Microflow chamber, as an example, is given below.

The tangent of the angle (θ)between the centre line and the wall is:

$$tg\theta = \frac{10}{87.5}$$ (see figure 3.8);

θ= 0.1143.

So that the overall angle of convergence is 2θ= 2(0.1143).

The width (W) of the channel at a distance, L, may be calculated by using the equation.

Width (W)= 2(0.1143) x (87.5-L) mm **(3.5).**

In here;

L= The distance from the inlet (mm).

When this W equivalent (equation 3.5) and

the value of the depth of channel (=D);

which is 1mm in first version of Microflow chamber

are inserted in equation 3.4 i.e:

A= DxW,

the cross section area (=A) at L distance would be:

$A= 0.2286(87.5-L)\ mm^2$ or

$A= 0.2286x10^{-6}(87.5-L)\ m^2$ **(3.6)**.

Now we can calculate the mean velocity. As given before mean velocity (=u);

$$u=\frac{V}{A} \qquad \textbf{(3.3)}$$

Since A is equal to $0.2286x10^{-6}(87.5-L)\ m^2$,

and flow rate (=V)

will be measured as $cm^3\ sec^{-1}$

u therefore would be;

$$u=\frac{4.37V}{(87.5-L)}\left(\frac{meter}{second}\right) \qquad \textbf{(3.7)}.$$

However since the shear stress at the centre of the channel is calculated at where velocity is maximum and

$u_{max}= u_{mean}x1.5$ **(3.8)** (Coulson et al 1990)

then at this point

$$u_{max}=\frac{6.56V}{(87.5-L)}\left(\frac{meter}{second}\right) \qquad \textbf{(3.9)}$$

The shear stress was given as $\tau=\mu x\frac{du}{dy}$ **(3.2)**

In here, $\mu= 1.2x10^{-3}\ Nsm^{-2}$;

$du=\frac{6.56V}{(87.5-L)}\left(\frac{meter}{second}\right)$, and

$dy=0.5x10^{-3}m$ then

The general formula for the shear stress calculation in the first version Microflow chamber can be obtained as

$$\tau=\frac{15.74V}{87.5-L}\left(\frac{N}{m^2}\right) \qquad (3.10)$$

A similar general formula for the third version of Microflow chamber can be obtained as;

$$\tau=\frac{13.15V}{73-L}\left(\frac{N}{m^2}\right) \qquad (3.11)$$

By using these equations, the critical shear stress can be calculated. When related values i.e.:

V= volumetric flow rate (cm^3s^{-1}) and

critical distance L= (mm) are inserted,

the shear stress (=τ)will be obtained as Nm^{-2}.

As an illustration the calculation of critical shear stress in the case of a flow rate of 1000 cm^3/min at different critical distances i.e. 30 mm, 40 mm, 50 mm, 55 mm, and 60 mm is given for the first version of Microflow chamber below.

In here the flow rate should be changed into cm^3s^{-1}

therefore V=1000/60=16.67 cm^3s^{-1}.

For example at a critical distance of 30 mm, L_1 ;

$\tau=\frac{15.74V}{87.5-L}\left(\frac{N}{m^2}\right)$ then

$$\tau = \frac{15.74x16.67}{(87.5-30)}$$ and

τ=4.56 Nm^{-2}.

In similar way the critical shear stress has been calculated for the other distances (see table 3.1).

Point	Critical Distance (mm)	Critical Shear Stress (Nm^{-2})
L_1	30	4.56
L_2	40	5.52
L_3	50	6.99
L_4	55	8.07
L_5	60	9.54

Table 3.1: The critical shear stress of detachment at different detachment points, at a constant flow rate of 1000 cm^3 $minute^{-1}$.

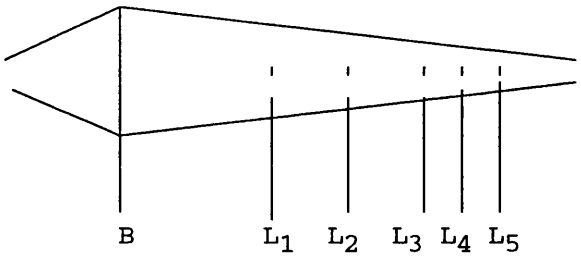


Figure 3.10: A Diagram to Show the Critical Shear Stress of Detachment at Different Points Along the Surface of a Test Substratum, at a 1000 $cm^3 minute^{-1}$ constant flow rate (B= begining of test section, for L values see table 3.1)

During the development of the Microflow chamber, reproducibility of the device was analysed. To do this the adhesion strength of

different mammalian cell lines has been measured by using the Microflow chamber, this is given below.

3.5. THE MEASUREMENT OF THE ADHESION STRENGTH OF DIFFERENT MAMMALIAN CELL LINES ON PLASTIC SUBSTRATUM.

To determine if the measuring of adhesion strength by Microflow chamber is reproducible, it was of initial interest to find out whether or not various cultured mammalian cell lines show similar or different critical shear stress (c.s.s) of detachment. Therefore different cell lines i.e. HeLa B, CHL, BHK-21, L929, and MDCK cells were grown under the conditions as illustrated in materials and methods. After 24 hours of growth, the adhesion strength of these cells on tissue culture grade polystyrene dishes were measured as described in materials and methods.

It was of interest to notice that each of these cells has a specific magnitude of the adhesion strength. That is the c.s.s. of detachment of L929 cells was 25.08±2.30 Nm^{-2} while it was 21.50±1.90 Nm^{-2} for MDCK cells. Adhesion strength of CHL cells and BHK 21 cells were much more lower than the above cells i.e. the c.s.s were 11.11±1.60 Nm^{-2} and 3.64±0.56 Nm^{-2} respectively (see figure 3.11).

The greater the critical shear stress, the tighter the cells are attached. It was encouraging that a high degree of reproducibility within a particular cell line was observed. That is, when any cell line was removed from liquid nitrogen storage and grown for 24 hours the measured c.s.s of detachment was always within the standard deviation. Moreover, the adhesion strength of a cell line measured after 1 month, 3 months or six months of removing cells from liquid nitrogen was also within the standard deviation. This shows that by

using the Microflow chamber, the adhesion strength of any monolayer cells can be measured with a high reproducibility.

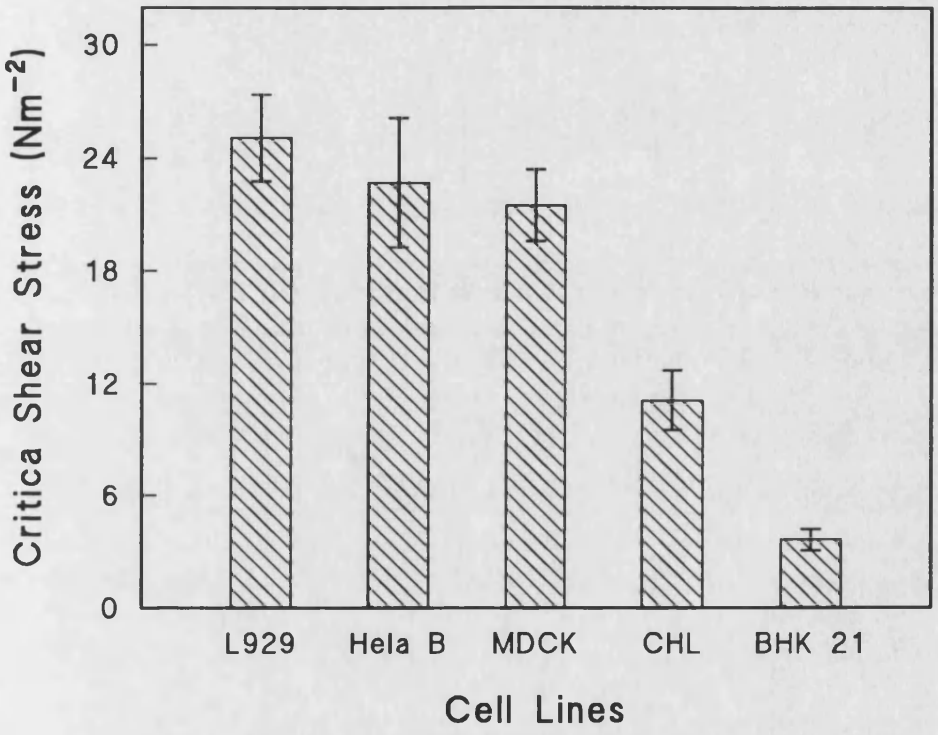


Figure 3.11 : Comparative Adhesion Strength of Different Cell Lines on the Plastic Substratum.

L929, HeLa B, MDCK, CHL and BHK 21 cells were grown on tissue culture grade plastic dishes (90 mm) as outlined in materials and methods. The adhesion strength of these cells in terms of the critical shear stress (c.s.s) of detachment was measured by using the Microflow chamber. Each data point is mean of 5 experiments each of which contains 20 measurements of c.s.s. of detachment. The error bars indicate the standard error of that mean. Further details may be found in the text.

3.6. DISCUSSION

Assays to measure the strength of cell adhesion to surfaces are vital if we are to understand the way in which cell interact with surfaces or with each other. As mentioned earlier in chapter 1, the previously published cell adhesion measuring techniques have been limited, complicated and often have remained qualitative. Some quantitative studies have been proposed but they all suffer from the need for complex equipment (Horbett et al 1988; Bowers et al 1989). Moreover, the lack of reproducibility of previous techniques render them of very limited use (McClay et al 1981; Owens et.al 1987; Lotz et.al 1989). Thus before we look at the mechanism of cell adhesion we had to develop a simple and reproducible quantitative method for the measurement of cell adhesion. In the present work these requirements have been met where a simple but reproducible technique for the measurement cell adhesion has been developed. The heart of this technique is a specially designed Microflow chamber which is the first of its kind in which a complete hydrodynamic laminar flow is achieved through a convergent channel. Before discussing the other methods which were used for cell adhesion measurement it is appropriate to discuss the mechanism of cell detachment by shear stress.

The exact mechanism of cell detachment is not known. However it has been reported (Van Kooten et al 1991 and 1992) that cells round up before detaching. This suggestion has been based on the scanning electron microscopic observation of cells during their experience of laminar flow. A model can be proposed for cellular detachment, in which spread cells first return to a round shape by retracting cytoplasm and leaving a network of membrane-enclosed tube like

structures which are still attached to the substratum. Subsequently, the contact sites may dissociate resulting in detachment (Van Kooten et al 1991). However, in the present study, there was no evidence for any change in cell morphology during the detachment period of ten minutes. No rounded-up cells were observed anywhere on the slide whether the shear was high or low. It is most likely that the force pushing against the front edge of the cell simply peels it from the surface (see figure 3.12).

Nevertheless to explain the cell detachment process due to shear stress two models have been proposed. Those are a uniform stress model (Hammer and Lauffenburger 1987) and a peeling model (Evans 1985). In the uniform stress model, the cell-substratum contact area is treated as totally rigid structure in which applied stresses are equally distributed in the focal contact simultaneously. Detachment is initiated when a single bond breaks, leading to fracture of the remaining receptor-ligand bonds. In this model cells detach when all the bonds break simultaneously (Hammer and Lauffenburger 1987; Lotz et al 1989; Ward and Hammer 1993).

In the peeling model it has been assumed that in the contact zone molecular bonds are stretched (Evans 1985). According to this model cell detachment occurs through a progressive breaking of bonds at the cell periphery and bonds do not break simultaneously. Only those bonds in a thin boundary layer at perimeter of the cell substrate contact area resist the detachment force. The cell membranes curves away from the substrate at the edge of the contact area, and only those bonds within this region are stressed vertically. When the force on the outer most bonds exceeds the maximum strength of the bond, the bond breaks and the boundary layer moves inward. The translation of the boundary layer results in peeling of the cell from

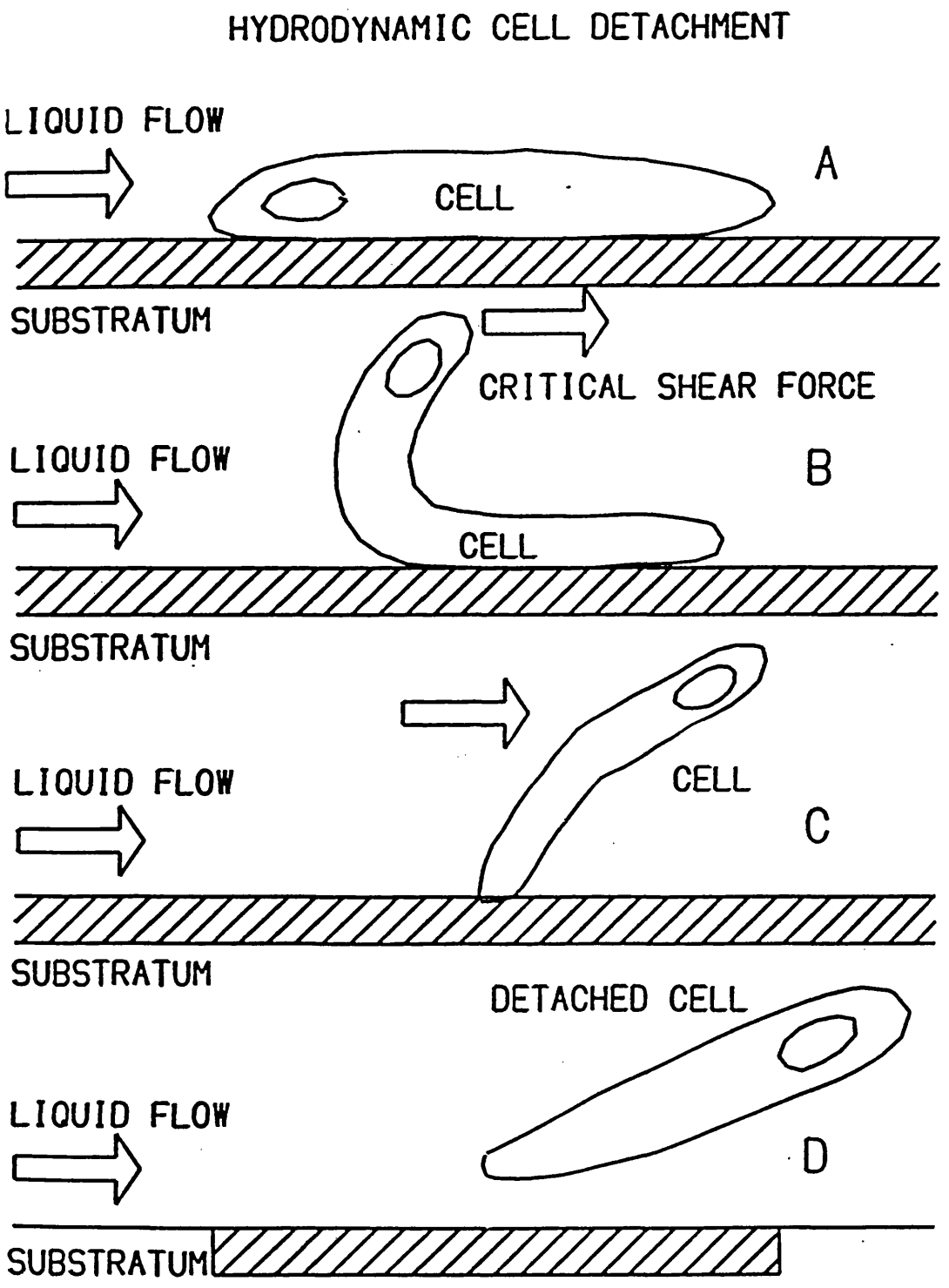


Figure 3.12. Schematic Representation of the Hydrodynamic Cell Detachment.

the surface (Evans 1985; Ward and Hammer 1993). However, these are speculative thoughts and the exact mechanism of cell detachment due to detachment forces is not yet known. What is absolutely clear is that a cell detaches from the surface when an applied detachment force exceeds the strength of the cell-substratum linkage.

Now perhaps it is worthwhile to outline the deficiencies of previously developed methods and to explain the advantage of Microflow chamber for the measurement of cell adhesion strength.

As previously, briefly, indicated some of these methods were unable to detach cells from the surface due to limited technical ability. For example, McClay at al (1981) have used a centrifugation method to study the adhesion of sheep erythrocytes and neural retina cells, however they have measured cell detachment at $4^{o}C$. These researchers were unable to detach cells from the surface. Because the maximum obtainable relative centrifugal force was 2000xg and this was not enough to dislodge cells from the surface (McClay et al 1981). Lotz et al (1989) have measured the adhesion of NIL cell fibroblast, glioma cell line and astrogloma cell line. But like McClay et al (1981) these authors have failed to measure NIL fibroblasts cells adhesion at $37^{o}C$ due to being unable to generate enough detachment force by centrifugation (Lotz et al 1989) It is not only the centrifugation methods which are deficient in measurement of cell adhesion strength. Owens et al (1987) used a rectangular parallel plates device (see figure in chapter 1) to measure detachment of Dictyostelium discodium amoeba, and E. Coli, and red cells from a glass substratum. In this device the maximum obtainable shear stress was 5.9 Nm^{-2} and it was capable off measuring the adhesion strength of E coli and red cells while the maximum obtainable detachment force was not sufficient to detach Dictyostelium discodium amoeba (Owens et

al 1987). That is to say the strength of the cell adhesion was so large that it exceeds this techniques ability to measure it. In another words the force required to separate the cells from the substratum exceeds the maximum obtainable detachment force. Considering this situation, any method developed for quantitative measurement of adhesion must produce a range of shear forces that are likely to be encountered in cells. That is; if cells are weakly attached, low shears will be needed whereas for tightly attached cells very high shear stresses will be needed. In the device described here these requirements are fulfilled by having the accelerated flow, described earlier and a pump which can produce both low and high volumetric flow rates. Thus the final strength of adhesion of any mammalian cell line on any surface can be assessed easily and accurately with the help of this Microflow chamber. In fact in this work different cells under various conditions have been measured. That is adhesion strengths which ranged from 2 Nm^{-2} to 30 Nm^{-2} has been measured (see figure 3.11).

Some of previous methods not only have a limited ability but also suffer from a need of complexity as well. For example, as described in chapter 1, Bowers et al (1989) have developed a micro mechanical technique which was very complex, although it was not suitable for round cells or small cells such as blood cells since the micro pipettes used were not suitable for small cells. Truskey and Pirone (1990) have developed a method by which they were able to obtain a range of shear stress from 5 to 14 Nm^{-2}. However it is time consuming since even 2 hours of fluid flow was applied. In present method detachment assay has been carried in 10 minutes and also by using fourth version, three channels Microflow chamber, it is possible to obtain three determinations for each run. Like Truskey

and Pirone (1990), Van Kooten (1991) et al have developed a parallel plate flow chamber. However in both of these methods a rectangular flow chamber has been used. In this type of flow chamber there is no fluid flow acceleration and of course there is no shear stress gradient over the channel and therefore over the test substratum. Therefore these researchers have expressed their results as a percentage of attached cells remaining after experiencing a defined shear force. While in the present system there is convergece channel. So that there is a shear stress gradient in the test section increasing from inlet to outlet. Therefore we are able to measure the critical cell distance at which cells start to come off. This convergent Microflow chamber, is the first of its type. However, recently it has been reported (Usami et al 1993) that a divergent parallel plate has been developed. In this chamber a linear variation of shear stress, starting from a predetermined maximum value at the entrance and falling to zero at the exit could be obtained. It is an advantage to have a varied shear stress along the test surface. Nevertheless, in a divergent channel there is a disadvantage, which is that the shear stress is maximum at the entrance. So cells will detach from this section and then could start to simply peel cells off as a sheet past the point where cells would be expected to remain attached. Hence the rolling of this detached cell sheet could disturb attached cells and of course this will affect the detachment assay. In the present convergent Microflow chamber there is no possibility of the disturbance of attached cells by peeling as a sheet, because beyond the critical distance cells will come off automatically since the shear stress increases from inlet to outlet.

It would be fair to say that the present work could not have been carried out with any existing hydrodynamic system. It would not

be immodest to suggest that this quantitative system outperforms anything published to date. In the following chapters, the reproducibility, accuracy and validity of this chamber will be discussed in detail and the results obtained used to understand the underlying mechanism of cell adhesion and its strength.

CHAPTER 4 THE ROLE OF ENERGY IN CHL CELL ADHESION.

4.1. INTRODUCTION

Cell substrate adhesion is a multistep process including initial cell contact to the substratum, attachment, spreading, and growth (Grinnel 1978; Lauffenburger and Delis 1983; Schakenraad and Busscher 1989). Despite extensive research concerning the adhesion of various cells to different types of substratum, the molecular requirements and mechanisms by which cells adhere to surfaces has not been elucidated. One of the approaches to evaluate the mechanism of cell adhesion is to find out the role of energy metabolism in cell adhesion (Grinnel 1974; Ueda et al 1976; Bereiter-Hahn et al 1990). Therefore the main question is whether cell adhesion is an active or a passive phenomenon. Passive adhesion does not depend on the cell being metabolically active i.e. it is not an energy requiring process and there is no subsequent step in which molecular reorganisation and spreading takes place. In active adhesion, a cell needs to be metabolically active and this process is followed by a second spreading step. In an effort to answer such questions, cell adhesion has been measured at different temperatures and in the presence of different metabolic inhibitors such as KCN, Na azide, and oligomycin (Klebe 1975; Juliano and Gagalang 1977; Nath and Srere 1977).

However, there are still some contradictions in these reports concerning the role of temperature in cell adhesion. Moreover most of these reports also only deal with cell attachment not cell adhesion strength. Cell adhesion strength being defined as the ability of cells to withstand a given hydrodynamic force (see chapter 3). In

other words cell adhesion strength is a measurement of the actual binding affinity between a cell and the surface on which it grows. In this chapter, the role of various factors including temperature and oxygen, cell-surface binding will be discussed, including an investigation of the role of serum and trypsinization. Finally the effect of temperature on CHL cell detachment will be discussed.

4.2. RESULTS AND DISCUSSION

4.2.1.TEMPERATURE EFFECT ON CHINESE HAMSTER LUNG CELL ATTACHMENT

Bacteria adhere to surfaces by a straightforward charge interaction between the surface and the cells (Ofek et al 1977; London,J 1991). Metabolism is not required for their adhesion. That is, total bacterial adhesion should be, to a large extent independent of temperature. That is the rate of attachment is different but the final degree of adhesion is the same at any temperature. Bacterial adhesion is somewhat analogous to, say, antibody-protein antigen binding where the rate of binding is temperature sensitive but the total binding is not. Of course the rate of bacterial attachment to a surface or the binding rate of an antibody to an antigen is affected by temperature, but systems will, given sufficient time, bind to the maximal amount. Thus the rate of charge-charge interactions or receptor mediated interactions are dependent on temperature, but total overall binding is not temperature dependent. It was with this in mind that these experiments with CHL cells were set up. The question being; " is the initial attachment of animal cells (not the subsequent spreading) receptor mediated"? That is, the rate of attachment will be temperature dependent, but given enough time 100% of the cells will stick?

To define the role of temperature in CHL adhesion, the attachment kinetics of this cell was studied between 4°C and 37°C and related to the strength of adhesion. The overall procedure is outlined in the material and methods (see 2.2). Briefly, before cell sub culturing and incubation, all buffers and media were brought to the temperature at which the related attachment study was performed. All unattached cells were removed with gentle washing and any cell not removed by a series of these washes was considered to be attached. The number of attached and non attached cells were counted in a haemocytometer (see materials and methods 2.3). Since the rate of attachment was temperature dependent the attachment studies have to be performed at different time intervals. This is because at one time point although attachment may be completed for one temperature, the attachment might only just have started at a lower temperature. For instance, at 15°C there is only 6%±2% attachment after 1 hour of incubation while it was almost complete (93%±3%) during the same period at 37°C (see figure 4.1.a and 4.1.c). Therefore for each temperature analysis the time scale has to be arranged over several trials. Results are expressed as the percentage of attached cells. Each data is the mean of 4 experiments each of which contains 2 measurements. The amount of attachment was, as expected, dependent on temperature i.e as the temperature increased so the amounts of attached cells increased. There was no attachment at 4°C and 9°C after 6 hours of incubation, and even after prolonged incubation for 24 and 48 hours there was only 2%±1% attachment (see figure 4.1.c). This result is itself suprising. As mentioned earlier the rate of a receptor-ligand binding interaction is temperature dependent but given sufficient time at low temperature ; the binding will proceed to near completion. This happens in antibody-antigen binding. These

findings therefore suggests that cell metabolism is required for cell

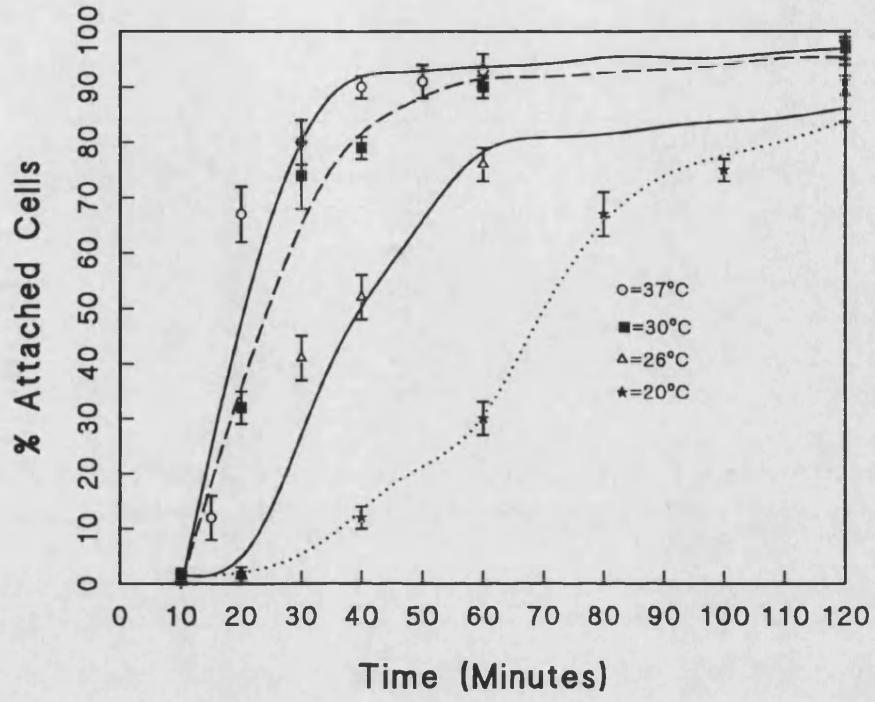


Figure 4.1.a. The Effect of Temperature on The Attachment of CHL cells.

