# Adhesion mechanisms in CLL and HCL


Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by Andrea M. Vincent

March 1996.

# ABSTRACT

Normal lymphocyte homing comprises the physiological processes by which lymphocytes seek out, and localise to, particular tissues - and to specific microenvironments therein. Homing mechanisms play a major role in the maintenance of specialised microenvironments and are critical for the dispersal and targeting of naive and memory lymphocyte populations that are required for effective immune surveillance. Migration is central to lymphocyte homing and is achieved through the processes of adhesion and de-adhesion.

The adhesive and migratory capacities of the abnormal clone in lymphoproliferative disorders result in accumulation of these lymphocytes in particular lymphoid compartments. While the hairy-cells (HCs) of hairy-cell leukaemia show a consistent and peculiar tissue distribution, the cells of chronic lymphocytic leukaemia (CLL) exhibit variable patterns of migratory behaviour, although lymphocytosis and lymph node infiltration are usual. This thesis investigates the adhesive capabilities of HCs and CLL cells and attempts to relate these to *in vivo* behaviour. The role of cell activation is highlighted as a major determinant of adhesive behaviour in CLL.

Chapter 2 characterised in detail the adhesion molecule expression in ten cases of CLL. The expression of integrins was variable between cases, but all cases expressed β2 and β1 heterodimers. Any combination of α3β1, α4β1, α5β1 or α6β1 could be present (α3β1 was most common, while only one case expressed α6β1). For the first time it was demonstrated that α4, when present in CLL, formed a heterodimer

with β1 and β7. Integrins of the β2 family were more consistent:-all cases expressed αLβ2, about half had moderate levels of αMβ2, and αXβ2 was rare. CLL cells were also shown to express L-selectin and CD31.

Chapter 3 investigated the ability of CLL cells to interact with endothelial ligands as a prerequisite for tissue entry, demonstrating generally poor adhesion via several potential receptor-ligand pairs (e.g. β2 integrins/ICAMs; α4β1/VCAM-1; E-selectin ligand/E-selectin) which was increased in constitutively or *in-vitro* activated cells. On tissue entry CLL lymphocytes interact with extracellular matrix, and this was the subject of Chapter 4. Binding to fibronectin varied between cases, and activation state influenced this adhesive behaviour. Adherence to vitronectin was more variable, and did not relate to activation. CLL cells did not constitutively make any cytoskeletal responses following adhesion to extracellular matrix proteins, unlike HCs which have previously been shown to attain polar (motile) morphology on vitronectin and flattened (adherent) morphology on fibronectin. CLL cells required exogenous cytokine stimulation to become motile, and following this stimulation they acquired the ability to migrate under endothelial cells in co-culture experiments.

In HCL, adhesive behaviour more closely correlates with adhesion molecule expression and distinct migratory properties can be attributed to particular adhesion molecules on the HC. This is probably due to their activated nature. Chapter 5 investigated the interactions of HCs with endothelial ligands. HCs bound generally better than did CLL cells under identical conditions; the interaction of α4β1 with endothelial VCAM-1 was shown to be particularly strong, and the implications of this are discussed in terms of its potential influence on *in-vivo* distribution of HCs.

# CONTENTS OF THESIS

## Acknowledgements

I would like to thank the many members of this Department for the help and encouragement they have provided during my studies. I have gratefully made use of the technical expertise of several of my colleagues:- they know who they are. Also, the staff in the Hospital phlebotomy clinic were extremely helpful in acquiring patient blood samples.

I particularly owe a great deal to Dr John Burthem, who provided the initial inception of the project; his continued interest and assistance were invaluable in the development of this work. Thanks also to my supervisor, Professor J.C.Cawley, especially for his advice during the preparation of the manuscript.

Finally, I would like to thank the Merseyside Bone Marrow Transplantation Trust who have funded this work; and those patients and their relatives, whose kind donations to the Department have purchased much of the equipment involved in this Thesis.