Attachment studies were performed on 35 mm dishes. 2 ml of cell suspension contains $1x10^6$ cells were added into these dishes. After incubations for indicated period, the number of attached and non attached cells were determined as described in materials methods. Each data point is the meaning of 4 different experiments in each of which 2 determinations were made. Error bars indicate the standard errors of that mean. Further information can be found in text.

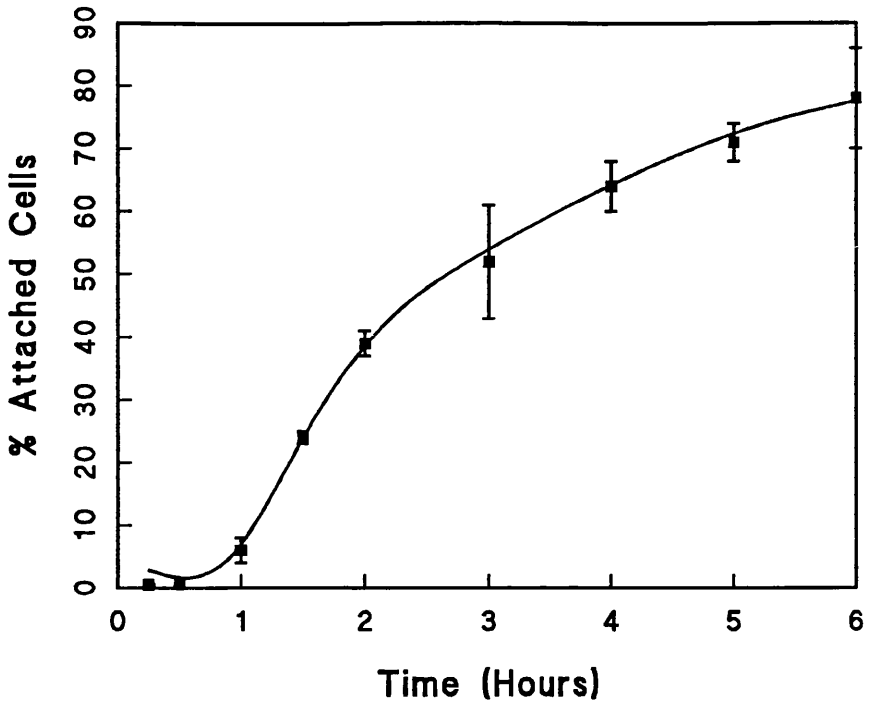


Figure 4.1.b: The Attachment of CHL Cells At 15°C.

Attachment studies were performed on 35 mm tissue culture grade plastic dishes. 2 ml of cell suspension contains $1x10^6$ cells were added into these dishes, after incubation at 15°C for a defined time period the number of attached and non attached cells were determined as described in materials and methods. Each data point represents the mean of 4 experiments in each of which 2 determinations were made. Error bars indicate the standard errors of that mean. Further details may be found in text.

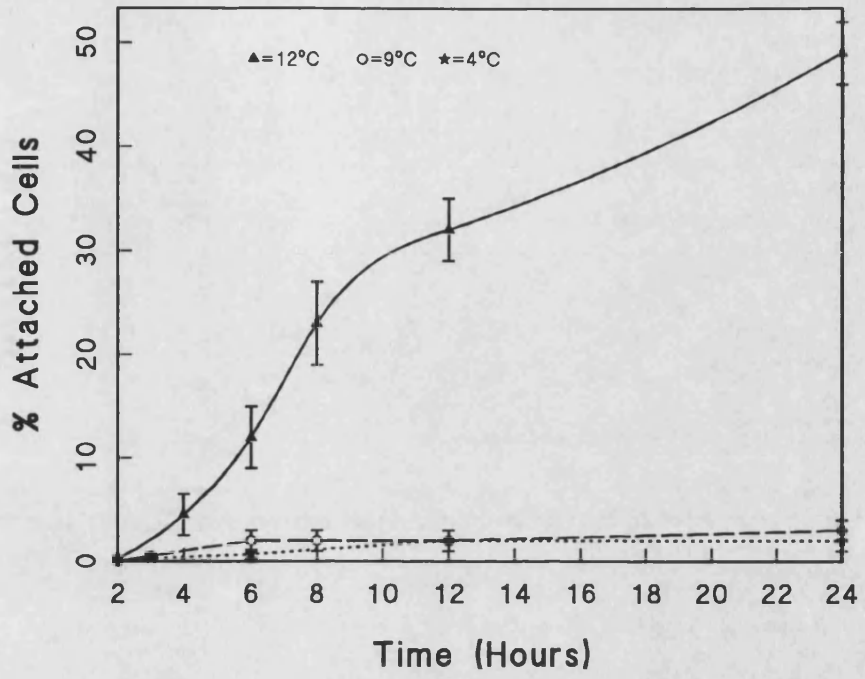


Figure 4.1.C. The Effect of Temperature on the Attachment of CHL Cells.

Attachment studies were performed on 35 mm tissue culture grade plastic dishes. 2 ml of cell suspension contains $1x10^6$ cells was added into this dishes. After incubation at defined temperature and for a indicated period, the number of attached and non attached cells were determined as described in materials and methods. Each data point represents the mean of 4 different experiments in each of which 2 determinations were made. Error bars indicate the standard errors of that mean.

adhesion at a very early stage. However, when the cells were transferred to 37°C, after 24 hours incubation at 4°C, they attached normally (94%±3%). Nevertheless, if cells were transferred down to 4°C, after being incubated at 37°C for 24 hours, they remanied attached even longer than if they were incubated at 37°C i.e. when CHL cells were cultured in a concentration of $2x10^5$ cells/ml they

start to come off after about 7 days of incubation if they were kept at 37°C, while in the identical conditions except but at 4°C cells remained attached for 10 days. This result suggest that although initial cell attachment is temperature dependent, once cells attached and spread they were not effected by low temperature. However, between 12°C and 37°C the rate of attachment was in fact temperature dependent. In all the attachment curves, except that at 4°C and 9°C, there is a lag phase in which there is about 5% cell attachment followed by a sudden rising phase where the number of attached cells increases dramatically (see fig 4.1.a,b,c). The length of the lag phase increased as the temperature decreased. For example at 12°C the duration of the lag period is 4 hours (4.5%±2% attachment) whereas it lasted for only 1 hour (6%±2) at 15°C, and after 12 hours of incubation there are 32%±3 and 78%±2% attachment at 12°C and 15°C respectively. However the length of the lag phase was only 30 minutes (4%±1%) and 20 minutes (2%±0.5%) at 20°C and 26°C, respectively. After 1 hour of incubation the amount of cell attachment reached to 30%±3% and 76%±3%. Nevertheless the lag phase was only 10 min at 30°C (2%±0.3% attachment) and 37°C (2%±1% attachment) and within 1 hour the percentage of attached cells were 90%±2% and 93%±3% at 30°C and 37°C respectively. Therefore at all temperatures there is this lag phase in which a very small number of cells attach to the substratum. The duration of this phase is, as mentioned earlier, temperature dependent. At 4°C and 9°C the lag phase is infinite. i.e. there was no cell attachment even after 48 hours. The question is why; this lag phase take place? One possible answer might be the settlement time, another words this lag phase difference could be due to different vicosities of the medium at different temperatures. This possibility will be discussed bellow.

4.2.1.1. THE ROLE OF VISCOSITY ON INITIAL CELL ATTACHMENT.

It is possible that this lag period is the required time for cells to reach the substratum. Since experiments were conducted in ifferent temperatures, the duration of lag period increased as the viscosity of

Temperature (°C)	Viscosity ($x10^{-3}$ Nsm^{-2})	Density of the cell (10^3 kg m^{-3})	Time required a cell to reach surface of the dish (minutes)
4	1.56	1.05 1.10 1.15	19.77 9.88 6.59
9	1.34	1.05 1.10 1.15	16.98 8.49 5.66
12	1.23	1.05 1.10 1.15	15.58 7.99 5.20
15	1.14	1.05 1.10 1.15	14.45 7.22 4.82
20	1.0	1.05 1.10 1.15	12.67 6.33 4.22
26	0.87	1.05 1.10 1.15	11.02 5.51 3.67
30	0.80	1.05 1.10 1.15	10.14 5.07 3.37
37	0.69	1.05 1.10 1.15	8.74 4.37 2.90

Table. 4.1. The Effect of Temperature on The Settling Time of Cells. Cells were assumed as 10 µm diameter spherical. The distance between the top of the medium to surface of dish is calculated as 2.07 mm distance. Time required for settling time was calculated by assuming that a 10 µm diameter spherical material will start to fall in onto surface of the dish. Since the density of the cell is not known calculations were made according to three different densities. The viscosity and the density of growth medium were assumed equal to water.

of the medium increased with decreasing temperature. However from general physics principles, the settling time of cells in different viscosity can be calculated. In all experiments, 2 ml of a cell

suspension was added to a 35 mm tissue culture grade polystyrene petri dish (see materials and methods 2.2.1). The distance between the surface of liquid and the substratum would be 2.07 mm. If one assumes a cell as a 10 μm diameter spherical body which will fall in from a 2.07 mm distance, in fact some of these cells would be only half distance from surface of dish and require less time than from table values to settle, the effect of viscosity on the settling time of cells could be calculated (see table 4.1). In this calculation it was assumed that the viscosity and density of growth medium is equal to water. Since density of cell is not known, calculations were made for three different densities. As it can bee seen from table 4.1. indeed the settling time of cell is effected by the viscosity. That is the the time required a cell to reach the surface increase as viscosity of medium increase. However, it appears that this settling time is not entirely depend on the viscosity of the medium in which cells settle. For example, the lag phase of cells is about 10 minutes at 37^{o}C (see figure 4.1.a), while if the density of cell is accepted as $1.05x10^{3}$ kgm^{-3} the settling time is 8.74 minutes and it is 4.37 minutes if CHL cell have a density of $1.10x10^{3}$ kgm^{-3} (see table 4.1). One might argue that these are close values and therefore it might be the case. Nevertheless, when the settling times of theoretical values compared with lag periods of experimental magnitudes for lower temperature it can be seen that it is difficult to draw a direct relationship between the viscosity of medium and lag period in cell attachment kinetics. For example, the theoretical settling time of cells at 12^{o}C and 15^{o}C, in the case of CHL cells density is $1.05x10^{3}$ kgm^{-3}, 15.58 minutes and 12.67 minutes respectively (see table 4.1). Whereas, the lag periods were 4 hours and 1 hours respectively (see figures 4.1.b and 4.1.c). In addition, the lag periods of attachment

graphics for 4°C and 9°C are greater than 48 hours although theoretical settling times were less than 20 minutes. Hence it seems that this lag period is not entirely because of the time needed for cells to settle to the surface. Therefore it seems fair to suggest that although viscosity might influence the duration of the lag phase it is not the major factor. One of the reasons for this lag phase could be (discussed previously in the introduction chapter 1.2) that the cells attach to the substratum after a brief initial contact with its surface molecules e.g. adhesion receptors. The cell might need the reorientation and reorganisation of these molecules to build adhesion structures, which is an energy requiring process. Since this might not take place below 9°C cell attachment did not occur below this temperature. At 12°C and above, the cells attached to the substratum and the amount of attachment is both temperature and time dependent. This point will be discussed widely later in the Membrane fluidity and cell adhesion section.

It is clear from these initial experiments with CHL cells that both the rate of attachment and final amount of attachment are temperature sensitive. This was a suprise. The result indicates that even the brief initial cell-substrate interaction is metabolically driven. However it must be said there are some contradictory reports in the literature. Some of these reports (Moscona 1961; Nath and Srere 1977; Klebe 1975; McClay et al 1981;) point out that cell attachment is a metabolically active process while the others suggest that initial cell attachment is independent of metabolic energy (Bereiter-Hahn etal 1990). Klebe (1975) measured attachment of CHO cells to collagen as the substratum. He found that there was only 9 percent attachment at 4°C after 1.5 hours of incubation. However

since he did not carry out his study beyond this period, it is an inconclusive finding for my work. The present study shows that cells need more time to attach to the substratum at low temperatures. But below 9°C there is no attachment. For example, as mentioned above, although 90%±4% of CHL cells were attaching within 40 min of incubation at 37°C, 1.5 hours of incubation was necessary to get 91%±4% attachment at 20°C. The attachment of BHK cells was studied by Nath and Srere (1977). However they have carried out their assay by centrifugation in which cells are forced onto the surface at 730xg. Therefore the actual initial contact is grossly abnormal. In this thesis, the actual moment of contact between the cell and its substratum is of critical importance and is not perturbed by increasing the g-force or other factors. Nevertheless, Nath and Srere (1977) found that after 30 minutes spinning at 730xg at 7°C there was a 5% attachment, while 80% of cells were attaching at 23°C in the same period. Breiter-Hahn et al (1990) have studied the adhesion of XTH-2 cells (a cell line drived from Xenopus laevis tadpole heart endothelia cells) on a glass substratum. Since inhibition of energy metabolism with antimycin A did not prevent cell attachment, although it inhibited subsequent spreading of XTH-2 cells, they have suggested that the initial cell attachment is independent of metabolic energy. However it is clear from these authors even initial cell attachment is a metabolically driven process. This will be discussed in the following sections.

Nevertheless most of the published data deals with only cell attachment and not with cell adhesion strength. Other studies reported above, were carried out with different cells and with a different aim in mind. In this thesis the aim was to determine the relationship between the temperature of attachment and subsequent

adhesive strength of this cell substratum link. This will be discussed below.

4.2.2. THE ROLE OF TEMPERATURE ON CHL CELL ADHESION STRENGTH

As mentioned earlier, cell adhesion is a multistep process which can be reduced to three centrally important mechanisms; initial attachment, spreading and growth. By studying each of these steps it is possible to determine the mechanism of the whole adhesion process. In the previous section the role of temperature in the initial CHL cell attachment has been discussed. In this section the role of temperature in CHL cell adhesion strength will be discussed.

Since it was of interest also to see whether the temperature effect is influenced by the chemical structure of the substratum, the temperature-adhesion strength relationship studies were conducted in glass and tissue culture grade polystyrene dishes. To find out the effect of temperature in CHL cell adhesion, detachment studies were carried out at various temperatures as described in materials and methods (2.4). Briefly, sub confluent monolayer cells are trypsinized and then the trypsin inhibited by serum containing culture medium. The resulting cell suspension was inoculated into complete growth medium, at the final concentration of $2x10^5$ cells/ml. This cell containing medium was poured into $144cm^2$ plastic dishes already containing five sterilised glass microscope slides (see materials and methods) or in tissue culture grade plastic dishes. As has been discussed earlier CHL cells were attaching onto tissue culture dishes in the presence of 10% foetal calf serum at 12^oC and above. i.e. only 32%±3% of cells were attaching after 12 hours of incubation at 12^oC and the amount of attached cells was 49%±3% after 24 hours incubation

(see figure 4.1.c), while 78%±2% of CHL cells were attaching in the incubation period of 12 hours at 15°C. However more than 95% of the cells were attaching in two hours of incubation in the presence of 10% foetal calf serum at 20°C and above. Thus CHL cell adhesion strength-temperature relationship studies were performed in the presence of 10% foetal calf serum at 15°C, 20°C, 26°C,30°C, and 37°C. Hereafter, unless it is stated, all adhesion related discussions indicate cell adhesion in the presence of 10% foetal calf serum. The cells were allowed to grow for 24 hours at the related temperatures then the adhesion strength of cells was determined by using the Microflow chamber, as has been described in chapter 3 (see 3.3 and 3.4).

It was observed that attachment as weel as spreading of CHL cells were temperature dependent. Thus, cells were attaching but not spreading, they had a round morphology even after 24 hours incubation at 15°C or 20°C while at 26°C the cells flattened. There was no detectable cell morphological difference between that cells were growing at 30°C and at 37°C at the light microscopic level. That is cells were spreading normally at both temperatures.

Cell adhesion strength at 15°C was too weak to measure. In other words, even at 18 mlminute^{-1} flow rate (minimum available flow rate) which is equal to a critical shear stress of 0.5 Nm^{-2}, all cells were coming off throughout the test section. Therefore it was impossible to measure a critical distance. That is although CHL cells were attaching into tissue culture grade dishes at 15°C this attachment was not strong enough to withstand even a 0.5 Nm^{-2} detachment force. Nevertheless it was possible to measure the cell adhesion strength providing that cells were incubated at 20°C or above.

The strength of adhesion was therefore measured at 20°C, 26°C, 30°C and 37°C. The latter being a normal control. The shear values measured were 5.66±0.44 Nm^{-2} at 20°C and 7.09±0.94 Nm^{-2} at 26°C and 9.50±0.90 Nm^{-2} at 37°C. Although critical shear stress was increased

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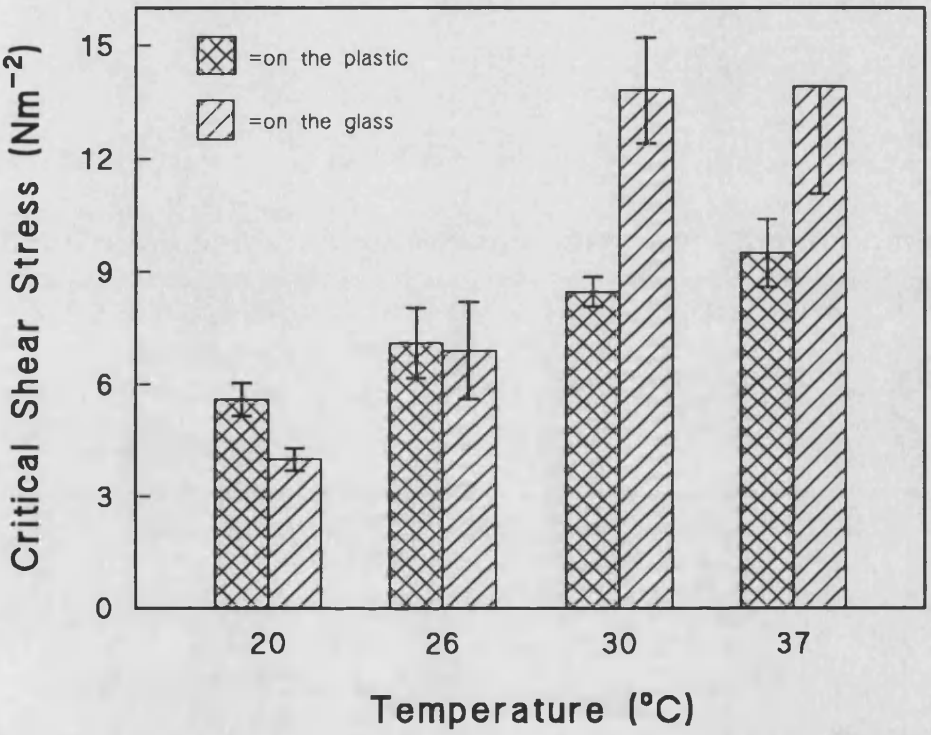


Figure:4.2. The Effect of Temperature on The Adhesion Strength of CHL Cells.

Mid-confluent cells were trypsinized, and subcultured on the round tissue culture grade polystyrene dishes which have a 72 cm^2 surface area or on a square tissue culture polystyrene dish which contains γ irradiated glass microscope slides. Cells were incubated for 24 hours at described temperatures. The adhesion strength of these cells in terms of critical shear stress (c.c.s) was determined. Each data point represents the mean of four experiments in each of which 10 determinations were made. Error bars indicate the standard error of that mean. Further details may be found in the text.

from 20°C to 26°Cthis was not statistically different (P=0.024). The 20°C and 26°C values are statistically significantly different from the 37°C shear value, where a P=0.0000 between 20°C and 37°C while P=0.0073 between 26°C and 37°C. However, the 30°C value was 8.46±0.40 Nm-2 which was not statistically different from the 37°C value. Here P=0.18.

Therefore it appears to be fair to suggest that there are some transition temperatures in the cell attachment and adhesion events. That is, below 10°C cells are not attaching at all (see figure 4.c). Between 12°C and 20°C cells were attaching e.g. 78%±2% of CHL cells were attaching in the incubation period of 12 hours at 15°C while more than 95% of the cells were attaching in two hours of incubation 10% foetal calf serum at 20°C. However below 20°C this attachment is very weak e.g. CHL adhesion strength onto tissue culture grade dishes at 15°C was not strong enough to measure. While at 20°C and above the cells have gained some adhesion strength. So presenting the data in a different way, at 20°C cells only gain 59% of their final adhesion strength, at 26°C this value is 75% and at 30°C culture has reached 90% of its maximal strength.

This is the first time that it has been possible to relate the temperature of cell attachment and the physical strength of the interaction between such a cell and its attachment surface. Although there are some reports (McClay et al 1981; Lotz et al 1989) in which the relationship between temperature and adhesion strength has been studied. Their techniques were inefficient and not quantitative. Thus such studies have failed to show the relationship between temperature and cell adhesion strength. For example, McClay et al (1981) studied embryonic chicken neural retina cell adhesion by using centrifugation. These authors were unable to determine the required

detachment force to detach cells which were incubated at 37°C since the maximum available force (2000xg) was not enough to detach the cells. Lotz et al (1989) have studied the adhesion strength of NIL (fibroblast) cells onto a fibronectin substratum. Although the adhesion strength of cells at 4°C was determined, to be 10^{-6} Nm^{-2}/cell, it was impossible to find out the cell adhesion strength when cells were incubated at 37°C. Moreover the presence of fibronectin on either glass or plastic surfaces causes a large increase in the adhesion strength of cells (Truskey and Pirone 1990). In fact, as it will be discussed in chapter 6, the coating of tissue culture plastic dramatically increased cell adhesion strength. However, the present study is the first time it is possible to relate the temperature of cell attachment and the physical strength of interaction between such a cell and its attachment surface. To an extent this results vindicates the significance of Microflow cell adhesion chamber (see chapter 3) and shows that meaningful quantitative numerical data can be obtained.

The effect of temperature on the initial cell attachment and on cell adhesion strength perhaps could be related to membrane fluidity. This possibility will be discussed below.

4.2.2.1. THE ROLE OF MEMBRANE FLUIDITY ON CELL ADHESION.

These temperature dependent adhesion strengths might well be correlated to the work of Wisniski et.al (1974). These workers found that there were four characteristic liquid phase transitions temperatures of the membranes of mouse fibroblast LM cells. That is, below 15°C all the lipids in the outer surface membrane are in a solid state. Above 30°C all of lipids are in a fluid state and between these temperatures separate defined and separate solid and

fluid phases form. Below 21°C all the lipids in the inner cell surface membrane are solid state and above 37°C all lipids of inner membrane are fluid (Wisniski et al 1974). A similar point has been made by Ueda et al (1976) using Baby Hamster kidney (BHK 21) cells. Here the authors found that membrane fluidity changed characteristically at 10°C, 20°C and 30°C i.e. at these temperatures the fluid state of the membrane lipids are increased in defined steps.

It is therefore possible that cell membrane fluidity has an important bearing on cell attachment. At low temperatures, the membrane lipids become increasingly viscous until a point is reached when several important mechanisms are perturbed. First, at low temperatures the cell becomes a rigid sphere. When it touches the surfaces the force of gravity is unable to deform the cell at the point of contact so that a time dependent increase in contact area between the cell and its substratum cannot occur. At these low temperatures it is likely that the cell membrane is so stiff that it cannot deform in response to the necessary programmed changes in the cytoskeleton, or if it is able to, processes requiring local rearrangements of adhesion molecules needed for firm attachment cannot take place. Then there will also be a lag phase in cell adhesion due to decreased flexibility or deformability of the cell membrane (Juliano and Gagalang 1977). It is possible that the "adhesion signal" that is, the signal set from the outside of the cell to the inside, cannot pass through this more viscous, stiffer membrane. Thus the cell and its cytoskeleton are unable to respond to the initial contact between cell and substratum (Gingell and Owens 1992).

A further consequence of the markedly decreased membrane fluidity, or its increased stiffness, is that transport through the membrane must be retarded. Here the cell has to transport large numbers of different proteins to its external surface so that adhesion receptors, plaques etc, and other adhesion structures can be formed, a process which must at least be retarded at low temperatures (Ueda et al 1977). Perhaps it is necessary to make a distinction that above argument is valid for the cell attachment in the presence of serum. Whereas, it is very unlikely that membrane fluidty has a role in cell attachment in the absence of serum (see later section 4.2.6).

However none of these possibilities are relevant if the first step of cell attachment is receptor mediated. The cell, at low or normal temperature will approach the substratum surface, touch it and limited receptor ligand binding will occur. Even at low temperatures, the receptor sites will be present on the surface of the cell even though they may be "frozen" there by the low temperature and high membrane viscosity. As stated earlier, the receptors must still bind at low temperatures and, even weakly, immobilise the cell to the substratum. However, even this initial binding could be a receptor-mediated phenomena. But,as it will be discussed in the signalling mechanism in Chapter 6, this receptor mediated binding is not simply linking a cell to the surface; this event is followed by sending a signal in to interior of the cell and then by reorganisation of cell functions e.g. cytoskelatal binding etc. Therefore, it might possible that a signal triggered by this binding at low temperatures is unable to activate further cell processes. Put another way the cell is simply unable to perform the required reorganisation to carry out adhesion functions due to lack of energy.

The objective behind this work is to dissect out the early adhesion process. The question is, is the first step mediated through simple ligand-receptor binding or through an active continuos metabolic process? The other possible factor is that cell metabolism which may play a significant role on the cell adhesion , will be discussed now.

4.2.2.2. THE ROLE OF CELL METABOLISM ON CELL ADHESION

Cell adhesion in the presence of 10% foetal calf serum is completely inhibited below 10°C. That is, there is no observable attachment. This indicates that all the steps from initial contact onwards are probably metabolically driven. Now, cell metabolism is of course an extremely complex process dependent on, amongst other mechanisms, metabolic pathways consisting of many series of enzyme reactions. For example, the oxidation of glucose to pyruvate requires 10 enzymatic reactions following one upon the other. Assume, for simplicities sake, that there is a four step metabolic pathway; A→B →C→D, in each step there is a 90% conversion of each substrate to product. Under normal conditions there will be about 73% conversion of A→D in a given time (A100→B90%→C81%→D72.9%). If however the temperature is lowered so that in the same unit time the conversion drops to 10% at each step, then the overall formation of product is also lowered, but this time to 0.1% (A100%→B10%→C1%→D0.1%) a factor of 730 times less when compared to the higher temperature. This is not a " worst case" scenario because no account has been taken of the additional reduction in conversion by the possible dramatic effects on Km and Vmax that the lowering of each substrate has on each enzyme. This is of course a gross oversimplification of the inter dependence of metabolism on temperature but it does illustrate why

there will be the an exquisitely critical temperature below which cellular metabolism stops.

It was noticed, in addition, cell adhesion strength was dependent on the structure of substratum. This will now be discussed.

4.2.2.3 THE ROLE OF SUBSTRATUM ON CHL CELLS ADHESION STRENGTH.

It was of interest to notice that the temperature effect on CHL cell adhesion strength was also dependent on the cells substratum as well.That is the critical shear stress was reduced by 50% at 26^{o}C and 70% at 20^{o}C on a glass surface where the 100% value is at 37^{o}C. On tissue culture polystyrene the reduction in adhesion strength was only 25% at 26^{o}C and 41% at 20^{o}C. Moreover, on glass, the final adhesion strength was 25% higher than the tissue culture plastic. The reasons for the difference between glass and plastic is probably due to the entirely different nature of these surfaces. The plastic is a hydrophobic (relatively), aromatic polymer whilst glass is a sodium borosilicate complex with quite different adsorptive properties. As has been discussed in chapter (6) glass adsorbs less protein than polystyrene. That is 40% of added radiolabeled fibronectin adsorbed onto the glass microscope slides while 70% of ^{125}I-fibronectin adsorbed onto tissue culture grade polystyrene. However it is possible that an adsorbed proteins conformation is more active for strengthening cell adhesion on glass than on the tissue culture plastic.

To define the role of metabolic energy in cell adhesion one of many approaches is to use metabolic inhibitors. The effect of metabolic energy inhibition on CHL cell adhesion will be discussed below.

4.2.3. OLIGOMYCIN EFFECT ON CHL CELL ADHESION STRENGTH.

The nitrogen effect will which be discussed later in this chapter (4.2.4), had a rapid and profound effect on adhesion. In an effort to be more specific, oligomycin was used to inhibit electron transport and thus cause ATP depletion (Klebe 1975).

To determine the oligomycin effect, the following procedure was applied. Cells are trypsinized and then the trypsin inhibited by 10% foetal calf serum containing culture medium which contained various concentration of oligomycin (0μM to 30 μM oligomycin). The resulting cell suspension was inoculated into complete growth medium which also contained 0 μM, 1 μM, 10 μM, 20μ or 30 μM oligomycin and the final concentration of cell was about $2x10^5$ cells/ml. The cells were allowed to grow on tissue culture polystyrene for 24 hours in a 5% (v/v) CO2 in air atmosphere at 37^oC; then the adhesion strength of cells were determined using Microflow chamber (see chapter 3.4).

It was noticed that in these experiments with oligomycin, the cells were flattening but not spreading. It appeared that the oligomycin effect is dose dependent i.e. at 1 μM oligomycin there was only about 18% reduction in adhesion strength and this value was about 21% at 5 to 20 μM and at 30μM oligomycin concentration adhesion strength was reduced by 37%. In other words in the control CHL cells the adhesion strength was 11.05±0.7 Nm^{-2} while the adhesion strength of CHL cells which were growing in complete medium containing 30 μM oligomycin was 6.93±0.59 Nm^{-2}. There is a statistically significant difference between these two shear values (P=0.0073). Nevertheless as will be discussed later in this chapter, the nitrogen atmosphere studies proved metabolic energy does have an important role in cell adhesion.

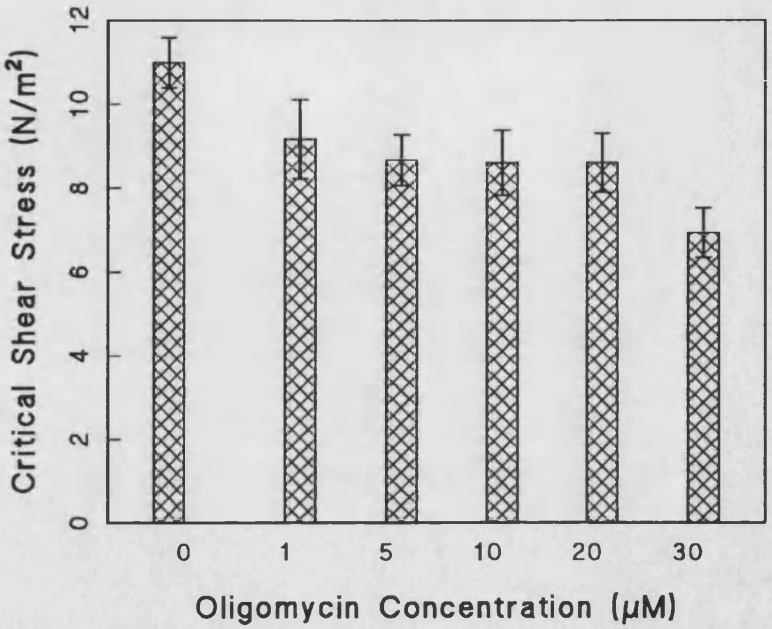


Figure 4.3. The Effect of Oligomycin on The Adhesion Strength of CHL Cells.

Mid-confluent CHL cells were trpsinized, and subcultured on 56 cm^2 tissue culture grade polystyrene dishes in the serum contains medium which has various concentrations (0-30 μM) of oligomycin. Cells were incubated for 24 hours at 37°C, then the adhesion strength of CHL cells were measured by using the Microflow Chamber. Each data point represents the mean of 30 different determinations, the error bars indicate the standard errors of that mean. Further details may be found in the text.

At this point it seemed worthwhile trying to dissect out the mechanism of the temperature effect in cell attachment. To do this, various studies were performed to discover the role of oxygen, serum, and trypsinization in the mechanism of cell attachment. These will be discussed below.

4.2.4 THE ATTACHMENT OF CHL CELLS IN NITROGEN ATMOSPHERE.

To define the role of ATP in cell attachment it is necessary to deplete ATP levels. The clearest and easiest way to deplete ATP levels in animal cells is to incubate them under anaerobic conditions. The integral partner to oxidation of glucose is of course oxygen (Alberts et al 1989) As mentioned earlier, without ATP and NAD the cells biosynthetic and catabolic pathways stop. For the anaerobic experiments, rigorous care has to be taken to exlude oxygen. The overall procedure is outlined in the material and methods (2.3.2). However to recall; cells were grown in normal medium until they reach mid-log phase and about 50% confluency. The cells were then trypsinized from the culture flask under a stream of special oxygen free nitrogen gas. That is during trypsinization culture flasks were kept in a polyethylene bag into which passed a continuos stream of special oxygen free nitrogen gas (materials and methods 2.3.2). The EDTA-PBS, and PBS, were also sprinkled nitrogen prior to use,and after trypsinization the cells were placed in the oxygen free medium. This had been made with boiled water and also sprinkled nitrogen prior to adding the serum. The complete medium could not be sprinkled with bubbling nitrogen because of the considerable foaming effect the serum proteins. For all these experiments 35 mm tissue culture grade plastic dishes were used and they were also kept in the nitrogen atmosphere for 30 minutes before 2 ml of cell suspension (contains $1x10^6$ cells) were added into them. The only remaining possible oxygen source would be cells themselves. However it can be calculated theoretically. That is, if we assume a cell as a 10 μm diameter spherical body then $1x10^6$ cells will occupy a volume of $0.52x10^{-3}$ ml. Since the solubility of oxygen in distilled water at 20^oC is 8.84

mgL^{-1} (Shreir 1976) it might be expected that there will be $0.14x10^{-6}$ mMol oxygen. However, this oxygen will last less than 3 minutes. (due to the oxygen utilisation rate which is for cultured cells , about $0.19x10^{-3}$ mMol oxygen per hour, Freshney 1987). Finally incubation was performed at 20°C in the nitrogen atmosphere. Then at various times, samples were removed so that the percentage of cells attaching could be measured using an haemocytometer (material and methods 2.3.2).The cells were also observed under an inverted microscope to determine cell attachment directly.

Under these conditions there was no measurable attachment even after 3 hours incubation in the nitrogen atmosphere at 20°C (see fig 4.4). A second series of experiments which were identical, except that cells were incubated in air after trypsinization instead of in nitrogen atmosphere, there was 15%±3% attachment within 3 hours (see table 4.2).This figure seems low but it must be kept in mind that the medium containing the cells was oxygen free and so the oxygen needed to dissolve to reach the cells. However in control experiment where the medium contained the normal amount of air, the attachment was 94%±4%. The results are summarised in table (4.2.).It is possible that the anaerobic conditions actually killed the cells. However this was not the case since over 90% of cells were still alive according to trypan blue exclusion even after 3 hours of incubation under a N_2 atmosphere. In further experiments cells were cultured as above and treated with the anaerobic conditions for 3 hours at 20°C. Again no cells adhered (see table 4.2). They were then transferred to a 95% (v/v) air 5% (v/v) CO_2 incubator at 37°C. Where 58%±4% of cells adhered within 24 hours.

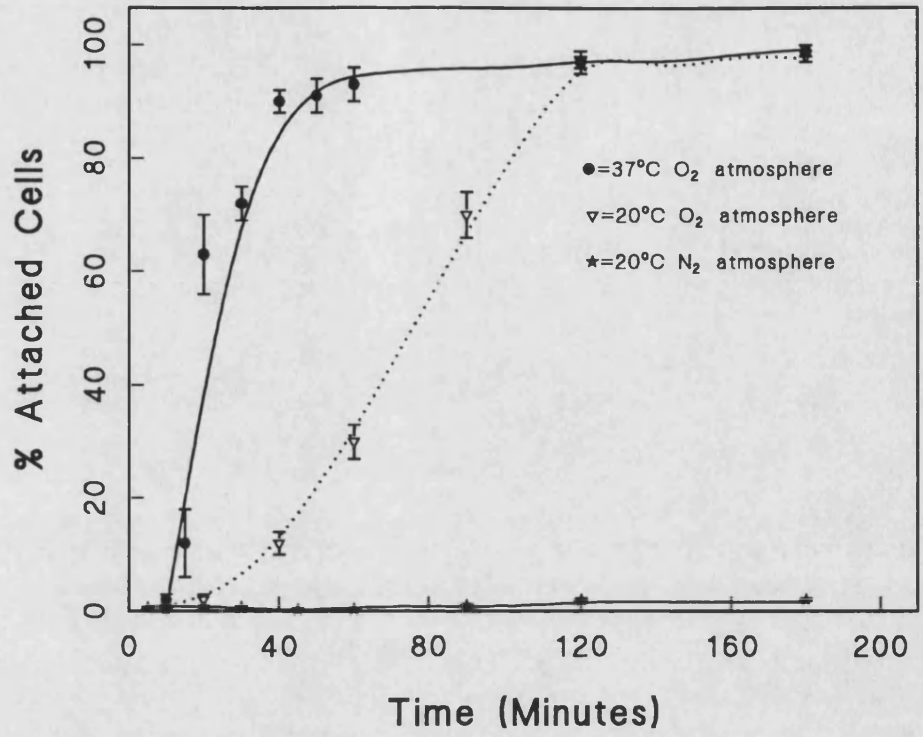


Figure 4.4. The Attachment of CHL Cells in N_2 Atmosphere in the Presence Of Serum.

The attachment studies were performed on 35 mm tissue culture grade plastic dishes. To compare the attachment of CHL cells on the air or N_2 atmosphere, the attachment studies were performed on both conditions at 20°C. As a control attachment studies were performed on 5% CO_2/air at 37°C. Each data point represent the mean of four different experiments in each of which two determinations were made. Error bars indicate the standard error of that mean.

One might ask why there was only 15%±3% attachment in 3 hours incubation although it rose to 58%±4% in 24 hours. It is likely that even after 3 hours there is insufficient dissolved oxygen in the medium which of course will effect cell attachment.

Medium+N_2	Medium+N_2	Medium+N_2	Medium+air
incubation in N_2, 3 hours	incubation in air, 3 hours	incubation in air, 24 hours	incubation in air, 3 hours
No attachment	15%±3 attach.	58±4 attach.	98±4 attach.

Table 4.2. The Role of Oxygen in CHL Cells Attachment.

These experiments confirm that all the steps in CHL adhesion are metabolically driven rather than being simply receptor mediated. Since in the absence of oxygen, ATP production could be depleted and this would prevent cell attachment. The Role of ATP in Cell adhesion is discussed below.

4.2.4.1 THE ROLE OF ATP IN CELL ADHESION.

There are contradictory reports on the role of ATP in cell attachment. It has been reported that inhibition of ATP synthesis profoundly inhibits cell attachment (Michaelis & Dalgarno 1971) A similar finding was seen by Klebe (1975) who inhibited electron transport and therefore ATP regeneration using oligomycin or sodium azide (Devlin 1986). However Klebe (1975) reported that oligomycin and sodium azide inhibited CHO cell attachment in the absence of glucose but not in the presence of glucose. That is, although cell attachment was inhibited more than 90% by both of these substances in the absence of glucose, there were only 35% and 15% reduction in attachment with sodium azide and oligomycin respectively in the presence of glucose. It is possible that ATP derived from glycolysis might reduced the effect of these electron transport inhibitors on cell attachment. Nevertheless the CHO cell attachment was only delayed although the ATP level was reduced to less than 5% of normal

by potassium cyanide, an inhibitor of electron transport (Devlin 1986), i.e. about 10% of cells were attaching in the presence of potassium cyanide in 30 minutes, and 80% attached in 60 minutes (Juliano and Gagaland 1977). According to Nath and Srere (1977) no correlation exists between cellular ATP concentration and the rate of BHK cell adhesion to the substratum. Hence, while ATP concentration was lowered by 95%, cellular adhesion is reduced only by 50%. It is possible that although there is a relationship between the rate of cell attachment and the level of cellular ATP this might not be linear. Therefore even a small amount of energy will be enough for a cell to carry out its attachment procedure. In fact it has been reported (Michaelis and Dalgarno 1971) that pig-kidney (PS) cells require only 12% of their endogenous ATP to attach to the substratum. It seems therefore that although there is a disagreement on the profile of the effect of ATP on cell adhesion, ATP probably has an important role in it. In this work we have found profound evidence that proves beyond doubt that oxygen consumption is needed for adhesion, which of course means, perhaps indirectly ATP.

The attachment studies discussed so far were performed on nitrogen or air atmosphere and in the presence of serum. However,as it will be discussed later, CHL cells attachment in the absence of serum is not a temperature dependent phenomena, therefore it was of interest to investigate the attachment of CHL cells in nitrogen atmosphere in the absence of serum. This will now be discussed.

4.2.5 CHL CELL ATTACHMENT IN NITROGEN ATMOSPHERE AT THE ABSENCE OF SERUM

To investigate the role of oxygen in CHL cells attachment in the absence of serum; an attachment assay under nitrogen atmosphere was conducted as described in materials methods (2.3.2) except that

serum free medium was used. Briefly, cells were grown in normal medium until they reach mid-log phase and about 50% confluent. The cells were then trypsinized from the culture flask under the stream of special oxygen free nitrogen gas. EDTA-PBS and PBS were sparged with nitrogen gas prior to use. Serum and oxygen-free medium was used which had been made with boiled water and which had been also sparged with nitrogen before use. To inhibit trypsinization, oxygen free soybean trypsin inhibitor was used which had been made with boiled water and which had been also sparged with nitrogen before adding the soybean trysin inhibitor crystals. After trypsinization it is necessary to inhibit trypsin activity otherwise, this proteolytic enzyme will damage the cells. In the case of serum, it is not necessary to use additional trypsin inhibitor since serum proteins are able to stop the trypsin activity. However in the case of serum free studies, trypsin activity must be stopped by a trypsin inhibitor. As before ,35 mm tissue culture grade dishes were used in this assay and they were kept in the stream of oxygen-free nitrogen gas for 30 minutes before cells were added to them. 2mls of a cell suspension which contained $5x10^5$ cells /ml were added into these dishes and incubated in the nitrogen atmosphere at 20^oC. Then at various times, samples were removed so that the percentage of cells attaching could be measured. As can be seen in figure (4.5), 15%±2% CHL cells were attaching after two hours of incubation at 20^oC and the amount of attached cell was 24%±2% after 3 hours of incubation. While in the control experiment serum free medium was prepared as described in materials and medium (2.2.1) but without nitrogen sparging in it and incubation was carried out in air atmosphere at 20^oC. In this case, the amounts of attached cells were 75%±3% and 77%±2% after two and three hours of incubation respectively. That is

there was 51% reduction of attachment in the absence of oxygen. These results suggest that CHL cells attachment in the absence of serum is not a temperature dependent processes while

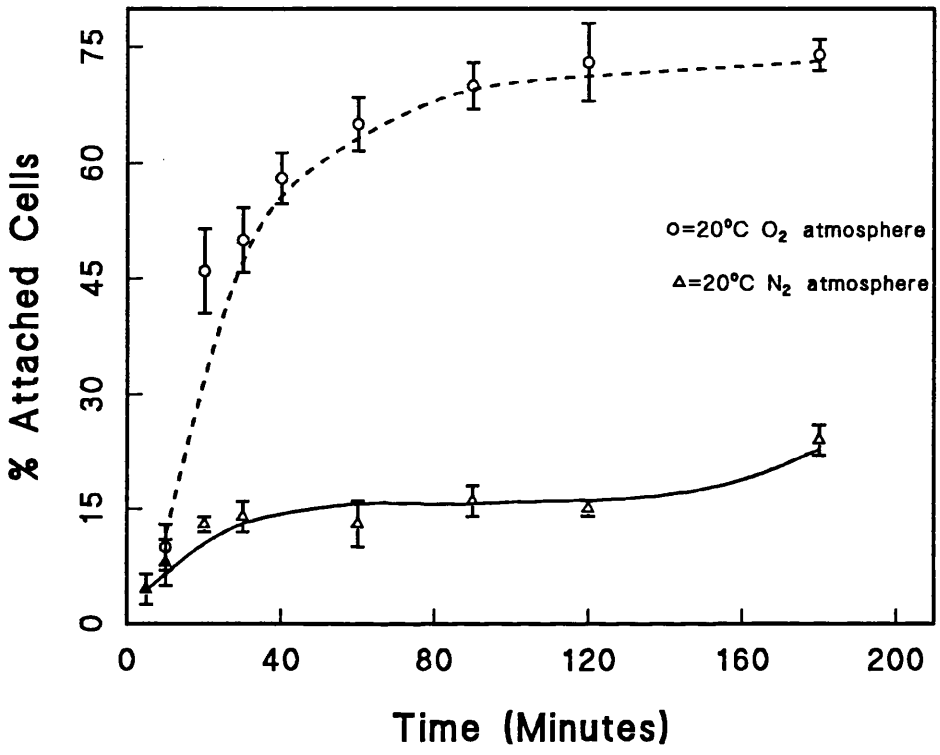


Figure 4.5 The Attachment of CHL Cells in N_2 Atmosphere .at the Absence of Serum.

The attachment studies were performed on 35 mm tissue culture grade plastic dishes. To compare the attachment of CHL cells on the air or N_2 atmosphere in the absence of serum, the attachment studies were performed on both conditions at 20°C. Each data point represent the mean of four different experiments in each of which two determinations were made. Error bars indicate the standard error of that mean. Further details may be found in the text.

cells do require oxygen to perform their adhesive function. In contrast, in the presence of serum, where there was no attachment in

nitrogen atmosphere, about 24% of cells were attaching under the same condition but in the absence of serum. Therefore it is possible to suggest that the cell uses different mechanism of adhesion in this two cases.

Since effect of nitrogen on cell adhesion was affected whether there was serum or not in the growth medium, it was of interest to investigate the serum affect in cell adhesion.

4.2.6. THE ATTACHMENT OF CHL CELLS IN THE ABSENCE OF SERUM.

To determine the effect of serum on CHL cell adhesion, attachment studies were performed in the absence of serum at different temperatures i.e. 4°C, 20°c and 37°C. Attachment studies were performed as described in section 2.3 except that after trypsinization of mid-confluent cells, trypsin was inhibited with soybean trypsin inhibitor and then cells were seeded in serum free medium. Results are given in figure 4.6. The amount of attached cells after one hour of incubation were 70%±2.6%, 67%±3.5% and 68%±4.2 at 37°C, 20°C and 4°C respectively. This is very much in contradiction to the attachment of CHL cells in the presence of serum in which attachment was dramatically affected by the change of temperature. For example, although more than 95% of cells were attaching at 37°C there was no higher than 2% attachment at 4°C (see figures 4.1a,b and 4.1.c). The other point was different from the attachment profiles of cells in the presence of serum was that there was no lag phase in the attachment of cells in the absence of serum (figure 4.6). While at the former case the duration of lag phase was dependent on the temperature at which attachment assay was performed (see figure 4.1).

These results suggest that the attachment of CHL cells in the presence of serum is energy-requiring process, while this process in the absence of serum is a passive phenomena. Similar findings for HeLa 71 cells were reported by Unhjem and Prydz (1973). While, Grinnel (1974) has reported that the low temperature inhibits the attachment of BHK-21 cells whether serum is absent or present in the incubation medium. It is clear that different cells are affected differently by temperature differences.

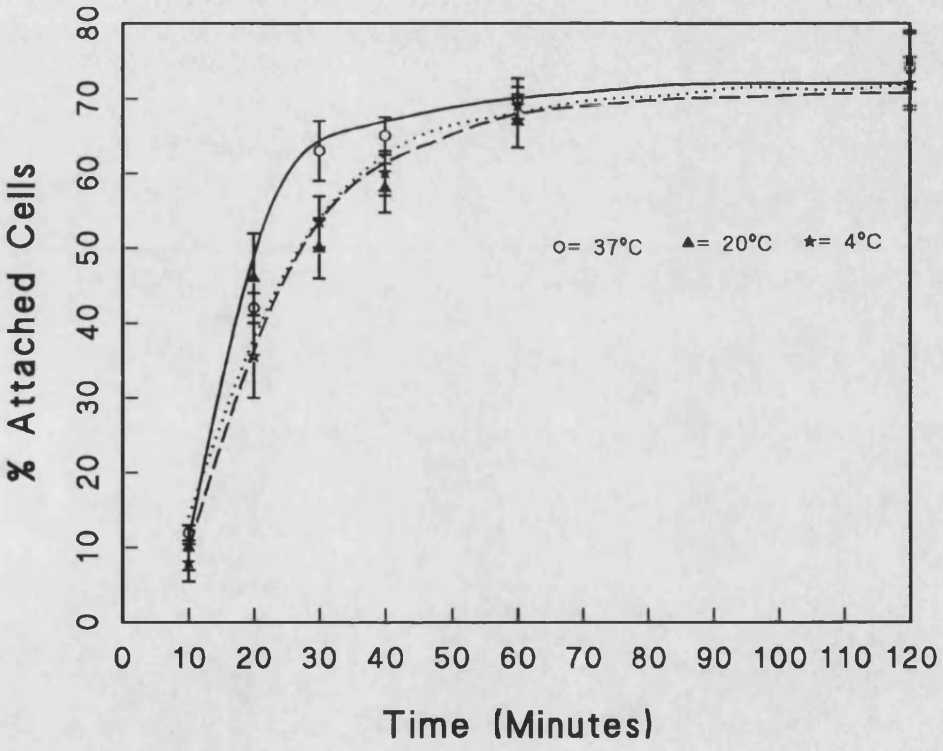


Figure 4.6 The Effect of Temperature on The Attachment of CHL cells in The Absence of Serum.

Mid-confluent CHL cells were trypsinized, trypsin then was inhibited by a trypsin inhibitor. Cells were seeded on 35 mm tissue culture grade plastic dishes in the absence of serum and cells were incubated at indicated temperatures. Each data point represents the mean of four different experiments in each of which two determinations were made. Error bars indicate the standard errors of that mean.

These different attachment behaviour of cells in different composition of medium,i.e either there is serum on it or not, could indicate that cell adheres to surface through different mechanisms. In the presence of serum, cells do not directly adhere to the surface instead they adhere to preadsorbed serum adhesive proteins via cell surface receptors. Whereas in the absence of serum there is no pre adsorbed proteins on the substratum and simply cells adsorb the surface non specifically (Steele et al 1992). In fact the adhesion mechanisms in these two different case will be discussed in more detail at chapter 5 and chapter 6. However, perhaps it is not out of place to suggest that in the presence of serum cell attachment involves different subsequent steps e.g. receptor ligand-binding, signalling and reorganisation of the other molecules which are involved in cell adhesion e.g., cytoskeletal proteins, these will of course take time and therefore there will be a lag phase in attachment. While in the absence of serum there are no such steps therefore as soon as cells touch the surface they adsorb to it. The fact that these cells will stick at low temperature ($4^{o}C$) in the absence of serum also suggest that my earlier proposal (see 4.2.2.1) that membrane temperature dependent fluidty and phase changes in the membrane lipids is responsible, or involved in, the lack of adhesion at $4^{o}C$ in the presence of serum cannot now be true. It is possible that serum lipids might adsorb to the outside of the cell membrane and they become viscous and cause the membrane to stiffen at $4^{o}C$, and inhibit or perturb cell attachment. However these are speculations and the exact reason is not known.

After finding that attachment of cells in the absence of serum is not completely inhibited at $4^{o}C$, while it was inhibited in the

presence of serum, it was of interest to evaluate the effect of serum on cell attachment. This will now be discussed.

4.2.6.1. THE ROLE OF SERUM ON CELL ATTACHMENT.

To determine the effect of serum in cell attachment following procedure was used to pretreat the culture dish with serum. Here, 2 mls of serum containing medium was added to 35 mm tissue culture grade polystyrene dishes, and these dishes were incubated at 37^{o}C for 24 hours. Afterwards, serum containing medium was discarded, dish rinsed with PBS, and the CHL cells were seeded on these dishes in the absence of serum at 4^{o}C. Even after 24 hours of incubation at this temperature there was no detectable cell attachment.

This result suggests that the pre absorbed serum proteins prevent the attachment of cells at 4^{o}C. The question is how? One of possible answer might be that some of the component(s) the serum is preventing cell attachment at this temperature. In fact it is known that serum contains adhesive proteins and anti adhesive proteins (Curtis and Forrester 1984). Therefore, it might be possible that these anti adhesive proteins are more active than adhesive proteins at low temperatures. However, it has been reported that a serum protein inhibits cell adhesion at low temperature (Curtis and Greaves 1965) while the inhibition of this protein overcome by cellular metabolism (Curtis and Greaves 1965; Moscona and Moscona 1966).

The other factor that is considered as a effect in the inhibition of cell adhesion in low temperature is trypsinization (Curtis 1973). This possibility was studied and willl be discussed below.