## Declaration

**The work presented in this thesis is my own, except for the immunoprecipitations from hairy-cell lysates presented as control data in Chapter 2; this work was performed by Dr. John Burthem and has been published (Burthem *et al*, 1994a).**

**The entire project was undertaken in the Department of Haematology, Royal Liverpool University Hospital under the supervision of Professor J.C.Cawley and Dr. J.Burthem.**

## Publications arising from this work

J.Burthem, A.M.Vincent, and J.C.Cawley: Integrin receptors and Hairy Cell Leukaemia. *Leuk Lymph* 21(3/4), pp211-215. (1996)

A.M.Vincent, J.C.Cawley, and J.Burthem: Integrin function in chronic lymphocytic leukaemia. *Blood* (in press).

A.M.Vincent, J.Burthem, R. Brew, and J.C.Cawley: Endothelial interactions of hairy cells: the importance of $\alpha 4\beta 1$ in the unusual tissue distribution of the disorder. (manuscript submitted).

# Figures and Tables

## Figures

## CHAPTER 4



## CHAPTER 5

# Tables

## CHAPTER 1



## CHAPTER 2



## CHAPTER 3



## CHAPTER 4

# Preface: Aims and content of the thesis

This study is intended to explore the hypothesis that adhesion receptors influence the *in vivo* recirculation behaviour of the lymphocytes of chronic lymphocytic leukaemia (CLL) and hairy cell leukaemia (HCL), and that particular adhesion structures may be associated with specific clinical features of the disorders.

Previous work in this Department has focused on the integrin-mediated functions of the hairy cells (HCs) of HCL and related the findings to the characteristic features of the disease (Burthem *et al*, 1994a and 1994b). The present study applies the same principles concerning the importance of adhesion molecules in lymphocyte function to the more complicated disease, CLL, where the behaviour of the abnormal cells is less predictable.

Chapter 1 provides a brief overview of the background to the project. In Chapter 2, the expression of adhesion molecules of ten cases of CLL is characterised using sensitive immunocytochemical techniques. The subsequent chapters analyse how these receptors are utilised in different aspects of lymphocyte recirculation processes. Interaction with endothelium is the subject of Chapter 3, and Chapter 4 is concerned with adhesion and responses to extracellular matrix ligands.

In Chapters 3 and 4, CLL cells are frequently compared with HCs because HCL had already been characterised in this Department with regard to integrin

expression/function and thus constituted a 'control', and also because HCL is a related lymphoproliferative disorder in which tissue involvement is very different. The thesis is therefore concerned with both CLL and HCL. The endothelial assays set up for CLL in Chapter 3 and the transmigration assays in Chapter 4 had not been previously applied to HCL. The examination of the behaviour of HCs in these assays led to novel findings concerning HC/EC interaction, and these are presented in Chapter 5. The thesis finishes with a brief overview of the findings of the thesis, and a discussion of further investigations which may arise from this work, set out in a Conclusions chapter (Chapter 6).

# Chapter 1

## General Introduction

As explained in the Aims, the thesis is concerned with both CLL and HCL, and both these diseases are therefore briefly reviewed. Given the hypothesis that adhesion receptors influence the *in vivo* recirculation and tissue distribution of the abnormal cells, particular emphasis is given to the different patterns of organ involvement in the two diseases.

Next, current understanding of B lymphocyte recirculation is explained and followed by a discussion of the many adhesion molecules involved in this process. Since lymphocyte movement is an important part of the tissue phase of lymphocyte recirculation, this subject is reviewed next.

Finally, lymphocyte activation is discussed since the concept is a recurring theme of the thesis. Thus many of the characteristics of HCs, including much integrin-mediated behaviour, have been shown to be related to the fact that HCs appear to be intrinsically activated cells (Katayama *et al* 1977; Braylan *et al* 1978). Furthermore, it is known that at least some of the heterogeneity of CLL is related to different degrees of activation present in different cases (Merle-Beral, 1990). Chapters 3 and 4 show that such activation influences the adhesive/migratory properties of CLL cells. The discussion of activation is therefore related to both HCL and CLL.