4.2.7. THE EFFECT OF TRYPSINIZATION ON CELL ATTACHMENT.

The possible effect of trypsin in the inhibition of cell attachment as low temperature considered was that trypsinization might damage the cell membrane which would not repair this damage at low temperature (Curtis 1973). Therefore, it was thought that if cells were given enough time in 37°C to recover from trypsin damage, then they might be able to attach at low temperature in the presence of serum. Bearing this possibility in mind, the following experiment was conducted. 5 mls of cell suspension containing $2x10^5$ cells/ml were incubated at 37°C for 24 hours at in a sterile polyethylene scintillation vials to which cells do not attach. After this incubation period the cell suspension was centrifuged (in a bench centrifuge at 500 rpm for 10 minutes) then cells were re suspended in 4°C serum containing medium and finally transferred into 35 mm tissue culture grade dishes. Even after 24 hours of incubation at 4°C the cells were not attaching. However, in the control cells, that is after centrifugation cells were re suspended in 37°C serum containing medium and incubated at 37°C, here more than 90% of cells were attached in two hours of incubation.

It is clear that even though the cells have been given 24 hours to recover from any trypsin damage, they still will not stick to a surface in the presence of serum at low temperature. In BHK cell adhesion Edward and Campbell (1971) and Vicker (1971) have proposed that trypsin damage is responsible for the observed low temperature effect, this cannot be the case in our experiment above.

CHAPTER 5 THE ROLE OF SERUM IN CHL CELL ADHESION STRENGTH.

5.1 INTRODUCTION.

It is generally believed that the growth of almost all types of mammalian cells in culture requires the presence of added serum in the culture serum. Serum is an extremely complex mixture of many molecules. Although many of the components of serum as yet are poorly characterised, the major functions of serum can be broadly defined: these are attachment and spreading, nutrition, stimulation, and protection (MacLeod 1988). However recently, rapid progress has been made in the identification and characterization of the serum proteins involved in cell adhesion such as fibronectin, vitronectin, laminin, thrompospondin. There are many poorly studied proteins (adhesive proteins) found to mediate cell adhesion (Hayman et al 1985; Underwood and Bennet 1989). In contrast,some serum proteins interfere with cell attachment (anti adhesive proteins) (Knox 1984; Van Wachem 1987; Tamad and Ikada 1993). For example, it has been shown that both α-1-antitrypsin and albumin reduce adhesiveness of BHK cells (Curtis and Forrester 1984) while immunoglobulin G (IgG) inhibits hepatocyte adhesion (Neumeier and Reutter 1985).

Considering the opposing roles of individual serum proteins in cell adhesion, it would be interesting to discover how serum as a whole affects cell adhesion. The approach made in the present chapter is based on attempts to understand the functions of serum as a whole in adhesion strength.

5.2 RESULTS AND DISCUSSION.

In order to define the quantitative role of serum in the adhesion strength of CHL cells the following strategies were adopted:

Firstly, the critical shear stress (c.s.s) of cell detachment was measured in the presence of various concentrations (between 0.05% and 10%) of foetal calf serum in the culture medium. The c.s.s was also measured in the total absence of serum.

Secondly, the effect of sera from different origins upon the c.s.s of detachment was studied. i.e. the CHL cell adhesion strength was measured in the presence of 10% foetal calf serum, foetal bovine serum or horse serum.

5.2.1. THE EFFECT OF DIFFERENT CONCENTRATIONS OF FOETAL CALF SERUM ON THE ADHESION STRENGTH OF CHL CELL

These experiments were based on the theory that one of the main functions of serum in cell adhesion is to provide a mixture of essential adhesion proteins such as fibronectin and vitronectin. These essential proteins are considered necessary for the attachment and spreading of cells. It is known that there are a large number of proteins in serum and that some of these proteins may support initial cell attachment while some of them may prevent cell attachment (Van Wachem et al 1987). However, there is as yet, no report which shows the role of serum in cell adhesion strength, hence the quantitative role of serum has not been established. By using the Microflow chamber which has been developed in present study, to measure cell adhesion we can evaluate the relationship between the strength of cell binding on a tissue culture dish and concentrations of foetal calf serum in culture medium. To find out this relationship CHL cells were grown for 24 hours at 37^{o}C with concentrations of 10%, 5%, 2.5%, 1%, 0.5%, 0.1%, 0.05% and 0.0% foetal calf serum (v/v) in the culture medium. The critical shear stress of detachment was measured

as described in materials and methods (3.4), and the results are presented in figure 5.1.

Cell adhesion strength increases with increasing serum concentration up to 1% of serum. while above 1% increasing serum concentration did not have any noticeable effect upon the strength of CHL cells adhesion (see figure 5.1). In the absence of serum the adhesion strength of CHL cells was very low; the c.s.s of detachment was 3.01± 0.25 Nm^{-2} . However, in the presence of very small amount of serum (0.05%) the c.s.s of detachment was 5.38±0.55 Nm^{-2} ; the adhesion strength was increased by 79%. When the serum concentration was increased further, the critical shear force required to detach cells from tissue culture dish increased as well. That is the values for thecritical shear stress of detachment were 7.22±0.54 Nm^{-2}, 8.14±0.74 Nm^{-2}, and 9.47±0.43 Nm^{-2} in the presence of 0.1%, 0.5%, and 1% foetal calf serum in the culture medium respectively. Upon comparison with the c.s.s of detachment in the absence of serum these adhesion strength represented an increase of 140%, 170%, and 216% respectively. Thus it is clear that upto a concentration of 1% serum, cell adhesion stress was serum-dosage dependent. Increasing serum concentration above 1% did not effect cell adhesion significantly. Although the c.s.s of detachment was 9.47±0.43 Nm^{-2} in 1% feotal calf serum it was 9.55±0.73 Nm^{-2} , 9.54±1.15 Nm^{-2} and 9.60±0.75 Nm^{-2} in the presence of 2.5%, 5%, and 10% serum respectively. Although there were slight difference between these values; these were not statistically significant. For instance there was no significant difference in the c.s.s of detachments even between 1% and 10% of serum (P=0.67).

These results indicate that at a serum concentration of 1% CHL cells gain a maximum possible adhesion strength,while even very low

concetrations of serum (0.05%) can significantly increase cell adhesion strength when compared to the absence of serum. The serum results are discussed and conveniently divided into following major sections.

1. The effect of lowering the serum concentrations from 10% to 0.05% upon the adhesion strength of CHL Cells.

2. The Effect of 0% of serum on the adhesion strength of CHL cells.

3. The Possible mechanism by which serum exerts its effects on the adhesion strengthening process.

5.2.1.1. THE EFFECT OF LOWERING THE SERUM CONCENTRATION FROM 10% TO 1% ON THE ADHESION STRENGTH OF CHL CELLS.

If serum is considered the only source of proteins for cell adhesion, it appears that foetal calf serum, even at a concentration of 1%, provided sufficient amounts of adhesive proteins for CHL cells to gain their maximum possible adhesion strength. Fibronectin and vitronectin are two major cell adhesion proteins in serum. Human plasma contains 300 µg/ml fibronectin (Ruoshlahti et al 1982) and 200-300 µg/ml vitronectin (Hayman et al 1985).

In our studies 20 ml of culture medium, containing different concentrations of foetal calf serum, was used in 100 mm petri dishes, each of which has a surface area of about 72 cm^2. If one assumes that foetal calf serum contains 250 µg/ml fibronectin (due to the binding of fibronectin to fibrin clot serum contains less fibronectin than plasma [Ruoshlahti et al. 1982]) then 20 ml of 1% serum containing medium is expected to have a 50 µg fibronectin and 40-60 µg vitronectin. Hence, the actual amount of fibronectin and vitronectin will be 0.69 $\mu g/cm^2$ and 0.55-0.83 $\mu g/cm^2$ respectively. One can

therefore suggest that these amounts of fibronectin and vitronectin are adequate for CHL cells to gain their maximum possible adhesion strength. In fact, as will be discussed in chapter 6, CHL cells

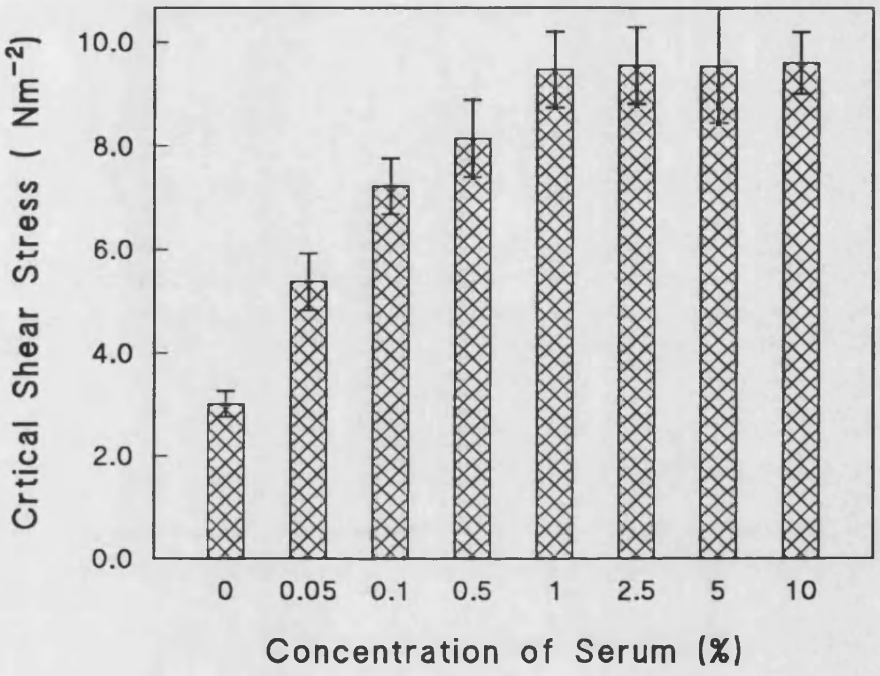


Figure 5.1. CHL Cells Adhesion Strength at the Various Concentration of Foetal Calf Serum.

Sub confluent CHL cells were subcultured and maintained inthe culture medium which was supplemented with 0% to 10% (v/v) serum. The adhesion strength of the CHL cells growing in these media is measured in terms of the critical shear stress (c.s.s) of detachment. Each data point represents four experiments in each of which 10 measurements were made . The error bars represents the standard error of mean. Full details may be found in the text.

reached 88% of their maximum possible adhesion strength when 0.14 $\mu g/cm^2$ fibronectin was adsorbed onto tissue culture dish.

It has also been reported that BHK cells were completely spreading on tissue culture dish on which 15 $\mu g/cm^2$ fibronectin was adsorbed (Grinnel and Feld 1981). Of course, this result is not directly comparable to our work since Grinnel and Feld (1981) studied different cell types and they did not carry out cell detachment studies. In addition a pure protein solution was used, whereas protein adsorption from serum, contains many different proteins and at different concentrations, is a complex phenomenon and it is therefore difficult to predict the amount of adsorbed proteins from serum. Some of the proteins in serum affect the adsorptive properties of the adhesive proteins. For example, albumin,and α-2-macroglobulin, serum anti adhesive proteins, reduce fibronectin adsorption (Curtis and Forrester 1984). Serum concentration therefore may have an influence on the adsorption of serum proteins. It was reported (Grinnel and Feld 1982) that at low concentrations of human serum in the incubation medium, the adsorption of plasma fibronectin increased as the serum concentration increased up to a concentration of 0.1% serum. The highest concentration of fibronectin adsorption was reached at 0.1% serum, which is equivalent to 12 ng/cm^2. This amount of fibronectin was sufficient to promote BHK cell spreading. Above a concentration of 1.0 % serum there was a marked decrease in the adsorption of fibronectin and at 10% serum very little adsorption occurred. This decrease in fibronectin adsorption at high serum concentrations could be due to competition between fibronectin and other serum proteins (Grinnel and Feld 1982). If fibronectin adsorption is very low at serum concentrations above 1% then it is possible to suggest that, at such concentrations, serum fibronectin has a very limited role or no role at all. This theory is supported by Knox (1984) who carried out BHK cell adhesion studies in which

fibronectin-depleted serum was used at various concentrations. It was observed that at serum concentrations of 3% and above, fibronectin depletion had no effect on BHK cell spreading. Therefore it was cocluded that at these serum concentrations the adsorption of fibronectin is completely masked by the other proteins and, at serum concentrations 3% or above, it is the vitronectin that carries out the adhesive function, because its adsorption is not inhibited by albumin and other proteins. At low serum concentrations adhesion is mediated by fibronectin, which is not inhibited at serum concentrations 0.1 % and 1% (Knox 1984). The reason why fibronectin adsorption is decreased as serum concentration is increased was perhaps due to the fact that, unlike the adsorption of fibronectin the adsorptions of albumin and high density lipoproteins (HDL) increases as serum concentration increases from 0.1% to 1% and 10% serum; at 10% serum the adsorption of these proteins reaches a maximum (Van Wachem et al 1987).

The relationship between serum concentration and the adsorption of fibronectin and vitronectin has been studied recently by Steele et al (1992) It was reported that the adsorption of fibronectin decreases a serum concentration of 2% or above while the vitronectin adsorption increases. We have not carried out an assay in which the relationship between adsorbed proteins and serum concentrations are studied, but the above reports, coupled with our CHL cell adhesion strength-serum concentration studies make it possible to suggest that at serum concentrations from 0.05% to 1% the major adhesive protein is fibronectin (which mediates cell-substrate interaction) while above 1% serum the main adhesive protein is vitronectin. Thus one might suggest that at low serum concentrations CHL cells gain their adhesive strength mainly via fibronectin, while at high serum

concentrations fibronectin has a limited role and at such concentrations CHL cells will attain their adhesion strength through vitronectin. This point supported by Knox (1984) who reported that the depletion of fibronectin did not have any significanct effect on BHK cell adhesion at serum concentrations of 3% or above.

It was interesting that at a serum concentration of 1%, the adhesion strength of CHL cells approached the maximum possible. At this serum concentration or below, mainly fibronectin-fibronectin receptors interactions are involved in cell adhesion, while at serum concentrations above 1% cells will perform their adhesive function mainly through vitronectin-vitronectin receptors. One might therefore suggest that the strength of cell adhesion does not significantly depend on whether the cell adhesion function is performed through fibronectin or vitronectin. Consequently, it is possible to speculate that, since fibronectin receptors and vitronectin receptors are integrin receptors (Akiyama et al 1990) both of these receptors might exert the same effect on the cell, as far as the strength of cell adhesion is concerned.

Even at a very low serum concentration (0.05%) there was a measurable and reproducible adhesion strength and therefore adhesion strength of CHL cells in the complete absence of serum was tested out of interest. In other words the c.s.s. of detachment of CHL cells was measured and it will be discussed below.

5.2.1.2. POSSIBLE MECHANISMS BY WHICH SERUM EXERTS ITS EFFECT ON ADHESION STRENGTH.

As was discussed in chapter 4,the mechanism by which a cell perform its adhesion function depends on whether there is serum in culture medium or not. In other words cell adhesion is mediated by

surface adsorbed serum adhesion proteins in the presence of serum, while in the absence of serum cell adhesion is performed by direct interactions between cell surface molecules and the substratum (Grinnel 1979; Steele et al 1991). Moreover, as was discussed in chapter 4,cell adhesion is an active process in the presence of serum whereas it is a passive phenomena in the absence of serum. It could therefore be said that cell adhesion, in the absence of serum, is just a linkage of cell surface molecules to the substratum and there is no subsequent further process. In fact there was no morphological difference, based on the observation by light microscopy, between the morphology of the cells that were incubated for 1 hour and those that were incubated for 24 hours i.e, in both cases the cells were circular. Consequently due to the lack of any subsequent process (e.g spreading) the cell adhesion strength in the absence of serum was 30% of that in the presence of 10% foetal calf serum. In the presence of serum the cells were spreading and the degree of spreading was dependent upon serum concentration . That is, there was little flattening in the presence of 0.05% serum but there was more flattening in the presence of 0.1% serum and at 1% serum the cells were spreading completely. Thus, there was no difference between the spreading of CHL cells at 1% serum and 10% serum, as was observed by light microscopy. It is therefore possible to suggest that at 0.05% serum there were not enough ligands to interact with related cell surface receptors and therefore the cells could not gain maximum possible adhesion strength. In contrast, at a serum concentration of 1% or above there was enough adsorbed proteins, (ligands) to interact with receptors and thus the cells were able to perform complete adhesion functions and subsequent spreading. Thus cells gained maximum possible adhesion strength. Adhesion receptors not only link

cells to surface coated proteins but, as discussed in chapter 1, they are connected to cytoskeletal proteins as well (Ruoshlahti 1988). Thus, these receptors might regulate whole cell processes by binding to related ligands and cytoskeletal proteins and effectively transducing signals from the exterior of the cell to the cell interior; these signals may trigger changes in other cell functions such as in gene expression (Juliano and Haskill 1993). Hence the type and concentration of adsorbed proteins may play a crucial role in the whole cell adhesion process. As will be discussed in chapter 6, if relevant proteins are provided then, even when serum is absent cells will achieve their final adhesion strength.

Another question related to serum was whether the origin of serum will have any influence in CHL cell adhesion. This will now be discussed.

5.2.2. THE EFFECT OF SERA OF DIFFERENT ORIGIN ON THE ADHESION STRENGTH OF CHL CELLS.

After examining the effect of serum concentration upon the adhesion strength of CHL cells it was of interest to discover whether or not sera from different origin exerted similar or different effects on the adhesion strength of the CHL cells. CHL cells were therefore grown in a 10% of foetal calf serum, new born calf serum, or horse serum, as illustrated in materials and methods (2.5.3). The c.s.s. of detachment of the CHL cells was measured according to the detachment assay described in materials and method (3.4). The CHL cell adhesion strength was highest in 10% horse serum and lowest in the foetal calf serum. The actual values for the c.s.s. of detachment were 11.73±0.80 Nm^{-2} 11.66±0.66 Nm^{-2} , and 10.96±0.73 Nm^{-2} in horse serum, new born calf serum and foetal calf serum respecively

(Figure 5.2). There was only a 7% difference between the adhesion strength of CHL cells grown in the foetal calf serum and horse serum. These results indicated that there is no significant effect of the origin of serum in cell adhesion strength, as far as above serum origins and CHL cells are concerned. In fact it has been reported that there is no detectable difference between horse serum fibronectin and calf serum fibronectin, as far as structure and functions are concerned(Ehrishman et al 1982). However, as has been previously discussed (5.2.1) that at 10% serum in the culture medium, vitronectin plays a major role, rather than fibronectin, in cell adhesion. Kitagaki-Ogawa et al (1990) have studied the serum vitronectin from different species including horse and bovine and they observed that six different animal vitronectins, purified by heparin affinity chromatography equally promoted the spreading of BHK cells. The maximum cell-spreading activity, around 80% of the total attached cells was morphology of the spread BHK cells in the presence of different vitronectins were indistinguishable from each other (Kitagaki-Ogawa et al 1990). These findings support our results, although there are as yet no reports which indicate the relationship between the origin of the sera and cell adhesion strength. In this work we were able to show that origin of serum has no significant effect on CHL cell adhesion strength.

Perhaps it should be mentioned here that the effect of serum in cell adhesion is not limited to the adhesion proteins. There are other serum components which might also have a role in cell adhesion. These components may include molecules such as transferrin and growth hormones which are described below.

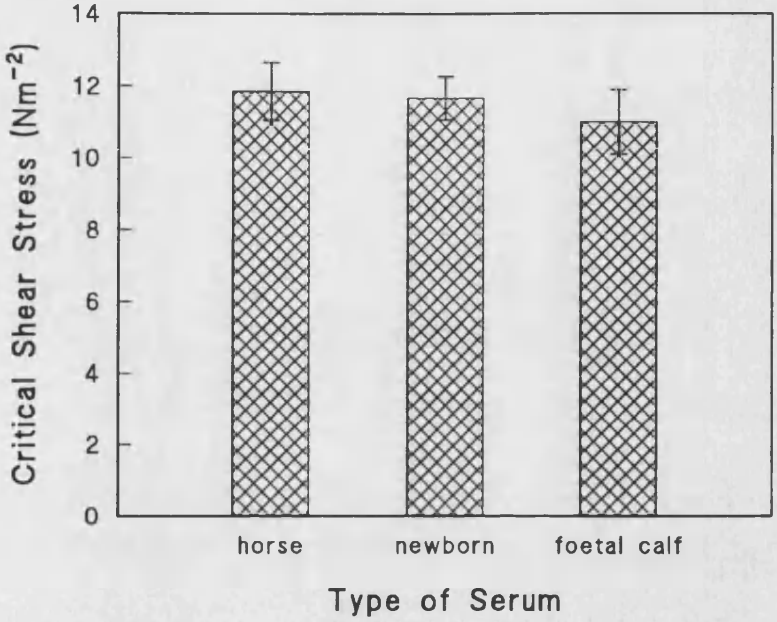


Figure: 5.2. The Effect of Origin of Serum In CHL Cells Adhesion strength.

CHL cells were seeded in the medium supplemented with 10% horse serum, or new born calf serum, or foetal calf serum on to plastic substratum. The adhesion strength of CHL Cells growing in these mediums is measured in terms of the critical shear stress (c.s.s) of detachment. Each data point represents four experiments in each of which 10 measurements were made. The error bars indicate the standard error of the mean. Full details may be found in the text.

5.2.3. GROWTH HORMONES.

Serum contains hormones and growth factors which are required for cells to proliferate (MacLeod 1988). However, transferrin and insulin enhance *in vitro* cell growth without serum (Mather and Sato 1979; Barnes and Sato 1980; Ito et al 1991). Serum hormones can alter the expression of affinity of receptors for proteins and might

therefore be expected to affect cell adhesion. Glucocorticoid is a serum hormone (Freshney 1989) and treatment of human fibrosarcoma cells with glucocorticoid increases the synthesis of fibronectin (Oliver et al 1983). Like hormones, growth factors can also affect cell adhesion. It has been reported (Blatti et al 1988) that the amount of fibronectin synthesised by AKR-2B mouse fibroblast cells was increased with treatment of epidermal growth factor. Therefore it is possible to suggest that although serum adhesive proteins are major factors in cell adhesion strength, hormones and growth factors also play a role.

CHAPTER 6 CELL ADHESION STRENGTH ON CHEMICALLY MODIFIED SUBSTRATUM

6.1. INTRODUCTION.

The previous chapter dealt with the effect of serum concentration on the adhesion strength of CHL cells. The influence of serum on cell adhesion strength was attributed to the presence of adhesion proteins, such as fibronectin and vitronectin, in the serum. However, serum contains a mixture of many adhesive and non-adhesive proteins (Curtis and Forrester, 1984). In order to further define the role of individual proteins, purified forms of adhesive proteins can be used in adhesion studies because they are commercially available. Normal plastic tissue culture dishes were therefore modified with a specific protein and studies undertaken to determine the effect of this modification upon cell adhesion.

The plastic surface of a tissue culture dish was coated with either fibronectin, collagen type IV, collagen type I, or polylysine. Apart from polylysine, cells usually bind to these proteins via a receptor-mediated mechanism that confers specificity to cell-protein interactions (Dedhar, et al 1987; Wayner and Carter, 1987; Herbst et al, 1988). Cells are able to bind to polylysine, which is positively charged, mainly via a non-specific interaction (Lauffenburger, 1993). Consequently, the effect of a polylysine-coating on cell adhesion strength was also of interest to this study.

6.2. RESULTS AND DISCUSSION

6.2.1. THE EFFECT OF PRE-ADSORBED FIBRONECTIN ON THE ADHESION STRENGTH OF CHL CELLS.

In order to further define the role of fibronectin in adhesion strengthening, tissue culture grade plastic dishes, each of which had a surface area of $72cm^2$, were each coated with various amounts of fibronectin, ranging from 0.1 µg to 50 µg . The fibronectin coating procedure was performed as described in Materials and Methods (2.6.1).

CHL cells were subcultured on fibronectin-coated dishes in serum-free medium. As a control, CHL cells were also seeded on non-coated dishes, again in serum-free medium. Finally, because it was also of interest to define the effect of the fibronectin coating on CHL adhesion strength in the presence of serum, CHL cells were seeded on dishes that had each been coated with 25 µg fibronectin in the presence of 10% foetal calf serum.

In all cases, the CHL cells were allowed to grow for 24 hours, before the c.s.s. of detachment was measured as described in chapter 3 (3.4).

A concentration-dependent spreading of CHL cells on fibronectin-coated dishes was observed. On dishes coated with less than 10 µg fibronectin, the cells were slightly flattened, and on dishes coated with 10 µg fibronectin the cells were completely flattened and spreading very well. The cells spread more even on dishes coated with 25 and 50 µg fibronectin in serum-free medium than on uncoated dishes in the presence of serum (See picture 6.1 and 6.3). The cells grown on non-coated dishes in serum-free medium were circular (picture 6.2). The data for these experiments are presented below.

The effect of fibronectin on the strengthening of CHL cell adhesion was concentration dependent. Nevertheless, even a very low amount of fibronectin significantly increased the strength of cell

adhesion. The c.s.s. of CHL cell detachment was 139% higher on dishes coated with 0.1 μg fibronectin, in the absence of serum, than on non-coated dishes; the c.s.s. values were 7.60±0.9 Nm^{-2} and 3.20±0.60 Nm^{-2}, respectively (figure 6.1). The cell adhesion strength increased with an increasing concentration of adsorbed fibronectin. For example, the force required to detach the CHL cells from dishes coated with 1 μg fibronectin was 8.40±0.94 Nm^{-2} while it was 9.56±1.62 Nm^{-2} on those coated with 10 μg fibronectin. This difference was not significant (P=0.13) but when the adsorbed fibronectin concentration was increased from 10 μg to 25 μg, the cell adhesion strength increased significantly, i.e. c.s.s. of detachment of CHL cells were 9.56±1.62 Nm^{-2} and 15.60±1.75 Nm^{-2} on 10 μg and 25 μg fibronectin-coated dishes, respectively (P=0.000).
Increasing the adsorbed fibronectin concentration above 25 μg did not change cell adhesion strength significantly: c.s.s. of detachment of CHL cells was 16.02±2.48 Nm^{-2} on 50 μg fibronectin-coated dishes (P=0.74, between 25 μg and 5 μg fibronectin coated dishes).

It was interesting to find that the c.s.s of detachment of CHL cells grown in serum-free medium on dishes coated with high concentrations of fibronectin (i.e. 25 μg and 50 μg) was significantly higher than that of cells grown on non-coated dishes in serum-containing medium (9.40±0.60 Nm^{-2} ;P=0.000). In addition, pre-adsorbed fibronectin was able to exert its effect on cell adhesion strength even in the presence of serum in the growth medium. For example, for CHL cells grown in serum-containing medium, the c.s.s. of detachment was 16.79±1.03 Nm^{-2} on dishes coated with 25 μg fibronectin while it was 9.40±0.60 Nm^{-2} on non-coated dishes (P=0.000). The results will now be discussed.

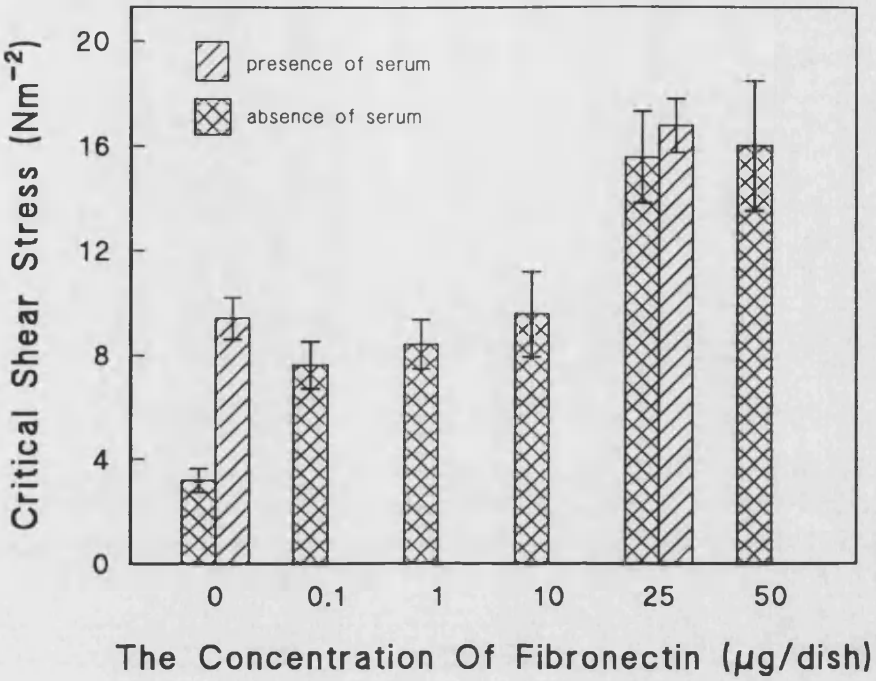
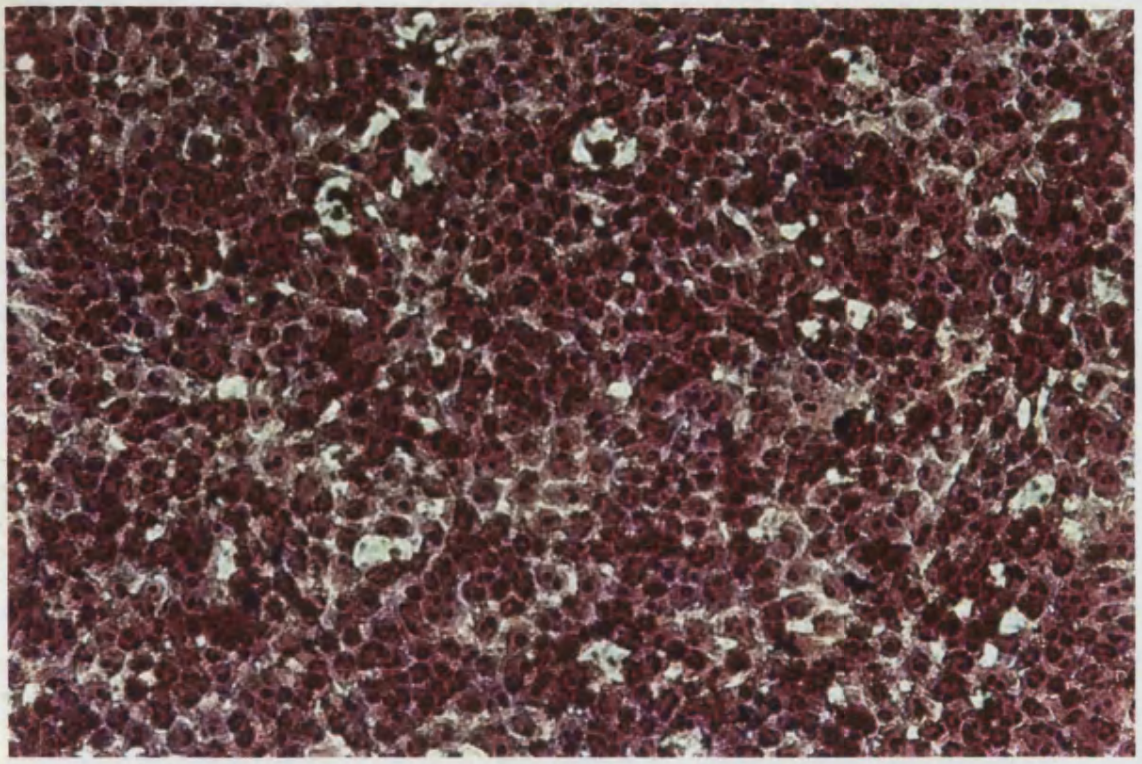


Figure 6.1 The Effect of Pre-adsorbed Fibronectin on the adhesion strength of CHL cells.

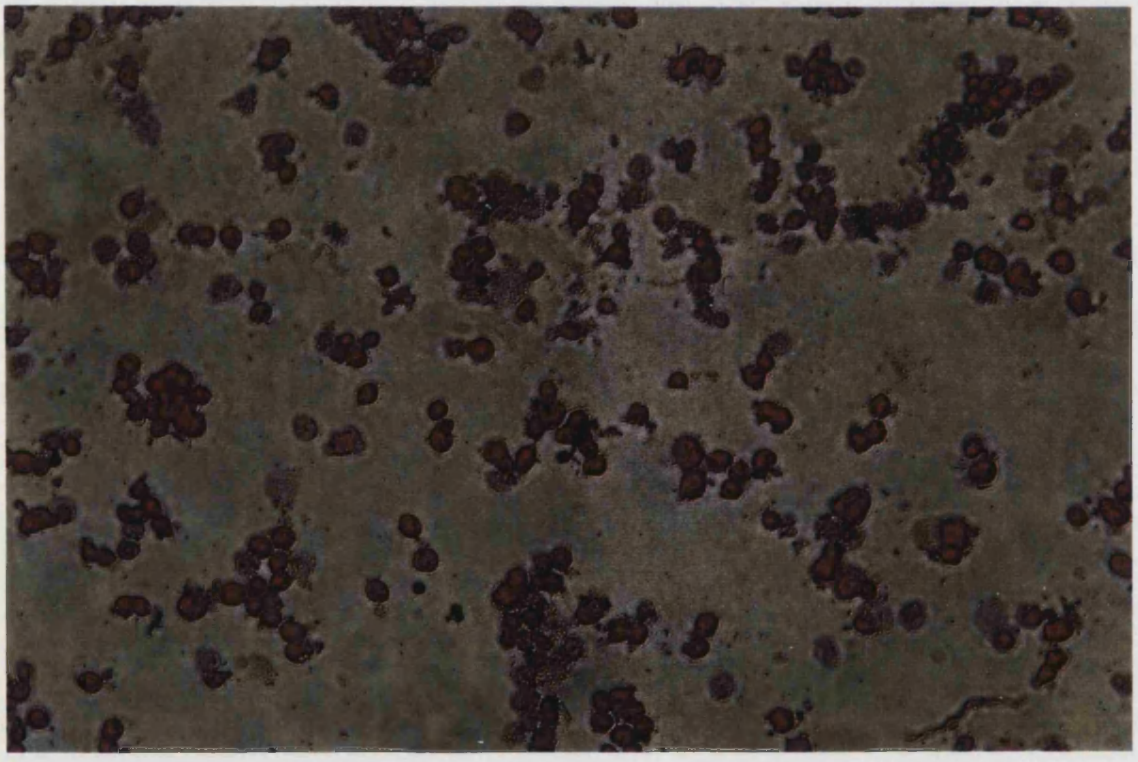
CHL Cells were seeded on various concentrations (0.1 to 50μg) of fibronectin-coated plastic tissue culture dishes, each of which had a 72 cm^2 surface area (Materials and Methods). The cells were allowed to grow for 24 hours, either in a serum-containing or serum-free medium. The adhesion strength of these cells in terms of the critical shear stress (c.s.s.) of detachment were measured in the Micro Flow Chamber. Each data point represents the mean of three different experiments, in each of which ten measurements were made. Further information can be found in text.

The results obtained in response to pre-adsorbed fibronectin indicate that even very low amounts of fibronectin (e.g 0.1 μg/dish) are able to significantly strengthen cell adhesion (see figure 6.1). However, because the fibronectin was only adsorbed to the plastic, it was not known how much of it remained fixed to the surface after extensive washing. Iodinated fibronectin (^{125}I-Fn) was therefore employed to determine the number of molecules adsorbed per unit area of the plastic tissue culture dish. The details of the procedure are described in Materials and Methods. Briefly, ^{125}I-Fn was coated onto the tissue culture plastic overnight and the procedure given above, for coating with non-iodinated fibronectin, was followed (also see Materials and Methods). The coated ^{125}I-Fn was extracted twice with 1M NaOH and counted using a gamma counter. CHL cells were seeded on various concentrations (0.1-50 μg) of ^{125}I-Fn. The counts per minute was used to calculate the amount of adsorbed fibronectin.

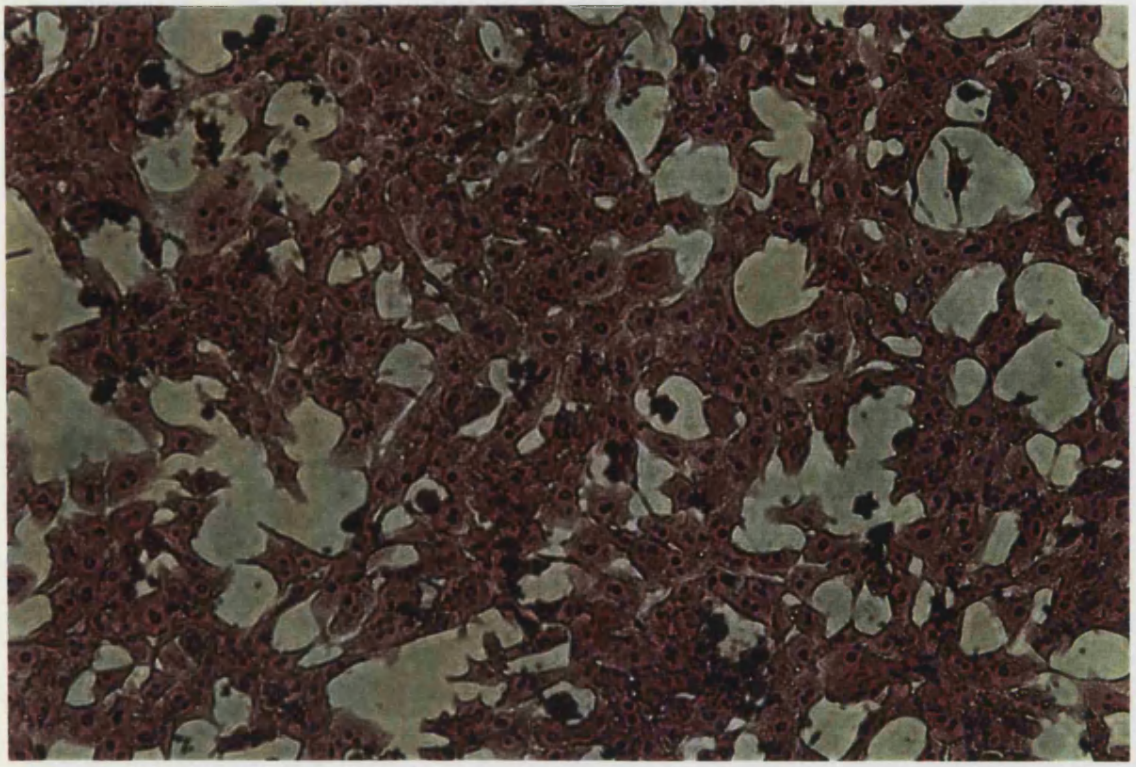
According to the ^{125}I-Fn experiments, about 70% (68.68%±3.99%) of the added fibronectin was adsorbed to the surface. For example, when 72 cm^2 dishes were coated with 0.1 μg and 25 μg fibronectin, the number of molecules which remained adsorbed was 13.2 and 3320 molecules mm^{-2} respectively. This meant that the presence of only 13.2 molecules of fibronectin mm^{-2} was sufficient to increase cell adhesion strength significantly. It would be interesting to find out whether this amount of molecules were covering the surface underneath the cell or not. For this purpose the following assumptions were made. Fibronectin is a rod shaped molecule with a length of 60-70nm and a width of 2-3nm (Ito et al, 1991). Assuming that fibronectin acquires a flat rectangular shape after its adsorption



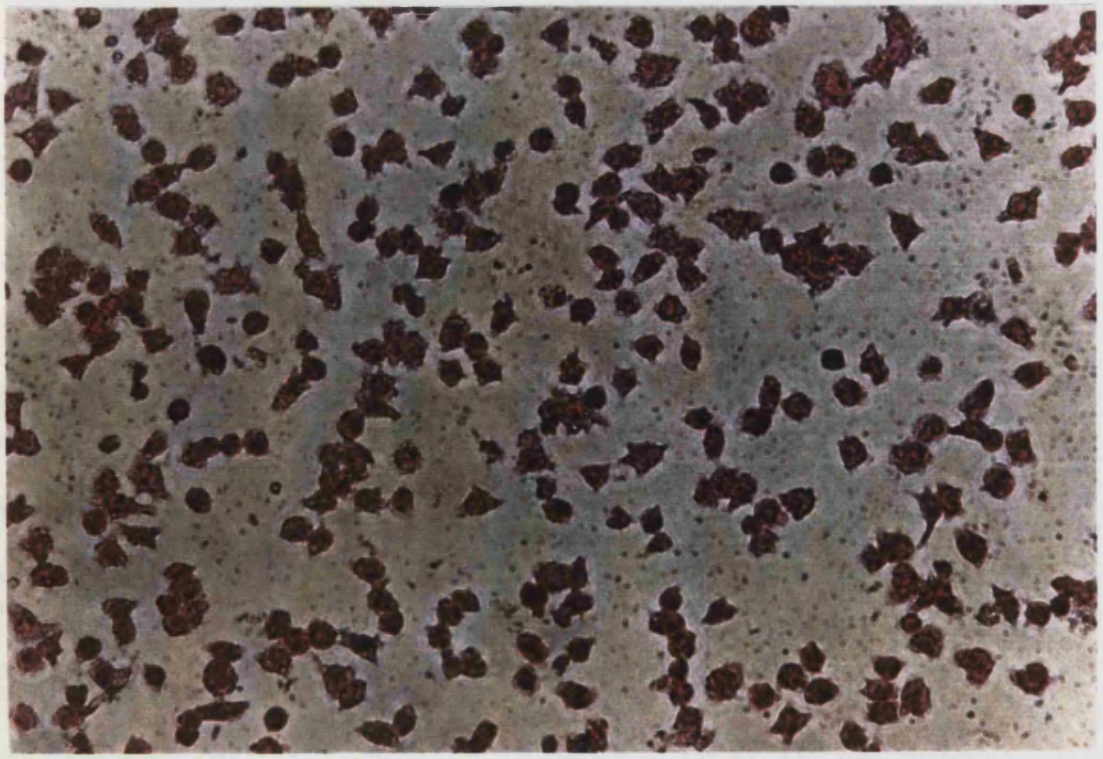
Picture 6.1. CHL Cells on Uncoated Dish in the Presence of Serum.



Picture 6.2. CHL Cells on Un Coated Dish in the Absence of Serum.



Picture 6.4: CHL Cells On Fibronectin Coated Dish In The Absence Of Serum.



Picture 6.4: CHL Cells On Polylysine Coated Dish In The Absence Of Serum.

onto the plastic surface, the area which the molecule will cover can be calculated as 195 nm^2 (=0.195 $x10^{-3}$ mm^2) as shown in Table 6.2.

As indicated above, and making an assumption about the size of the surface bound fibronectin, there are 5.3 x 10^5 molecules required per cell (also assuming a CHL cell is a 10 μm sphere and covers the area of 104 μm^2).

Applied FN per dish (=72 cm^2)	Adsorbed FN Molecules/cell	C.S.S. (Nm^{-2})
0 μg	0	3.17±0.60
0.1 μg	1373	7.60±0.90
1 μg	13728	8.40±0.94
10 μg	138216	9.56±1.62
25 μg	345280	15.50±1.75
50 μg	690560	16.02±2.48

Table 6.1.: The Relationship Between Adsorbed Fibronectin and C.S.S.
^{125}I-Fibronectin was coated on the tissue culture grade plastic dish as illustrated in Materials and Methods. The number of adsorbed fibronectin (FN) molecules/cell was calculated as described in Materials and Methods. The Critical Shear Stress of detachment (c.s.s.) of CHL cells growing on this adsorbed fibronectin was measured in terms of Nm^{-2}. Further details may be found in the text.

L (nm)	W (nm)	A (nm^2)	Molecules required/cell
65	3	195	533333

Table 6.2: The Size of A Fibronectin Molecule.
On the basis of theoretical estimated size of adsorbed fibronectin, number of molecules required to cover the underneath of the cell surface was calculated by assuming the size of cell 104 μm^2. Further details may be found in the text.

As can be seen in Table 6.2, in order to cover the underneath of a cell, the application of more than 25 μg fibronectin is required on a 72cm^2 dish. However, the application of 0.1 μg was sufficient to strengthen cell adhesion significantly and it is therefore possible suggest that fibronectin is acting as an activator rather than a mediator. Although the fibronectin effect was concentration dependent, there was not a linear relationship between the concentration of fibronectin and the adhesion strength of the CHL cells. For example, when fibronectin concentration was increased 2.5-fold (1.38 x 10^5 molecules/104 μm^2 to 3.4 x 10^5 molecules/104 μm^2), the cell adhesion strength increased only 1.6-fold (9.56±1.62 Nm^{-2} to 15.50±1.75 Nm^{-2}, respectively). Increasing the fibronectin concentration above 3.4 x 10^5 molecules/104 μm^2 did not have a significant effect on the cell adhesion strength. Therefore, it is possible to gest that at this fibronectin concentration, at which 65% of a CHL cell surface is occupied, the CHL cells gained a maximum possible adhesion strength and increasing the amount of fibronectin would not make a significant change to the CHL cell adhesion strength. A similar point was made by Truskey and Pirone (1990). These authors investigated the number of cells remaining attached on fibronectin-coated microscope slides after exposing a constant shear force (4.7 Nm^{-2}) for 2 hours. It was reported that the adhesion of 3T3 cell was maximal at 10ng fibronectin cm^{-2}, and that above this concentration adhesion was independent of fibronectin concentration.

It was interesting to notice that there were two points of sudden increase in the plot of c.s.s. of CHL cells versus adsorbed fibronectin concentration (Figure 6.1). One was between non-coated dishes and 0.1 μg fibronectin dishes and the other was between 10 μg and 25 μg fibronectin. A possible explanation for this could be that

at a concentration of 0.1 μg fibronectin, although there were not enough fibronectin molecules to bind all fibronectin receptors of the CHL cells, the molecules present were able to activate cells (see following Section) enabling them to increase their adhesion strength. Alternatively, at concentrations of fibronectin of 25 μg or above, almost all of the receptors might be occupied by fibronectin molecules. Therefore, at these higher concentrations (cells were spreading very well) cell adhesion strength was even greater due to both increased receptor-ligand bonds and activation. Thus it would be convenient to divide the adhesion strengthening effect of fibronectin into two discussion topics: the role of receptor-ligand bonds and signalling.

6.2.1.1. THE ROLE OF RECEPTOR LIGAND BONDS IN CELL ADHESION

As it can be seen in Figure 6.1, cell adhesion strength increases with increasing ligand (fibronectin) concentration. Therefore, it could be said that an increased concentration of ligands leads to an increase in the number of receptor-ligand bonds that will eventually strengthen cell adhesion. This was also suggested by Cozens-Roberts et al (1990) in their theroetical studies and reinforced by Truskey and Prolux (1993). In the detachment process (see Chapter 3) bonds formed between the cell and the substratum will be pulled apart or broken by the applied force; hence, as the number of bonds inreases, the force required to break the cell-substratum linkage would also increase. However, it would be wrong to simply assume that adhesion increases with an increasing number of bonds because the receptor is not a simple passive antenna-like component whose function is restricted to recognizing certain molecules. Since cells are bound to ligands through their

transmembrane receptors, the binding of a receptor to a ligand will send a message to the interior of cell; the cell would act according to this signal and possibly be able to perform further processes (Simon 1992). The effect of signaling on adhesion will now be discussed.

6.2.1.2. SIGNALLING AND CELL ADHESION

As stated above, it seems likely that a signaling mechanism mediates fibronectin-enhanced adhesion strength. Recently, Curtis et al (1992) reported that fibronectin is able to incease the adhesiveness of BHK cells through a signaling mechanism. BHK cells, in suspension, were exposed to 2.8 μm beads which had been covalently derivatized with fibronectin. Attachment of even a single bead significantly increased cell adhesion and spreading on a solid surface onto which haemoglobin had been adsorbed. The BHK cells poorly adhered to the haemoglobin-coated surfaces in the abscence of fibronectin-coated beads. It was therefore suggested that the attachment of fibronectin-coated beads triggers a systematic increase in adhesiveness (Curtis et al 1992).

Signals can be generated in adhesion by adhesion receptors. Cell contact with the substratum causes receptor clustering; this clustering at the site of contact generates signals and these signals can regulate adhesion (Gingell, 1992). The exact mechanism of integrin-mediated signaling is not known but there are two different views on this issue. The first one is that integrins are true receptors capable of giving rise to biochemical signals within the cell. In this case, the effects on cytoskeleton are mediated by small molecules such as cAMP (Gingel and Owens 1992). The second view is that integrins transmit signals by organizing the cytoskeleton, thus

regulating the shape and internal cellular architecture of the cell (Juliano and Haskil, 1993).

The determination of the signaling mechanism was not an aim of this study but from the above reports it is possible to suggest that fibronectin binding via receptors gives a signal to the cell and this will lead to a strengthening of cell adhesion. However, this may be oversimplifying the situation and it could be that a variety of stimuli are involved in strengthening cell adhesion. Some of the possible stimuli are discussed below.

6.2.1.3. ADHESION STRENTHENING VIA FORMATION Of FOCAL ADHESIONS AND CYTOSKELETALl ORGANIZATION.

It is known that b subunits of integrins connect the outside of the cells to cytoskeletal molecules (Chapter 1.2.2.). This assembly may, therefore, trigger the biochemical events responsible for the formation of focal contacts and cytoskeletal organization (focal contacts are areas of the cell surface where cytsokeletal molecules and extracellular components combine to produce stable cell-matrix interactions [Woods and Couchman, 1988]). The signaling mechanism that facilitates focal adhesion and the accompanying cytoskeletal reorganization are not completely understood (Romer et al 1992). However, some of the possible mechanisms can be discussed:

a) cAMP.

It is known that elevating cAMP levels, by cAMP activator agents such as dibutyril-cAMP (dbcAMP), can increase cellular adhesion and spreading (Hsie et al 1975). For example, it was reported that AD^{V} F11 cells (CHO variants) which were unable to adhere fibronectin-coated dishes, were able to do so after treatment with dbcAMP (Cheung and Juliano, 1985). cAMP exerts its effects

through cAMP-dependent protein kinases. Increasing cAMP concentration increased protein kinase activity and, consequently, protein phosphorylation (Cheung et al, 1987). Thus it could be said that the binding of fibronectin with its receptor induces conformational changes in the cytoplasmic portion of fibronectin receptors, which in turn increases cAMP levels in the cell resulting in the activation of protein kinases. The protein kinases may be stabilizing the adhesion protein-receptor-cytoskeleton via the phosphorylation of some of the molecules in this complex, as is now discussed.

b) PHOSPHORYLATION.

It has recently been reported that fibronectin coating results in an increase of protein phosphorylation of 3T3 cells (Burridge et al 1992) and previously it had been reported that phosphorylation stabilizes integrin-cytoskeleton interactions (Suzuki et al 1987). In addition, when fibroblasts were spread on fibronectin, cytoskeletal proteins but not integrin receptors became phosphorylated (Gingel 1993). Fibronectin-integrin interactions may therefore cause protein phosphorylation of cytoskeletal proteins, which in turn may strengthen the fibronectin-integrin complex binding and thus strengthen cell adhesion.

6.2.2 EFFECT OF PRE-ADSORBED FIBRONECTIN ON THE ADHESION STRENGTH OF CHL CELLS IN THE PRESENCE OF SERUM.

It was interesting to find that, when the cells were grown in serum-containing medium, cell adhesion strength was significantly higher on dishes pre-adsorbed with fibronectin than on non-coated surfaces. However, serum fibronectin did not increase cell adhesion as much as pre-adsorbed fibronectin. As was discussed in Chapter 5, in medium containing 10% feotal calf serum, only 38ng cm^{-2}

fibronectin was adsorbed on tissue culture dishes (Steele et al, 1991), whereas in this study 243ng cm^{-2} (25 µg $dish^{-1}$) fibronectin was pre-adsorbed, i.e. about 15 times more than fibronectin would adsorb when present in serum-free medium. It is therefore probable that cell adhesion was lower on non-coated dishes than on fibronectin-coated dishes because less fibronectin was adsorbed to the former.

It was suprising to find that the adhesion strength of CHL cells on surfaces pre-adsorbed with 25 µg fibronectin was not significantly affected by the presence or absence of serum in the growth medium (c.s.s. 16.79±1.03 N m^{-2} and 15.50±1.75 N m^{-2}, respectively; P=0.23). This could suggest that once fibronectin-activated events have begun and the sequence of events leading to the final adhesion strength is initiated, then the adhesion strength is independent of the presence of serum.

Having established that fibronectin dramatically enhances the strength of cell adhesion, it was considered interesting to investigate what effect other adhesion proteins would have on cell adhesion. Collagen type IV and collagen type I were selected for investigation.

6.2.3.EFFECT OF PRE-ADSORBED COLLAGEN TYPE IV ON THE ADHESION STRENGTH OF CHL CELLS.

CHL cells were seeded on the collagen type IV-coated tissue culture dishes instead of fibronectin-coated dishes. The details of collagen type IV coating were outlined in the Materials and Methods (2.6.2). Briefly, various amounts of collagen type IV (0.1 µg to 50 µg) were dissolved in sterile double distilled water and placed in 72 cm^2 dishes which were left to coat overnight in a laminar flow

cabinet. Immediately before use, unadsorbed collagen type IV was removed by extensive washing with PBS and serum-free medium. In order to determine how much collagen type IV was adsorbed on the surface, the protein could have been iodinated and the adsorption determined as described for fibronectin. Unfortunately, financial constraints ruled this out. Nevertheless, the collagen type IV-coated dishes were used to determine the effect of pre-adsorption of collagen type IV on the adhesion strength of CHL cells. CHL cells were seeded on the coated dishes and grown for 24 hours at 37^{o}C in serum-free medium. Finally, detachment of CHL cells was measured as described in Chapter 3.4. As a control, cells were grown in the presence and absence of serum on uncoated dishes. In addition, cells were seeded on the 25µg collagen type IV-coated dishes in serum-containing medium to see whether the protein coat could exert its effect(s) in the presence of serum. The results are now given.

Like fibronectin, preadsorption of collagen type IV increased CHL adhesion strength significantly. The effect of the coating was concentration dependent, although this relationship was not linear (Figure 6.2). Even a very low amount of collagen type IV was able to significantly increase cell adhesion strength. The c.s.s. of detachment of CHL cells was 3.20±0.40 Nm^{-2} on non-coated dishes while it was raised to 5.54±0.7 Nm^{-2} on 0.1 µg collagen IV-coated dishes (P=0.001). The strength of cell adhesion increased as the amount of collagen type IV used for pre-adsorption increased from 0.1 µg to 10 µg. Above 10 µg, the cell adhesion strength was not affected significantly. The c.s.s of detachment of CHL cells was 13.06±1.80 Nm^{-2} on 10 µg collagen IV-coated dishes while it was 13.29±0.88 Nm^{-2} on 25 µg collagen IV-coated dishes (P=0.76).

It was interesting to observe that a coating of collagen type IV enabled a strengthening of cell adhesion even in the presence of 10% foetal calf serum. The c.s.s. of detachment of CHL cells grown in the presence of serum was 13.95±0.80 Nm^{-2} on 25 μg collagen type IV-coated dishes while it was 9.60±0.80 Nm^{-2} on non-coated dishes. The explanation for this enhanced cell adhesion strength could be that fibronectin, present in the serum, mediated a strengthening of collagen-cell adhesion (Grinnel and Minter 1978) or, alternatively, that serum anti-adhesive proteins (Curtis and Forrester 1984) might be less active on collagen coated surfaces than on non-coated tissue culture dishes. Therefore collagen coated surfaces are biologically more favourable than non-coated dishes in the presence of serum. Thus cells are able to perform their adhesive functions more efficiently, resulting in an increased cell adhesion strength. The results indicate that CHL cells are able to adhere directly to collagen type IV. In other words, CHL cells are expressing adhesion receptors which are able to mediate CHL cell-collagen type IV interactions. The cell surface receptors for the interaction of collagen type IV are members of integrin family i.e. $\alpha_1\beta_1$ and $\alpha_2\beta_1$ (Vanderberg et al, 1991).

Therefore, as in the case of fibronectin, CHL adhesion to collagen type IV, via these integrin receptors, may trigger further activities which result in a dramatic increase in the adhesion strength of CHL cells; for example, the coating of dishes with 25 μg collagen type IV increased cell adhesion strength by 4-fold.

After finding that the modification of tissue culture dish enhanced CHL cell adhesion strength, it was considered interesting to determine whether modification of surfaces with collagen type I - a fibrillar collagen whereas collagen type IV is a non-fibrillar collagen (Hulmes 1992) - will exert similar effects on cell adhesion.

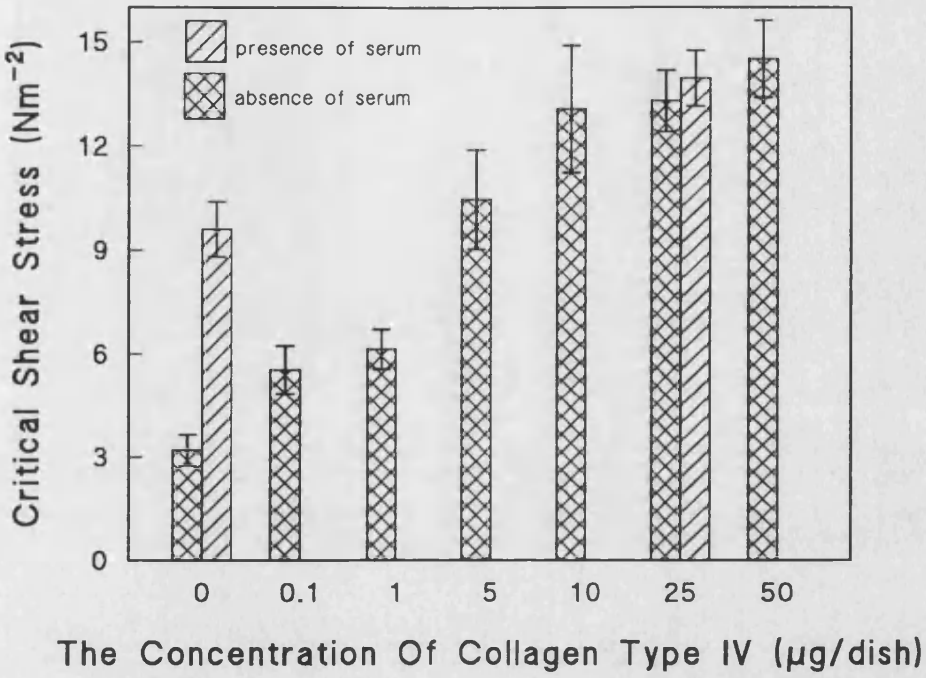


Figure 6.2 The Adhesion Strength of CHL cells on the Collagen Type IV Pre-adsorbed Plastic Surface.

CHL Cells were seeded on various concentrations of collagen type IV (0.1 to 50µg) -coated plastic tissue culture dishes, each of which had a 72cm2 surface area. The collagen type IV coating was carried out as described in Materials and Methods. Cells were allowed to grow for 24 hours, either in a serum-containing or serum-free medium. The adhesion strength of the cells were then measured in the Micro Flow Chamber. Each data point represents the mean of three different experiments, in each of which ten measurements were made. Further information can be found in text.

6.2.4. EFFECT OF PRE-ADSORBED COLLAGEN TYPE I ON THE ADHESION STRENGTH OF CHL AND HELA B CELLS.

Collagen type I from calf skin was used to coat tissue culture dishes as described in Materials and Methods (2.6.3). Briefly, 10-200 µg collagen type I was dissolved in 0.1 M acetic acid and added to 72

cm^2 culture dishes, which were left to coat overnight in the laminar flow cabinet. Immediately before use, unadsorbed collagen was removed by extensive washing with PBS followed by serum-free medium. As with collagen type IV, financial constraints ruled out the determination of the amount of protein adsorbed on the surface using 125-iodination. Nevertheless, the collagen type I coated dishes were used for the determination of the effect of pre-adsorbed collagen type I on the strength of cell adhesion. Therefore, CHL and Hela B cells were seeded on collagen-coated and non-coated (control) dishes in serum-free medium. In addition, CHL cells were seeded on 25 µg collagen type I in the presence of serum and both CHL and HeLa B cells were seeded on non-coated surfaces in the presence of serum.

6.2.4.1.THE EFFECT OF PRE-AFDORBED COLLAGEN TYPE I ON CHL CELL ADHESION.

In dramatic contrast to dishes coated with fibronectin and collagen type IV, dishes coated with collagen type I in the absence of serum did not have any significant effect on the adhesion strength of CHL cells, even when coated with collagen type I amounts as high as 100 µg. The c.s.s.of detachment of CHL cells was 3.60±0.45 Nm^{-2} while it was 3.90±0.55 Nm^{-2} and 3.90±0.43 Nm^{-2} on 25 µg and 50 µg collagen I coated dishes, respectively (P=0.50). Further increasing the collagen concentration to 100 µg resulted in c.s.s of CHL cells of still only 4.05±0.43 Nm^{-2}.

Although collagen type I pre-adsorption did not affect CHL adhesion strength in the absence of serum, it was able to significantly strengthen CHL cell adhesion strength in the presence of serum. The c.s.s. of detachment of CHL cells was 9.90±1.13 Nm^{-2} on

non-coated plastic dishes while it was 14.60±1.25 Nm^{-2} on 25 μg collagen I-coated dishes (P=0.0064).

There was no morphological difference between cells that were grown on non-coated or collagen type I-coated dishes. In both cases the cells remained spherical, even after 24 hours of incubation. If we assume that 70% of the added collagen type I was adsorbed (i.e. in equal proportion to fibronectin), then the concentration of adsorbed collagen in the case of 200 μg collagen per dish would be about 1944ng cm^{-2} (tissue culture dishes have surface area 72 cm^2) which is about 100 times higher than that required for BHK cell spreading (BHK cells spread on a concentration of 15-20 ng collagen type I/cm^2 [Hanski et al, 1986]). Therefore, it would appear that there is no possibilty of a requirement for less ligand concentration before CHL cell spreading can occur, and in any case, it was indeed observed that Hela B cells were spreading very well on 25 μg collagen type I coated dishes. Although there was no increase in the adhesion strength of CHL cells on collagen-coated surfaces in the absence of serum, a significant enhancement of the adhesion strength of CHL cells on collagen-coated dishes in the presence of serum (Figure 6.3) might suggest that CHL cells are unable to bind to collagen directly. Therefore, pre-adsorption of collagen type I did not make a significant difference to adhesion strength in the absence of serum. Fibronectin, present in serum, might mediate collagen type I-CHL cell interaction and thus strengthen adhesion to a collagen type I-coated surface.

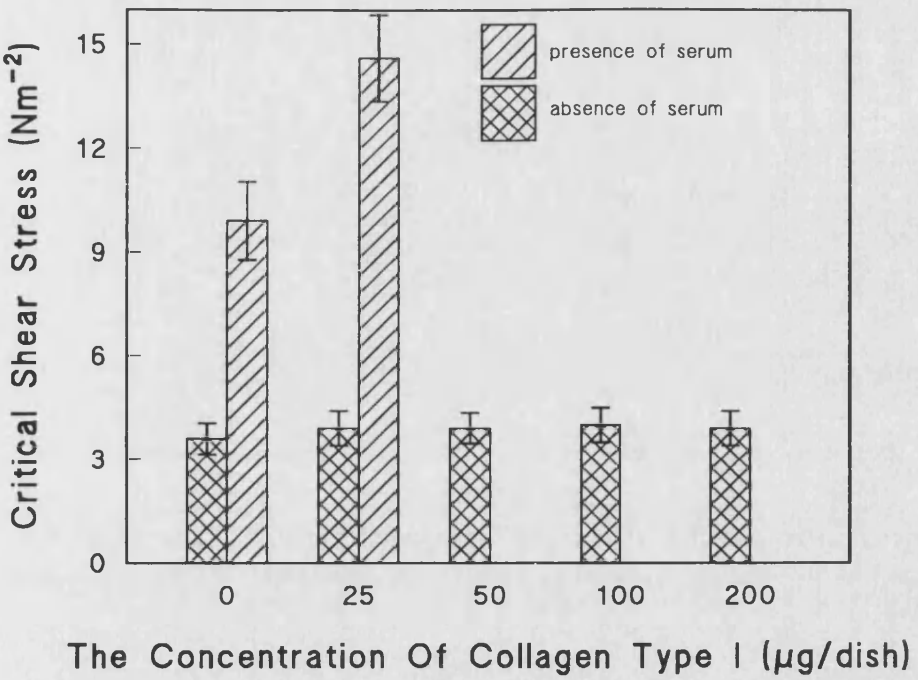


Figure 6.3. The Effect of Pre-adsorbed Collagen Type I on the Adhesion strength of CHL cells.

CHL cells were seeded on various concentration of collagen type I (25 to 200 µg) coated tissue culture grade plastic dishes each of whic has a surface area of 72 cm^2. Cells cells were allowed to grow for 24 hours either in serum free medium or in serum contains medium. Then the adhesion strength of cells in terms of c.s.s. of detachment of cells were measured in Microflow Chamber. Each data point represents the mean of three different experiments in each of which ten measurements were made. Error bars indicate standart error of that mean, Further information can be found in text.

It has, in fact, been reported that some cells require added fibronectin in order to mediate adhesion to collagen - for example, SV-3T3 cells (Klebe, 1974) and CHO cells (Kleinman et al, 1979,1981) - while other cells can interact directly - for example

BHK cells (Grinnel and Minter, 1978) and HeLa B cells (Schor and Court, 1979). However, some cells do not need exogenous fibronectin in order to perform their adhesive function on collagen-coated surfaces since they can adhere to the substratum via cellular fibronectin (Scott et al, 1983). It was shown that the adhesion of Bovine Corneal Endothelial (BCE) cells on collagen-coated surfaces was inhibited by antibovine fibronectin antibody, while in the absence of antibody cells were able to adhere and spread on the collagen-coated dishes (Scott et al, 1983). Hence it is possible to suggest, in our case, that CHL cells could neither express cell surface receptors which were able to mediate CHL cell-collagen type I adhesion nor synthesise enough fibronectin to mediate CHL cell adhesion. As discussed in the fibronectin-coating Section, even very low amounts of pre-adsorbed fibronectin significantly strengthened the adhesion of CHL cells. This might also suggest that in the absence of serum, CHL cells were unable to synthesise fibronectin. Further evidence for the role of cellular fibronectin was that BHK cells, which have a high level of fibronectin, were able to adhere very well on collagen-coated surfaces while derivatives of BHK cells (PyBHK), possessing no detectable fibronectin, were unable to adhere to this substratum without serum or fibronectin (Pearlstein, 1976)

One might ask why, in the absence of serum, CHL cells are unable to spread and strengthen their adhesivity on collagen type I-coated dishes when the adhesion strenth of CHL cells on type IV-coated dishes increases dramatically. Although both of these collagen ligands can bind to the same integrin receptors, $\alpha_2\beta_1$ and $\alpha_1\beta_1$, $\alpha_2\beta_1$ shows better binding with collagen type I and, likewise, $\alpha_1\beta_1$ with collagen type IV (Kramer and Marks, 1989). Although CHL cells might synthesise adhesion receptors, for instance a_1b_1, which may mediate

CHL cell-collagen type IV adhesion, these receptors may be unable to mediate collagen type I adhesion. Moreover, it is possible that CHL cells are unable to synthesise cell surface receptors which can mediate CHL cell-collagen type I adhesion, since different cells are known to express different collagen receptors; e.g. primary Rat Hepatocytes express $\alpha_1\beta_1$, while rat cells express $\alpha_2\beta_1$ (Gullber et al, 1992).

In contrast to the uneffectiveness of pre-adsorbed collagen type I in the absence of serum, CHL adhesion strength was significantly increased on 25 μg collagen-coated dishes in the presence of serum. The c.s.s.of detachment of CHL cells was 9.90+1.13 Nm^{-2} and 14.60+1.25 Nm^{-2} on non-coated and 25 μg collagen-coated surfaces, respectively (P=0.006). This strengthening could be caused by serum fibronectin mediating collagen type I and CHL cell adhesion, thus increasing the CHL cell adhesion. It could also be caused by serum anti-adhesive proteins being less effective on collagen type I-coated surfaces than non-coated surfaces. CHL cells would therefore be able to perform their adhesion function more efficiently than on non-coated dishes.

After finding that collagen type I did not effect the adhesion strength of CHL cells in the absence of serum, it was interesting to discover the effect of pre-adsorbed collagen type I on the adhesion strength of HeLa B cells, since it was known that these cells possess collagen receptors (Lu et al 1989). The effect of pre-adsorbed collagen I on HeLa B cell adhesion strength will now be discussed.

6.2.4.2. EFFECT OF PRE-ADSORPTION OF COLLAGEN TYPE I ON THE ADHESION OF HELA B CELLS.

HeLa B cells were seeded on collagen type I coated dishes in the absence of serum, as described for CHL cells (6.2.3). As controls, HeLa B cells were cultured on non-coated dishes both in the absence and presence of serum.

Unlike CHL cells, HeLa B cells were able to spread on collagen type I-coated dishes. HeLa B cells were also able to strengthen their adhesion on this substratum. HeLa B cell adhesion strength was significantly increased, even on 10 µg collagen type I-coated dishes. The c.s.s. of detachment of HeLa B cells was 2.87±0.35 Nm^{-2} on non-coated dishes while it increased to 4.98±0.35 Nm^{-2} on 10 µg collagen type I-coated dishes (P=0.0001). As the pre-adsorbed collagen type I concentration increased from 10 µg to 50 µg, cell adhesion strength increased as well. (Figure 6.4). However, increasing collagen type I concentration above 50 µg did not increase the adhesion strength of HeLa B cells. The c.s.s. of detachment of Hela B cells was 9.38±0.96 Nm^{-2} and 9.55±0.95 Nm^{-2} on 50 and 75 µg collagen type I-coated dishes, respectively (P=0.75). Therefore, more than 75 µg collagen type I was not used.

The results indicate that, unlike CHL cells, HeLa B cells are able to interact directly with collagen type I. In other words, HeLa B cells have receptors which mediate cell adhesion to collagen type I. Thus, the modification of the tissue culture dish with this protein enhanced the adhesion strength of HeLa B cells. In fact, collagen receptors of HeLa B cells have been identified and characterized by Lu and co-workers (Lu et al, 1989).

It was somewhat suprising to find that the adhesion strength of HeLa B cells was significantly lower on 75 µg collagen type I-coated dishes (at which c.s.s of detachment of HeLa B cells was maximum in the absence of serum) than on uncoated dishes in the presence of

serum. The c.s.s values were 9.55±0.95 Nm^{-2} and 20.83±1.86 Nm^{-2}, respectively (P=0.000). This was different from the activation of CHL cell adhesion strength by pre-adsorbed fibronectin or collagen type IV, in which CHL adhesion strength was significantly higher in the absence of serum than
on non-coated surface in the presence of serum (Figure 6.1 and 6.2). This might suggest that unlike fibronectin and collagen type IV activation of CHL cell adhesion, the enhancement of HeLa B cells by collagen type I is mainly due to the number of receptor-ligand bonds (Cozens-Roberts et al 1990b). Hence, at a concentration of 50 µg collagen type I, almost all receptors were occupied and a further increase in ligand density did not significantly alter Hela B cell adhesion strength.

Fibronectin and collagens are adhesive proteins and cells adhere to these proteins through adhesion receptors (Hynes, 1987). In contrast, cells adhere to polylysine, an acidic amino acid, without receptors, via charge-charge interactions. The effect of polylysine coating on CHL cell adhesion was therefore invesitgated.

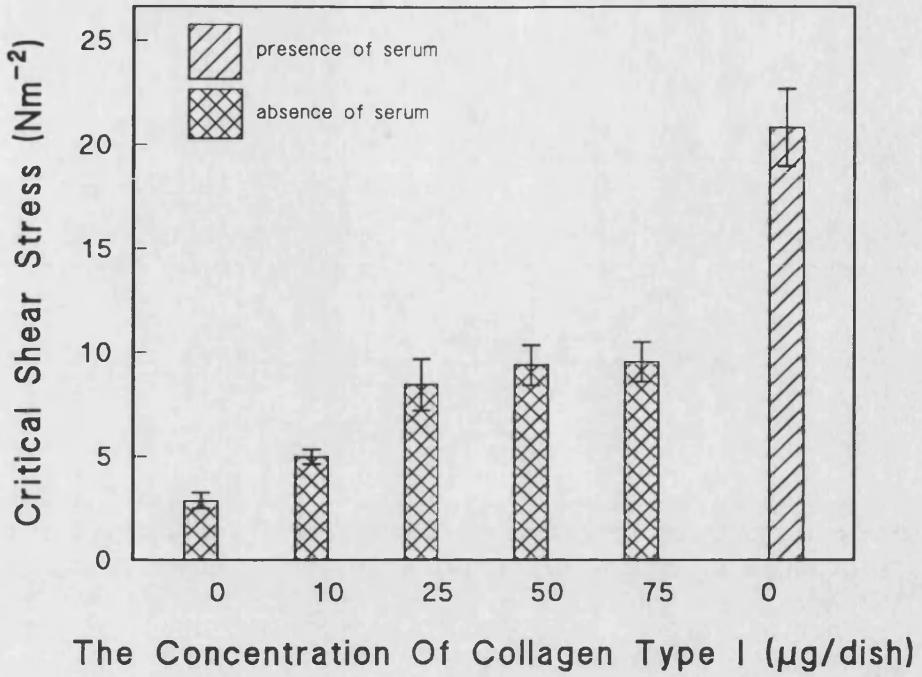


Figure 6.4. The Effect of Pre-adsorption of Collagen type I on HeLa B Cell Adhesion strength.

Hela B cells were seeded on various concentrations of collagen type I-coated plastic tissue culture dishes, each of which had a 72 cm2 surface area. Cells were allowed to grow for 24 hours, either in serum-free or serum-containing medium. The adhesion strength of the cells, in terms of the critical shear stress, was measured in the Microflow chamber. Each data point represents the mean of 30 different determinations experiments, the error bars indicate thestandart errors of that mean.. Further information can be found in text.

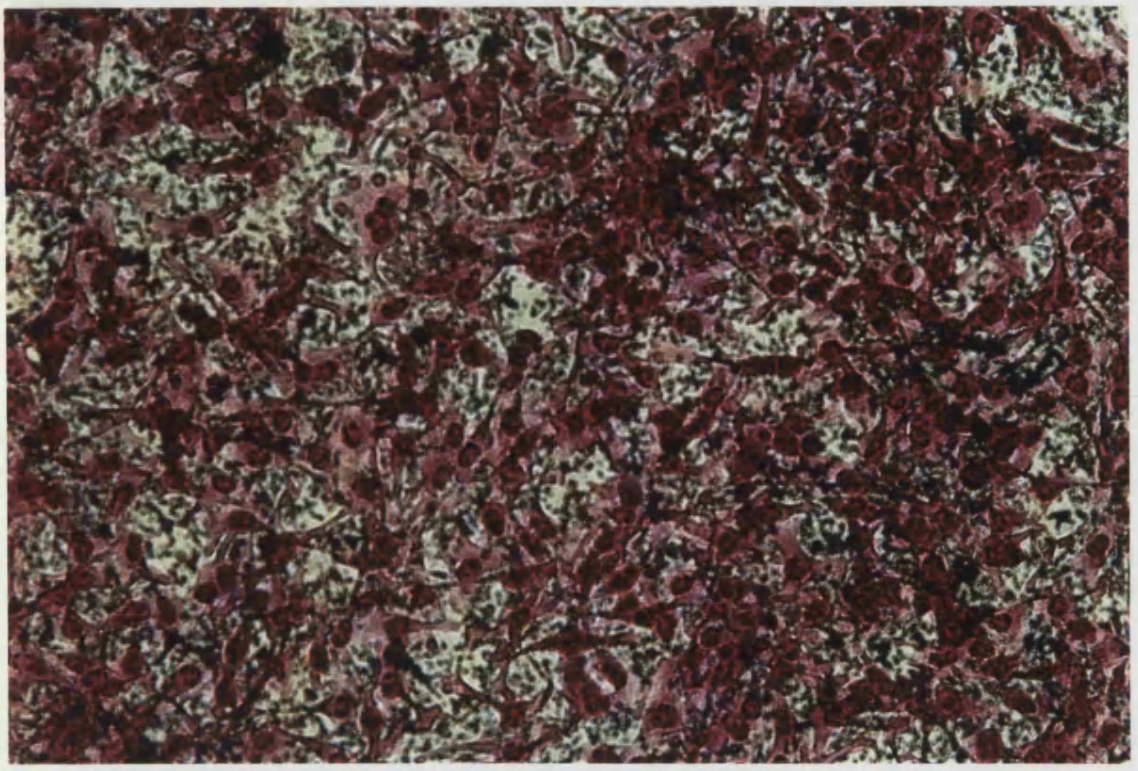
6.2.6. THE EFFECT OF PRE-ADSORPTION OF POLY-D-LYSINE ON THE ADHESION STRENGTH OF CHL CELLS.

Poly-D-lysine M_w 300,000 was obtained from Sigma and 72 cm^2 tissue culture grade plastic dishes were coated with the various amount (5 µgto 50 µg) of polylsine. Coating was proceeded as described in materials and methods.

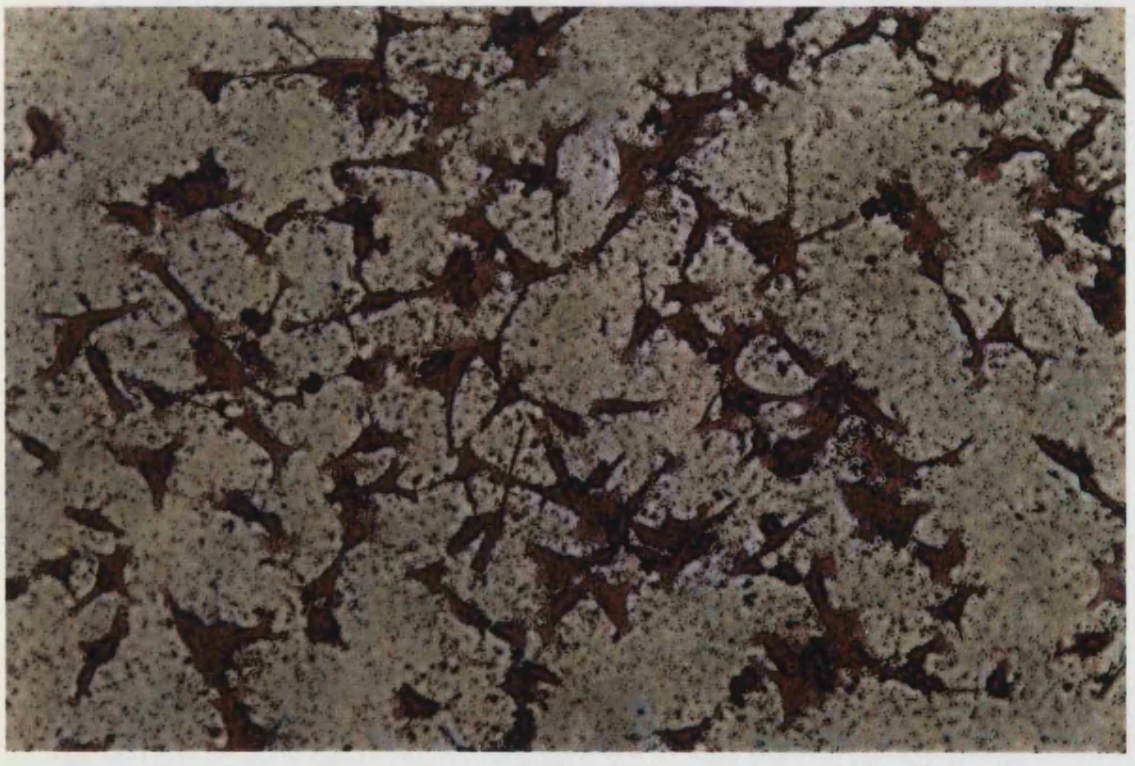
CHL cells were seeded on this modified dishes and cells were allowed to grow for 24 hours in serum free medium. As controls cells were seeded on uncoated dishes in both serum free and serum contains medium. In addition cells were seeded on 25 µg polylysine coated dishes in the presence of serum, to find out whether the adhesion strength of CHL cells is effected by the presence or absence of serum. Cell detachment studies were performed by Micro flow chamber as outlined in chapter 3.4.

The results indicate that pre-adsorption of polylysine strengthened the adhesion strength of CHL cells significantly. For example, the c.s.s. of detachment of CHL was 2.80+0.30 Nm^{-2} and 4.11+0.54 Nm^{-2} on non coated and 5 µg polylysine coated surfaces , respectively (P=0.0001). As it can bee seen from figure 6.5 the adhesion strength of CHL cells increased as the amount of pre-adsorbed polylysine increased. However, above 25 µg, increasing of the concentration of the

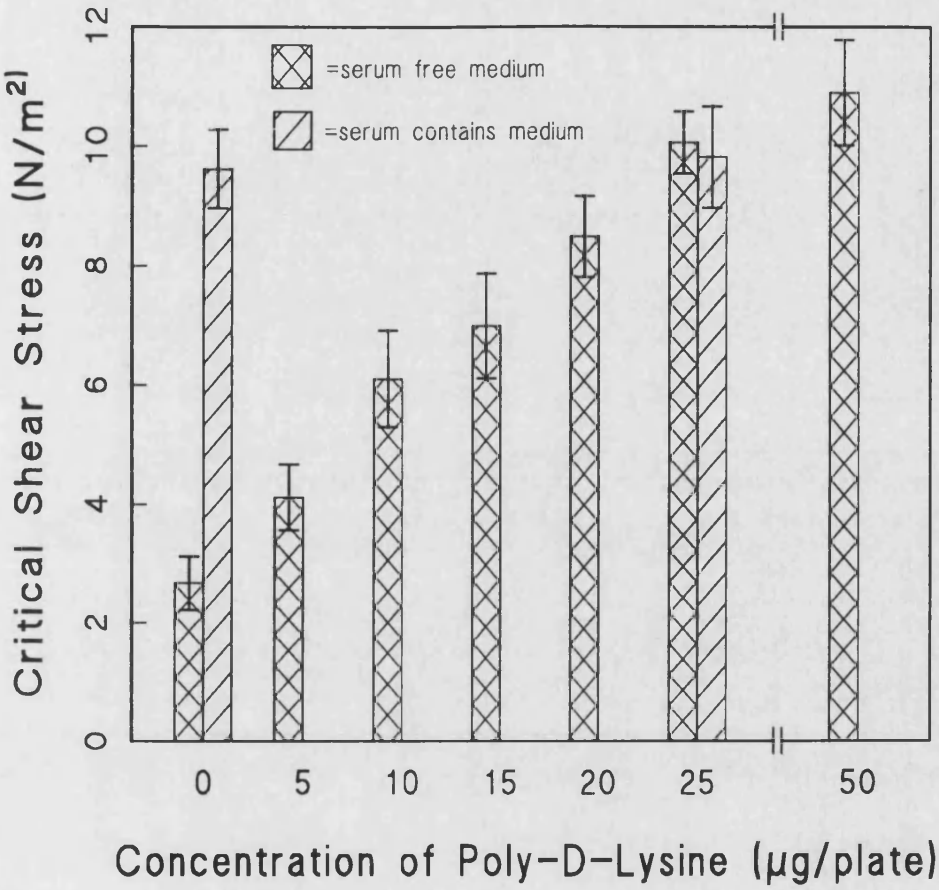
coated polylysine did not increase cell adhesion strength significantly. That is the c.s.s. of detachment of CHL cells was 9.94+0.57 Nm-2 and 10.90+0.88 Nm-2 0n 25 µg and 50 µg polylysine coated surfaces, respectively (P=0.020).



Picture 6.5: HeLa B Cellls On Uncoated Petri Dish In The Presence of Serum.



Picture 6.6: HeLa B Cellls On Collagen Type I Coated Petri Dish In The Absence of Serum.



(P=0.020) ((9 **Figure 6.5: The Effect of Pre-Adsorbed Poly-D-Lysine on CHL Cells Adhesion Strength.**

The tissue culture grade plastic dishes (72 cm2) were incubated with (5-50 µg) polylysine. Polylysine coating was proceeded as illustrated in materials and methods. CHL cells were grown on these polylysine coated dishes for 24 hours. The adhesion strength of cells in terms of the critical shear stress (c.s.s.) was measured by using Microflow Chamber. Each data point represents the mean of 30 different determinations, the error bars indicate the standart error of that mean. Further details may be found in text.

The adhesion strength of CHL cells on polylysine coated surface was not affected by the presence or absence of serum in culture medium.

Therefore the c.s.s. of detachment of CHL cells on 25 µg polylysine coated surfaces was 9.94+0.57 Nm^{-2} and 9.82+0.85 Nm^{-2} in the serum free medium and serum contains medium, respectively (P=0.72).

Although cell adhesion strength was strengthened by the pre-adsorbed polylysine, cells were not spreading even on 50 µg polylysine coated surfaces. This was a contradiction to the morphological behaviour of cells on fibronectin or collagen type IV coated dishes at which increased cell adhesion strength was related to cell spreading as well, as far as observation by the light microscopy. Therefore, one might possibly get some idea about the effect of these pre-adsorbed proteins on cell adhesion strength. Whereas in polylysine case this is not possible. This indicates that cell adhesion strength is not necessarily dependent on cell spreading.

Perhaps it is not out of place to make a clarification which is that unlike cell adhesion to fibronectin or collagen (receptor-ligand binding involves, as discussed previously in related sections), cells adhere to polylysine through electrostatic interactions (Yavin and Yavin 1974; McKeehan and Ham 1976). Polylysine treated surfaces present positively charged surfaces, while cell surface has negative charges. Therefore non-specific cell substrate may result regardless of the availability of the receptors and complementary ligands (Lauffenburger et al 1993). In polylysine case, adhesion strength increases as the density of the adsorbed polylysine increases and hence increases the number of ionic bonds between cell and substratum (Clapper 1991). Thus it seems possible to suggest that most of the cell surface negative charges were occupied on 25 µg polylysine coated surfaces therefore increasing of density of polylysine did not increase cell adhesion strength significantly.

CHAPTER 7.THE ROLE OF ENDOGENOUS PROTEINS IN CHL CELL ADHESION

7.1. INTRODUCTION

The role of serum (a complex mixture of various adhesive and anti-adhesive proteins) and purified adhesive proteins (e.g. fibronectin) in CHL cell adhesion was discussed in the previous chapter. However, according to the investigations described in Chapter 1, the cell adhesion process involves not only extragenous proteins but also endogenous proteins. To summarize, when a suspension of cells in a serum-containing medium is poured into a tissue culture dish, serum proteins adsorb immediately to the surface of the dish; subsequently, a cell-surface contact is established and the adhesion process begins. After adhesion the cell secretes its own proteins, which mix together with the pre-adsorbed serum proteins to form an extra-cellular matrix. This matrix forms the foundation for cell spreading and adhesion strengthening (Couchman et al, 1983; McDonald, 1988; Schakenraad and Busscher, 1989).

The role of endogenous proteins in cell adhesion could be studied using inhibitors of protein synthesis such as cycloheximide or emetine (Farsi et al, 1985), or by using inhibitors of protein secretion such as monensin (Sanders and Chokka, 1987). Although the role of endogenous proteins in various cells has been investigated in the past, most published studies deal solely with initial cell attachment (Flickinger et al, 1990) or cell morphology (i.e. whether cells are spreading or not) (Pizzey et al, 1983). However, the present study describes the use of a Microflow chamber to investigate not only the relationship between endogenous proteins and initial cell attachment, but also the role of endogenous proteins in cell adhesion strength. The results of these investigations are now presented.

7.2. RESULTS AND DISCUSSION

7.2.1. THE DETERMINATION OF EFFECTIVE DOSE OF EMETINE OR CYCLOHEXIMIDE FOR PROTEIN SYNTHESIS INHIBITION

In order to determine the concentration of emetine or cycloheximide required to effectively arrest cell growth, a family of growth curves was obtained for each inhibitor: cells were grown in 10% foetal calf serum in the presence of various concentrations of cycloheximide or emetine, between 0 and 5μg ml^{-1} . Although 0.1μg ml^{-1} and 0.5μg ml^{-1} of cycloheximide or emetine, respectively, substantially inhibited the growth of the CHL cells, growth was almost completely halted when the concentration of either drug was 1μg ml^{-1} (Figure 7.2 and 7.3). In order to confirm that these drugs actually inhibit protein synthesis in CHL cells, the cells were labelled with [^{35}S]-methionine and the incorporation of this radiolabelled amino acid was determined in the presence of either drug, as described in Materials and Methods. It was observed that the protein synthesis was inhibited by 97% and 95% in the presence of 1μg ml^{-1} emetine or cycloheximide, respectively (Figure 7.3 and 7.4). It was interesting to observe that even after five hours, a residual protein synthesis was maintained in response to the above drugs (3% and 5% of original protein synthesis in the presence of emetine and cycloheximide, respectively). By combining results from the growth experiments with those from the biosynthetic labelling study, it was concluded that 1μg ml^{-1} of either drug was an appropriate concentration with which to inhibit protein synthesis.

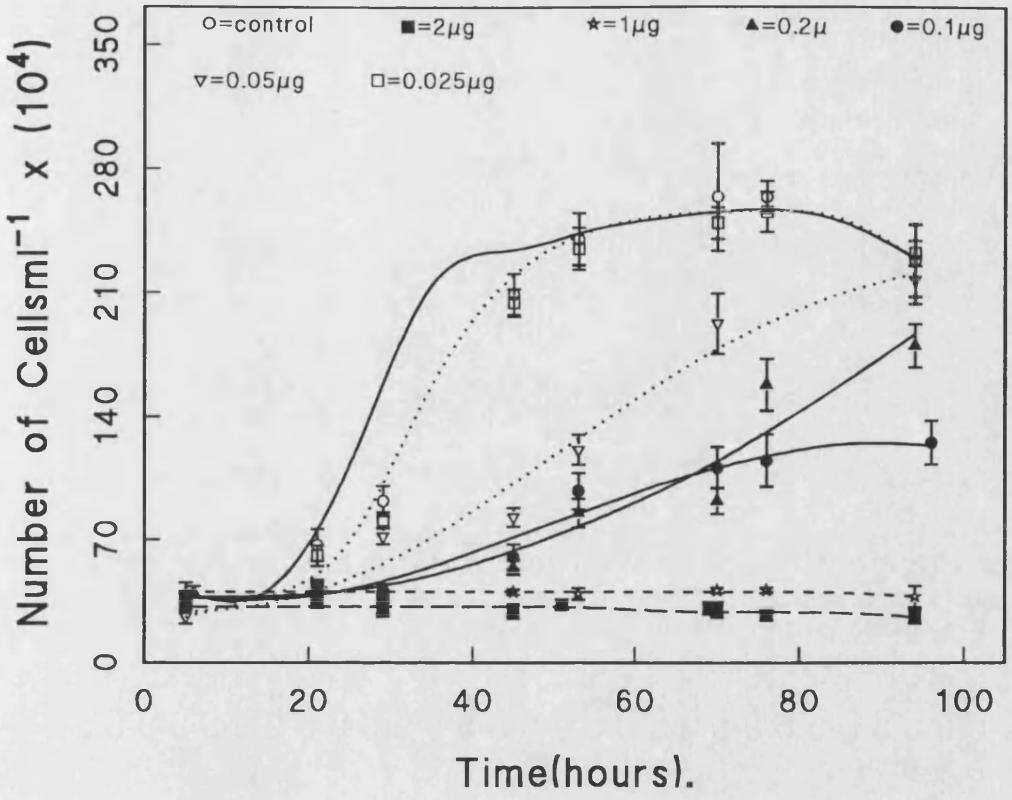


Figure 7.1: The Effect of Cycloheximide On the Growth of CHL Cells

Sub-confluent CHL cells were trypsinized and inoculated in tissue culture flasks (25 cm^2 each). The dose of cycloheximide which effectively inhibited the growth of these cells was determined by adding different concentrations of the drug at the beginning of the experiment, which lasted for 94 hours. Each data point represents five different experiments; in each experiment the cells were counted three times. Each error bar represents the standard error of the mean. Where an error bar does not appear, it is smaller than the symbol. Further details of procedure may be found in the text.

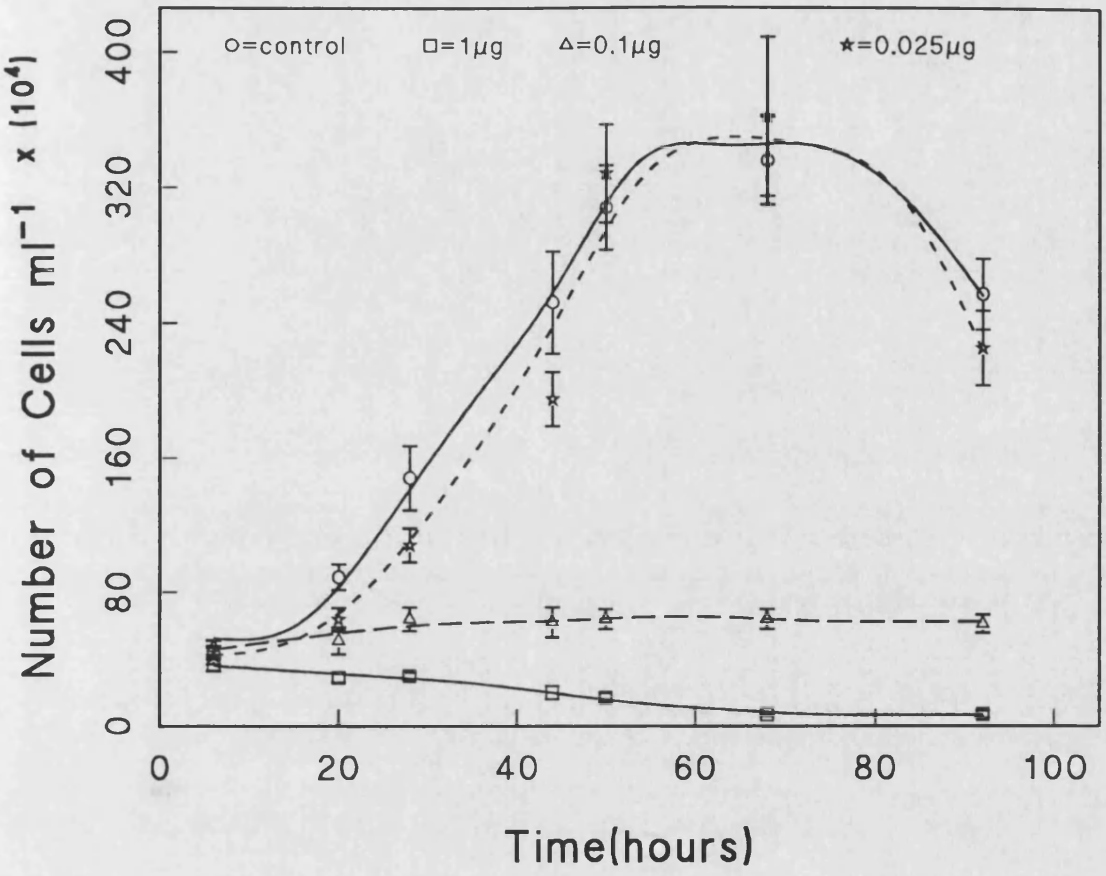


Figure 7.2: The Effect of Emetine on the Growth of CHL Cells

Sub-confluent CHL cells were trypsinized and inoculated in tissue culture flasks (25 cm^2 each). The dose of emetine which effectively inhibited the growth of these cells was determined by adding different concentrations of the drug at the beginning of experiment, which lasted for 96 hours. Each data point represents five different experiments; in each experiment the cells were counted three times. Each error bar indicates the standard error of the mean. Where an error bar does not appear, it is smaller than the symbol. Further details of procedure may be found in the text.

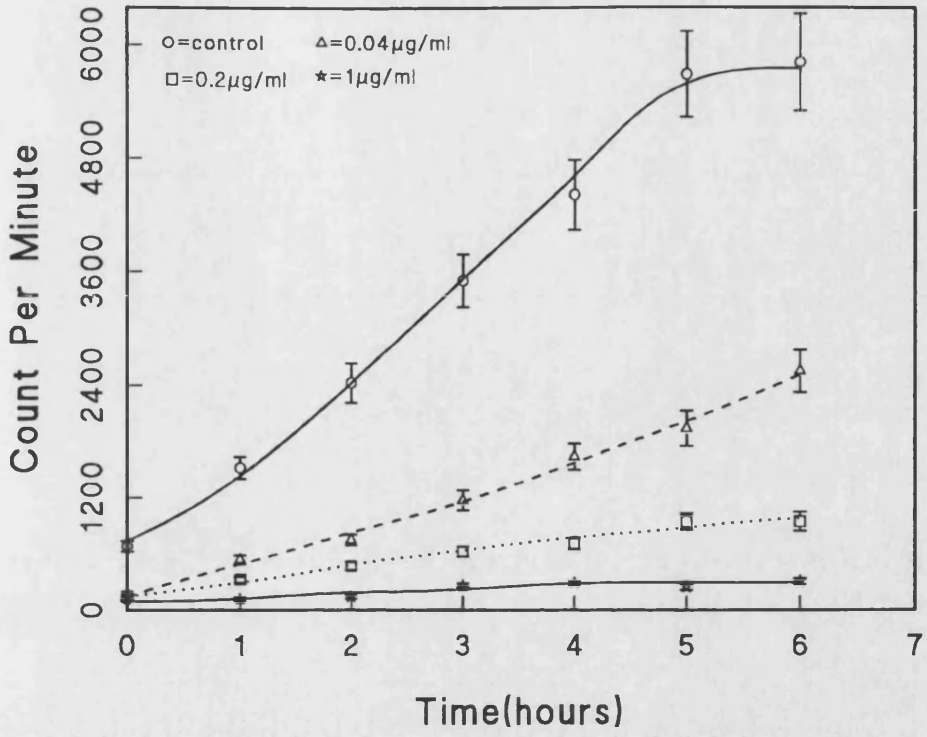


Figure 7.3: Protein Synthesis in CHL Cells in Response to Cycloheximide.

Adherent cultured CHL cells were plated at a density of $5x10^5$ cells ml^{-1} in the presence or absence of cycloheximide, and allowed to attach to the 24-well tissue culture plate for 2 hours. At this stage, the cells were metabolically labelled with [^{35}S]-methionine, incorporation being followed over a period of 6 hours. At times ranging between 0 and 6 hours, the cells were sequentially prepared for scintillation counting as described in Materials and Methods. Each data point represents five different experiments; in each experiment the effect of different concentrations of cycloheximide was examined in triplicate. Each error bar indicates the standard error of the mean. Where an error bar is not apparent, it is smaller than the symbol. Further details may be found in the text.

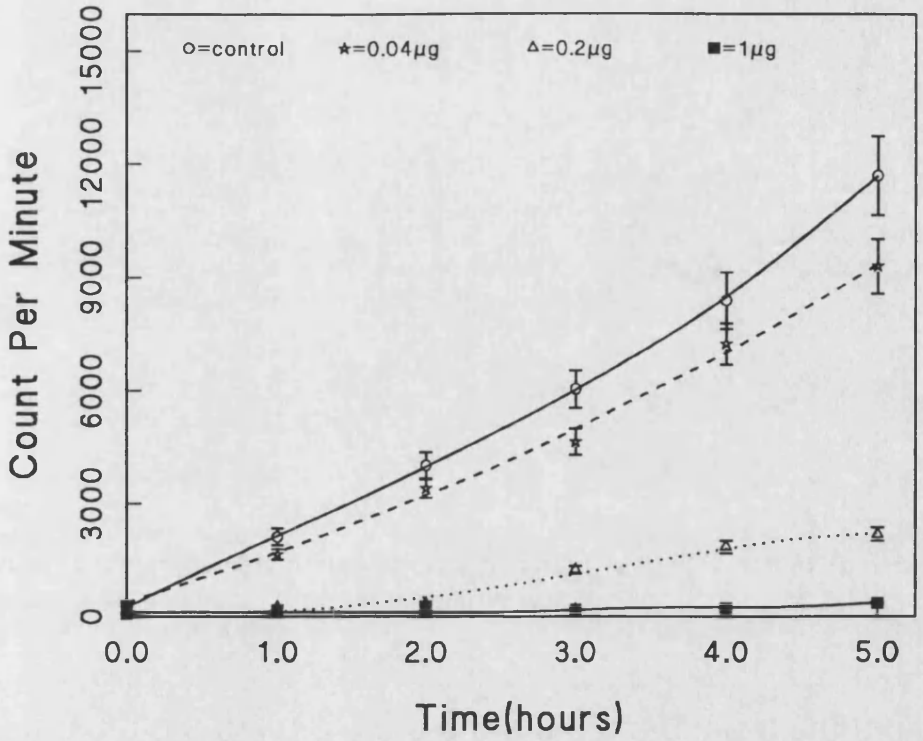


Figure 7.4.: Protein Synthesis in CHL Cells in Response to Emetine.
Adherent cultured CHL cells were plated at a density of $5x10^5$ cells ml^{-1} in the presence or absence of cycloheximide and allowed to attach to the 24-well tissue culture plate for 2 hours. At this stage the cells were metabolically labelled with [^{35}S]-methionine, incorporation being followed over a period of 6 hours. At times ranging from 0 to 6 hours, the cells were sequentially prepared for scintillation counting. as described in Materials and Methods. Each data point represents five different experiments; in each experiment the effect of different concentrations of cycloheximide was examined in triplicate. Each error bar indicates the standard error of the mean. Where an error bar is not apparent, it is smaller than the symbol. Further details may be found in the text.

In order to discover the effect of endogenous protein synthesis on CHL cell adhesion, the following approaches were made:

1. The initial attachment of CHL cells was measured in the presence of emetine and cycloheximide.

2. The adhesion strength of CHL cells was measured, both with and without pre-treatment with inhibitors by adding 1μg ml^{-1} emetine or cycloheximide.

3. The adhesion strength of the CHL cells was measured during the inhibition of protein secretion by monensin.

Each of the above points is discussed below.

7.2.2. THE ROLE OF PROTEIN SYNTHESIS IN CHL CELL ATTACHMENT.

When using inhibitors of protein synthesis to study its role in cell adhesion, it is important that the inhibition is specific. Emetine is a well-known specific protein synthesis inhibitor (Pestka, 1971). It stabilizes the 80S eukaryotic ribosomes so that they cease to move along mRNA, effecting an irreversible inhibition of protein synthesis (Oleinic 1977). As stated above, 1μg ml^{-1} emetine inhibits 97%±2% of CHL cell protein synthesis within 5 hours (Figure 7.3). However, it has previously been reported that cultured cells contain protein pools, so that even if protein synthesis is totally inhibited, a cell may continue to secrete proteins from these pools (Grinnel and Feld, 1979). In an attempt to remove proteins secreted even in the presence of emetine, cells were grown in normal complete medium (Materials and Methods 2.2.1) until mid-log phase and this medium was then replaced with fresh complete medium, also containing

1μg ml^{-1} emetine. The incubation was continued for a further 6 hours at 37°C, after which the cells were trypsinized and the attachment assay performed as usual (Materials and Methods 2.3.1) except for the presence of 1μg ml^{-1} emetine in the medium. As seen in Figure 7.5, there was a marked reduction in cell attachment in the presence of emetine: only 8.5±2% of the cells attached after 20 minutes of incubation at 37°C, whereas 67±6.5% of the control

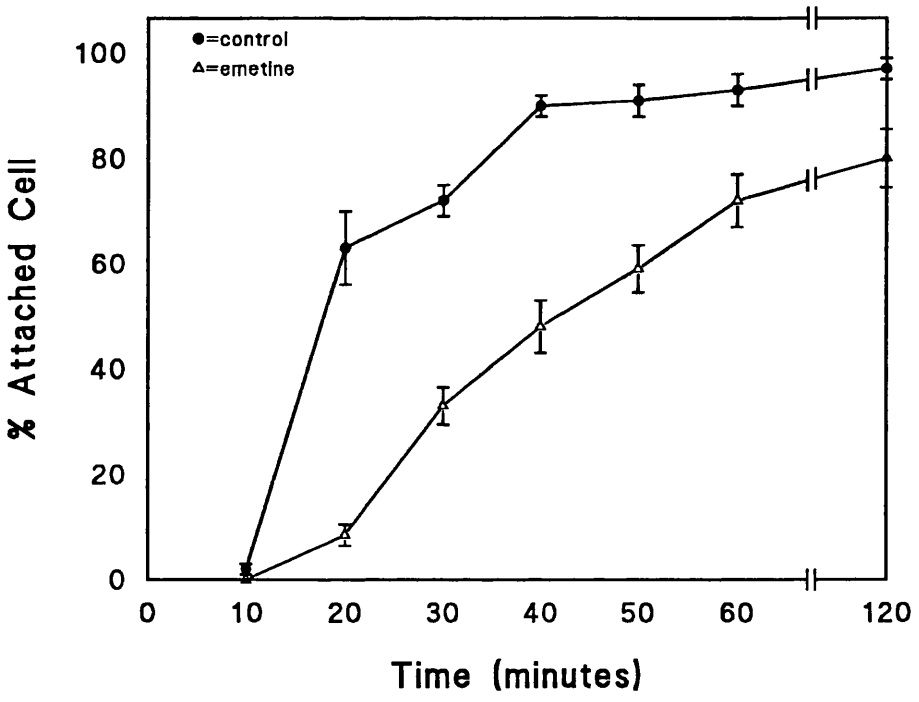


Figure 7.5: The Effect Of Emetine on the Attachment of CHL cells
Sub-confluent CHL cells were pre-treated with 1μg ml^{-1} emetine for 6 hours before trypsinization. After trysinization, cells were seeded on 35mm tissue culture grade dishes in medium containing 10% foetal calf serum in the presence of 1 μgml^{-1} emetine. Control cells were seeded in the absence of emetine without pretreatment. At the time points indicated, the cell attachment was measured. Each data point represents the mean of five different experiments, in each of which two determinations were made. Each error bar represents the standard error of the mean.

cells had attached during the same period. However, after 30 minutes of incubation, cell attachment in the presence of emetine had grown to 33±3.7%, with 72±4.6% of cells attaching in the absence of emetine. After one hour of incubation, although there was a statistically significant difference ($p=0.0005$) between the amount of attachment in the presence and absence of emetine (75±4.5% and 93±3%, respectively), the difference was not as large as that observed after a 20 or 30 minute incubation period. Nevertheless, after 2 hours 80± 5.6% of the emetine treated cells attached, while the percentage of attached control cells was 97±2%.

From the above results it could be said that although inhibition of protein synthesis delayed cell attachment in the initial period of incubation, most of the cells were able to attach within 2 hours of incubation. This was surprising because, as discussed previously (Introduction 1.2), a cell attaches to the substratum via proteins on its surface (adhesion receptors); in order to perform an adhesive function, a cell therefore needs to synthesize proteins. Hence the question: how do cells attach to a surface if they are unable to synthesize the necessary proteins? The answer could be that since protein synthesis is not completely inhibited (97%), a residual amount of protein synthesis is sufficient for cells to carry out attachment. An alternative explanation could be that only a few of the adhesion proteins are involved in adhesion at any one time, and although many may be broken upon trypsinization many others are still available, either whole or in subunit form (Kolodony, 1972). A final explanation could be that the cell can attach to a substratum without the need for specific cell surface proteins (Grinnel, 1978).

Although the above explanations are merely speculative, we found that the effect of protein synthesis inhibition on CHL cell attachment was less than might have been expected. Similar unexpected results have been reported. Kolodony (1972) reported that inhibition of the protein synthesis of 3T3 cells with 80ug ml^{-1} of emetine did not affect cell attachment to a plastic substratum during the 1 to 2 hour period of the attachment study. However, after 6 hours of incubation most of the cells had either been disrupted, contracted or detached from the plastic surface. It was concluded that the initial process of adhesion did not require protein synthesis. Although the study was not qualitative, cells were observed using inverted light microscopy to see whether they were adhering or spreading. In contrast to our results and those of Kolodony (1972) it has been reported that inhibition of protein synthesis, by cycloheximide, actually increases adhesion of Ehrlich-Lettre hyperdiploid ascites carcinoma (EAT) cells to plastic surfaces (Weiss and Chang, 1973). A similar observation was also made by Antoni et al (1987) using emetine and Thymic cells. Both reports suggested that the increased adhesion was due to the inhibition of anti-adhesion protein synthesis by these drugs. Emetine inhibited only 65% of protein synthesis in Thymic cells; by assuming that the synthesis of mainly anti-adhesive proteins was inhibited, it was suggested that one might expect inhibition of protein synthesis to increase cell attachment (Antoni et al, 1987). Although these are just speculations, it would not be out of place to consider briefly the effect of anti-adhesion proteins on cell adhesion, as follows.

7.2.1.1. ANTI ADHESIVE PROTEINS

Although some cellular proteins (e.g. fibronectin) are able to promote cell adhesion (Ruoshlahti, 1988), others can interfere with

cell-substrate adhesion and are simply called anti-adhesive proteins; examples are tenanscin, thrompospondin and SPARC (Saga and Bonstein 1991). These anti-adhesion proteins may exert their effects in different ways. They could interfere with the interaction between integrin receptors and adhesive proteins, either by binding to integrin receptors or to adhesive proteins (Ehrishman et al 1988; Sipes et al 1993). Alternatively, anti-adhesive proteins could bind to the specific cell surface receptors, which presumably transduce information to the cell. This information could then trigger a cytoplasmic response that would alter the functions of the integrin receptors (Lightner and Erickson, 1990; Sipes et al, 1993). Another possible mechanism for anti-adhesion proteins is a simple steric interference: these proteins adsorb to the surface and in doing so prevent the subsequent adsorption of adhesive proteins (Lightener and Erickson, 1990).

In the present study, 97% of protein synthesis was inhibited and, in contrast to Antoni et al (1987), adhesion of CHL cells was reduced in the presence of emetine. The contrasting results may be due to the different cell systems used, which possibly behave differently during the attachment process. As far as this work is concerned, protein synthesis inhibition reduced cell attachment but did not prevent it completely.

At this stage it was considered of interest to determine the effect of protein synthesis inhibition on the adhesion strength of CHL cells, as will now be discussed.

7.2.2. THE EFFECT OF EMETINE AND CYCLOHEXIMIDE ON THE ADHESION STRENGTH OF CHL CELLS.

In order to determine the role of protein synthesis on the adhesion strength of CHL cells, two approaches were taken:

1. Sub-confluent cells were trypsinized, and the trypsin then inhibited using a serum-containing medium with 1 μg ml^{-1} emetine, cycloheximide or both of these drugs added. Cells were then seeded on tissue culture surfaces in identical media and the adhesion strength of these cells measured after 24 hours incubation using a Microflow chamber, as outlined in Chapter 3.

2. In order to avoid the possible secretion of cellular proteins from protein pools, the medium of sub-confluent cells was replaced with medium containing 1μg ml^{-1} emetine, cycloheximide or both of these drugs, and after six hours of incubation (chosen because, as demonstrated using [^{35}S]-methionine labelling, 1 μg ml^{-1} emetine or cycloheximide inhibits protein synthesis within 5 hours) with drug-containing medium, cells were trypsinized and subsequently treated as above. The inhibition of protein synthesis by cycloheximide or emetine reduced the adhesion strength of the CHL cells, the critical shear stress of detachment for the cells being 8.18±1.03 N m-2 and 6.70±0.30 N m-2, respectively, while that of the control cells was 10.48±0.78 N m-2. In other words, cycloheximide treatment reduced cell adhesion strength by 21%, while it was reduced by 36% in the presence of 1μg ml-1 emetine. It was interesting to note that the presence of 1μg ml-1 of both emetine and cycloheximide further reduced cell adhesion strength: the c.s.s was 5.66±0.36 Nm-2 (45% inhibition). Thus it is possible to suggest that emetine and cycloheximide act synergistically to inhibit protein synthesis. In fact, it is known that these two drugs inhibit different stages of protein synthesis: cycloheximide primarily acts on the initiation step of synthesis, while the elongation step is most sensitive to inhibition by emetine (Oleinick 1977).

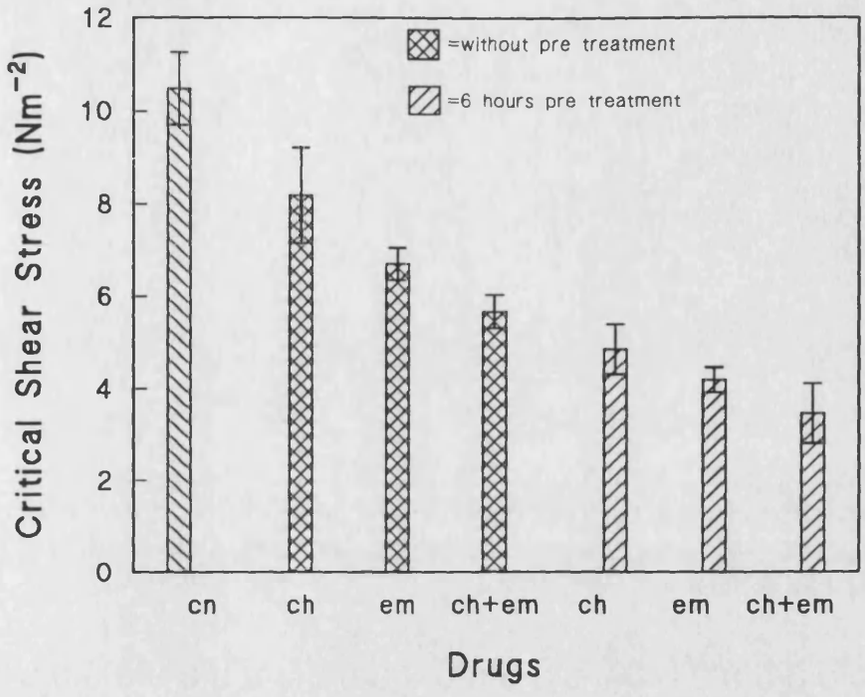


Figure 7.6: Adhesion Strength Of CHL Cells on Plastic Substratum in Response to Cycloheximide (ch) or Emetine (em) or cycloheximide plus emetine (ch+em); cn = control.

CHL cells (either pre-treated with the indicated drug for 6 hours or with no pre-treatment) were inoculated in the culture medium containing 1µg ml^{-1} drugs (indicated on the X-axis) or without drugs. After 24 hours of incubation the adhesion strength of the cells was measured in terms of the critical shear stress (c.s.s.) of detachment. The error bars indicate the standard error of five different experiments in each of which ten measurements were made. Each error bar represents the standard error of the mean.

Hence, one might expect a mixture of these two drugs to be more effective in reducing the strength of cell adhesion. The pre-treatment of CHL cells with the above drugs resulted in a further reduction in adhesion strength. For example, cycloheximide reduced adhesion by 53% when cells were pre-treated for 6 hours before

trypsinization and seeded in medium containing 1 μg ml^{-1} cycloheximide. Without pre-treatment, as indicated above, cell adhesion strength was reduced by 21% in the presence of cycloheximide. The c.s.s. of detachment of CHL cells was 4.84±0.54 N m^{-2} and 8±1.03 N m^{-2} with and without cycloheximide pre-treatment, respectively - a statistically significant difference (P=0.0007). The similar pre-treatment effect, i.e. further reduction in cell adhesion strength, was observed for medium containing emetine alone or both emetine and cycloheximide (Figure 7.6).

These results support the theory that the cell contains protein pools which are used in the absence of cellular protein synthesis (Grinnel and Feld, 1979). It is therefore possible to suggest that when protein synthesis inhibited, the cell would use these proteins to perform its limited adhesive function. In the case of pre-treatment, the cell might use up most of its the stored proteins during the treatment period. Hence, in latter case cell adhesion strength was significantly lower than that in the former case. This point was reinforced by Flickinger and Culp (1990) who reported that spreading of human fibroblasts on collagen was inhibited after 18 hours of pre-treatment. It was suggested that, after this long period of incubation, cells could deplete collagen receptors. Nevertheless, as indicated previously, not all cellular proteins promote cell adhesion. Some of these proteins have negative effects on cell adhesion (Ehrismann et al, 1988) and it could be that, during inhibition of protein synthesis, the inhibitory effect of anti-adhesive proteins is more pronounced. In fact, Hasselaar et al (1991) reported that the anti-adhesive effect of SPARC (secreted protein acidic rich in cysteine), an anti-adhesive protein, is not blocked by

cycloheximide in bovine aortic endothelial (BAE) cells. Therefore, cell spreading was still inhibited by SPARC.

Another way of studying the effect of endogenous proteins in cell adhesion is to inhibit the protein secretion process. Monensin inhibits the secretion of proteins (Sanders and Chokka, 1987) as discussed in the following.

7.2.3. THE EFFECT OF MONENSIN ON CHL CELLS ADHESION STRENGTH.

As a first step to determine the effect of monensin on the strength of CHL cell adhesion, the inhibitory concentration of monensin, which effectively arrest the growth of cells, was determined. For this purpose, as for emetine and cycloheximide, a family of growth curves was obtained, in which varying concentrations of monensin (0 to 1μg ml^{-1}) were used. As can be seen in Figure 7.7 although 0.1μg ml^{-1} monensin substantially inhibits the growth of CHL cells, a concentration of 1μg ml^{-1} monensin almost completely halted the growth of CHL cells. Therefore, in studying the effect of monensin on CHL cell adhesion strength, a concentration of 1μg ml^{-1} monensin was used. As in the emetine and cycloheximide studies, two approaches were taken in the monensin studies: in the first case, CHL cells were seeded on tissue culture grade plastic (polystyrene) dishes in 10% foetal calf serum and medium containing μg ml^{-1} monensin. In the second case, cells were seeded on the plastic dishes after 6 hours of pre treatment with monensin. In both cases, cell detachment studies was performed, as described in Chapter 3, after 24 hours of incubation.

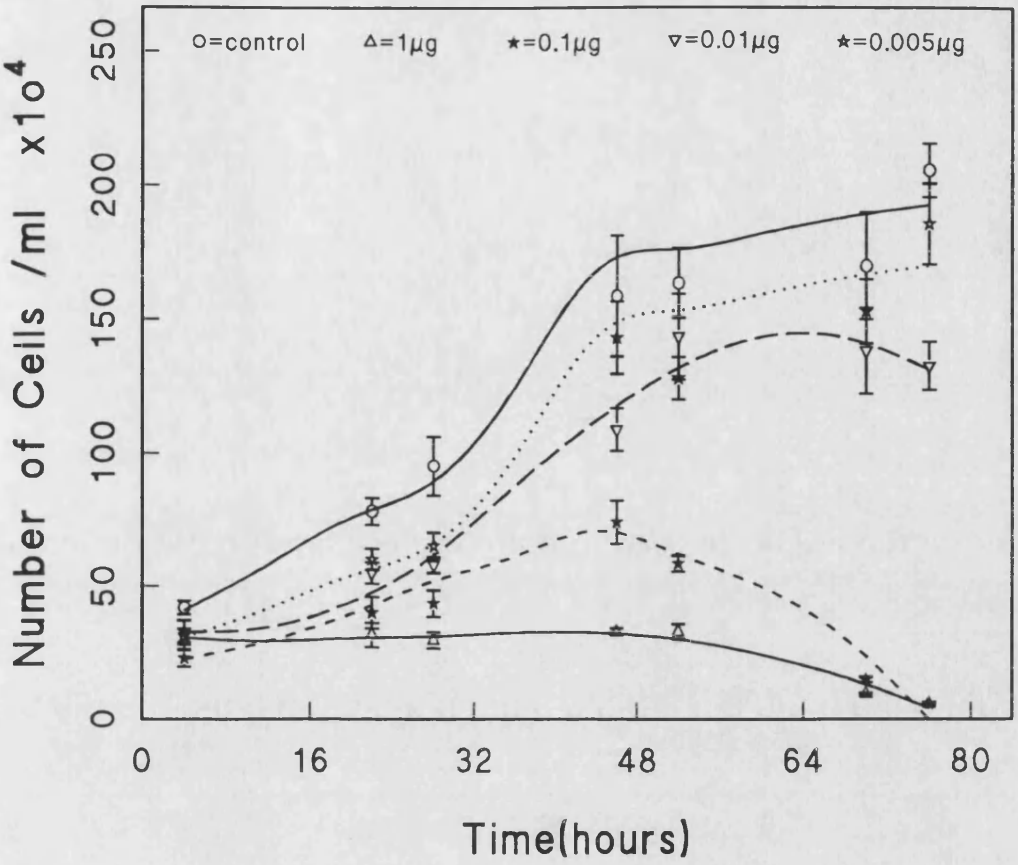


Figure 7.7: Effect of Monensin on the Growth Of CHL Cells

Sub-confluent CHL cells were trypsinized and inoculated in tissue culture flasks (25 cm^2 each). The dose of monensin which effectively inhibited the growth of these cells was determined by adding different concentrations of the drug at the beginning of the 76 hour experiment. Each data point represents five different experiments; in each experiment the cells were counted three times. Each error bar indicates the standard error of the mean. Where an error bar does not appear, it is smaller than the symbol. Further details of the procedure may be found in the text.

As in the case of the protein synthesis inhibitior studies, pre-treatment with monensin was more effective in the inhibition of cell adhesion strength. That is, the c.s.s of detachment of CHL cells was 10.35±o.50 N m^{-2} in control cells, while it was reduced to 5.05± 0.35 Nm^{-2} and 5.89±o.67 Nm^{-2} in the presence of emetine with and without pre-treatment, respectively. Despite the added effect of pre-treatment, the difference in adhesion strength with and without pre-treatment was not statistically significant (P=0.13).

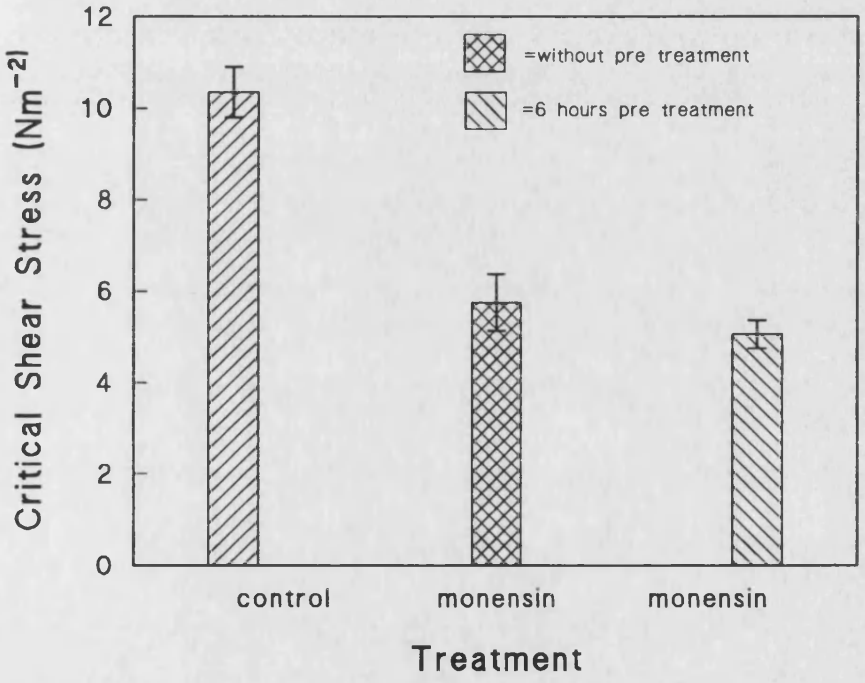


Figure 7.8: Adhesion Strengt Of CHL Cells on Plastic Substratum in Response to Monensin.

CHL cells (either pre-treated for 6 hours with monensin, or else without pre-treatment) were inoculated in culture medium containing 1 µg ml^{-1} monensin. After 24 hours of incubation, the adhesion strength of the cells was measured in terms of critical shear stress of detachment. Data was collected from six duplicate experiments, in each of which ten measurements were made. The error bars represent the standard error of that mean.

It was interesting to note that the inhibition of endogenous protein synthesis or secretion did not inhibit cell adhesion completely. This could be due to the presence of serum proteins, i.e. the cell may able to perform a limited function by using serum adhesive proteins. Hence, monensin-treated human fibroblast cells did not spread in the absence of serum, but did do so in medium containing serum (Pizzey et al, 1983 and 1984). In contrast, CHL cells did not spread either in the absence or presence of serum when they were monensin treated, although some flattening was evident. It was nevertheless considered of interest to evaluate the serum effect on the adhesion of CHL cells in the presence of protein synthesis or protein secretion inhibitors. Unfortunately, this was not possible since in these cases the cells lysed.

The above result supports reports that indicate that the real effect of emetine and cycloheximide on cell adhesion could be masked by serum proteins. The monensin could prevent the formation of focal adhesion and therefore reduce the adhesion strength (Virtanen et al 1982; Lehto and Virtanen 1985).

The above results could suggest that although cells are able to attach under the inhibition of synthesis or secretion of cellular proteins, due to a lack of cellular adhesive proteins and adhesive receptors, they are unable to perform required functions such as signalling, response to these signals, reorganization of cytoskeletal proteins and the formation of focal adhesions. Therefore cell adhesion strength is significantly reduced by the above drugs.

CHAPTER 8 CONCLUSIONS

The aim of research presented here was the development of a simple and reproducible method for the measurement of cell adhesion and the application of this technique to understand the underlying mechanism of cell adhesion.

In general cell adhesion has been studied qualitatively. However, some methods have been developed for the quantitative measurement of cell adhesion. Nevertheles, some of these methods have a limited ability, while the others suffer a need of complex equipment and/or they are time consuming (see chapter 1 and chapter3). Thus it was necessary to develope a simple, quantitative, and reproducible method to evaluate the underlying mechanism of cell adhesion. These requirements are largeley met in the present study with the development of a cell adhesion measuring device "Microflow chamber". At the present four versions of Microflow chamber have been designed. The first version was able to measure the adhesion strength of cells on the glass or the glass size plastic surface, while the second and third version of device are able to measure the critical shear stress (c.s.s) of detachment cells from glass or plastic petri dishes. By using any of above chambers only one measurement can be made from one cell growing substratum. However, three measurements can be made and therefore statistically more data can be obtained by using three channels in the fourth version of the Microflow chamber. Another advantage of this technique over previous methods is the existence of convergent channel(s) in Microflow chambers. Thus under precise hydrodynamic control, in a defined flow rate, a range of well defined shear forces over the attached cells could be set up.

The accuracy and reproducibilty of the Microflow chamber was checked by measuring the adhesion strength of different cell lines. Each cell has a specific and constant adhesion strength, critical shear stress (c.s.s). When the applied shear stress exceeds the adhesion strength of cells, cells would come off. The reproducibilty of the method was determined by measuring the c.s.s of cell deatchment in a small standard deviations.

The Microflow chamber is not only able to measure cell adhesion strength quantitatively, but it also able to determine the role of various parameters which are involved in cell adhesion. The factors examined with the help of the Microflow chamber include cell themselves, temperature, serum, purified adhesion proteins, and endogenous adhesion proteins.

Cell adhesion is a multi step phenomenon. These steps include the initial contact of cell to substratum, attachment, spreading and strengthening of cell adhesion. Temperature is an important factor in the cell adhesion. It does not only determine the strength of cell adhesion, but even initial attachment as well. To gain possible maximum adhesion strength, CHL cells needed to be incubated at 37^{o}C. Therefore, when they were incubated at 20^{o}C for 24 hours CHL cells were able to obtain only 60% of adhesion strength of those incubated at 37^{o}C. The requirement of metabolic energy in the gaining of adhesion strength was determined more specifically by using the electron transport inhibitor, oligomycin. The presence of olgomycin significantly reduced adhesion strength of CHL cell ($P=0.0073$, between the presence and absence of 30μM oligomycin). The effect of temperature on the initial cell attachment was dependent on the presence or absence of serum in cell medium. That is in the former case the attachment of cells did not take place at 9^{o}C or 4^{o}C even

after 48 hours of incubation, whereas in the latter case temperature did not have significant effect in the attachment of CHL cells. This indicates that cells have different mechanisms of attachment on these two cases. The requirement of energy in cell attachment was further proved by showing that lack of of oxygen inhibited cell attachment (chapter 4).

The strength of CHL cells adhesion was dependent on the serum concentration in the growth medium up to 1%. Thus, increasing serum concentration from 0% to 1% increased critical shear stress of detachment of CHL cells. While at the presence of 1% fetal calf serum cells reached possible maximum adhesion strength and above 1% increasing serum concentration up to 10% did not effect cell adhesion strength. This could indicate 1% of serum is able provide sufficient amount of adhesion proteins and the other molecules which cells need to gain possible adhesion strength. However, the origin of serum did not have significant role on the adhesion strength of CHL cells. Thus there was no statistically significant difference between the adhesion strength of cells which were grown in 10% fetal calf serum and those grown in horse serum or in new born calf serum (chapter 5).

By using the Microflow chamber the effect of the modifications of plastic dish by pure adhesive proteins on the adhesion strength of cells were determined. Preadsorption of fibronectin strengthened the adhesion strength of CHL cells in a concentration dependent fashion up to 25µg fibronectin per plate. Above this amount increasing fibronectin concentration did not change adhesion strength significantly. It was surprising that even a very small amount of fibronectin, 0.1µg which will only cover 0.25% cell, was able to strengthen cell adhesion significantly. ($P=0.0001$, between non coated

and coated with 0.1μg fibronectin). This could indicate that fibronectin not only promotes cell adhesion simply, acting as a an adhesive ligand, but also acts as an activator to strengthen cell adhesion. The increasing critical shear stress of detachment with the increasing fibronectin concentration could also indicate that the number of ligand-receptor bonds are an important factor in the gaining of cell adhesion strength. Like fibronectin, coating of plastic dish with collagen type IV increased adhesion strength of CHL cells dramatically. Whereas, preadsorbtion of collagen type I was uneffective in the increasing adhesion strength of CHL cells. Thus even modification of plastic dish with 200μg collagen type I did not increased cell adhesion strength significantly. However,HeLa B cells were able to enhance significantly the strength of adhesion on collagen type I coated surface. This might indicate that although CHL cells have surface receptors which mediate in cell collagen type IV interaction, there are no receptors to mediate a cell-collagen type I interaction. Although it has been previously determined that HeLa B cells have collagen receptors, there is so far no report to show whether CHL cells have collagen receptors. However, the present work indicates that CHL cells have collagen receptor(s). Although this receptor(s) is/are unable to mediate cell-collagen type I interaction they are able to promote cell-collagen type IV interaction. The determination and characterization of this receptor could be interesting. Since this will then be able to prove once more the usefulness of Microflow chamber in the studying of cell adhesion. The adhesion strength of cell does not always necessarily correspond to the spreading of cells. Hence,CHL was able to strengthen adhesion on 25μg polylysine coated petri dish in the absence of serum although cells were not spreading and there was no significant difference

between the critical shear stress of detachment of CHL cells that were grown under above conditions and those that were grown on non coated dishes in the presence of serum at which cells were spreading very well (P=0.72).

The requirement of endogenous proteins in CHL cell adhesion has been determined. The inhibition of protein synthesis, 95% with 1μg/ml cycloheximide or 97% with 1μg/ml emetine (as shown by [^{35}S]-methionine labelling), prevented CHL cells from gaining possible maximum adhesion strength. Although the effect of protein synthesis inhibition on the attachment of different cells has previously been studied there is no data to show the relationship between the adhesion strength and endogenous proteins. In the present study the requirement of protein synthesis or secretion for CHL cells to reach possible maximum adhesion strength was showen by means of the Microflow chamber. Newertheles, to be able show the role of spesific endogenous proteins in cell adhesion it would be interesting to use specific antibodies for that particular adhesion proteins then measure cell adhesion strength.

The mechanism of strengthening of cell adhesion starts to emerge. The spreading and formation of focal adhesions, which lead cells to enhance their adhesion, is now commonly believed to occur through integrin receptors signalling. Either integrin can act as a signal molecule or stimulant for the other second messengers e.g. cAMP, Ca^{2+}, which could result in the regulation of cellular functions including reorganization of cytoskeletal proteins, protein phosphorylation. By the means of the Microflow chamber the role of these factors in cell adhesion can be evaluated.

In conclusion, the succesful development of the Microflow chamber has provided a simple but useful way of elucidation of underlying mechanism of cell adhesion.

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