# 1.1 Chronic Lymphocytic Leukaemia

## 1.1.1 Definition

CLL is a lymphoproliferative disorder in which there is a clonal expansion of B cells arrested at an intermediate stage of differentiation (Freedman and Nadler, 1993). A small proportion of cases of CLL involve monoclonal T lymphocytes. These, however, are most commonly considered to be a different disease and are excluded from this thesis. The abnormal lymphocytes of CLL are small apparently mature B cells; they express surface immunoglobulin (sIg) which is restricted to a single light chain (κ or λ; Han *et al*, 1982), and also express CD19, CD20, CD22, CD23, and CD24 (Freedman 1990).

CLL cells resemble naive B cells following release from the bone marrow; they show no evidence of affinity maturation (Dameshek, 1967). CLL is the most commonly occurring form of leukaemia in North America and Europe, accounting for almost one third of all cases, but is extremely rare in the Orient. The disease typically occurs in patients over the age of fifty years and is distinctly unusual in individuals younger than thirty years; the ratio of male:female cases is between 2 and 3 to 1 (Gale and Foon, 1985).

The expression of CD5, which is normally associated with T cells, is unusual among normal and neoplastic B cells and is therefore of diagnostic importance in CLL. The presence of CD5 on CLL cells has been a focus of the debate regarding a normal counterpart for the CLL cell. Populations of normal CD5-positive B lymphocytes exist, and these cells have many features in common with CLL cells, but also many striking differences. CD5-positive B-cells were originally considered to be

a separate lineage to CD5-negative B- cells, however, Wortis (1992) showed that CD5 expression could be induced on CD5-negative cells by appropriate cross-linking of the surface IgM receptor. Conversely, normal CD5-positive B cells can be induced to differentiate to CD5-negative cells with features in common with germinal centre cells (Caligaris-Cappio *et al*, 1989). Thus CD5 expression in CLL may be a feature of their activated, undifferentiated nature (see later). Other candidate cells for a normal counterpart of CLL are mantle zone B lymphocytes, peripheral blood and spleen CD5-negative cells, and the CD5-positive population of foetal blood cells. Some groups regard the criteria of peripheral B-cell lymphocytosis plus bone marrow infiltration as sufficient for diagnosis of CLL (Binet *et al*, 1989). More often, CD5 positivity is now regarded as an essential part of the diagnosis, and lack of this antigen is usually considered to indicate a CLL variant (Dighiero, 1993). For the purposes of this thesis a tight definition of CLL was employed, with a high proportion of the B cells in all cases expressing CD5, CD22 (low-moderate intensity), and CD23 (moderate-high intensity).

### 1.1.2 Pattern of disease

The mechanisms by which CLL lymphocytes accumulate are not clear. A review by Strykmans *et al*, in 1977, concluded that both an increased rate of production and an increased life span probably contribute to steady accumulation of the abnormal cells. Also, blood CLL lymphocytes are capable of rapidly leaving and returning to the vascular system, but despite this, recirculation does not proceed normally in CLL. The reason for this phenomenon is not known.

The clinical course of CLL is highly variable between patients. The clinical features of CLL may be related to (1) leukaemic, (2) local, and (3) immunological

manifestations of the disease, or a combination of these (Sweet *et al*, 1977). Leukaemic manifestations are the result of peripheral blood and bone marrow replacement of the normal elements, and of the associated metabolic problems and their effect. A local problem denotes an anatomical aberration due to a specific regional involvement. Immunological manifestations relate to CLL as a defect in immunoglobulin synthesis.

CLL may involve any organ system including the skin, lymph nodes, liver, spleen, gastrointestinal tract, lungs, nervous system, skeleton, and genitourinary tract (Sweet *et al*, 1977). However, the most common features which are clinically significant are lymphocytosis, bone marrow infiltration, lymph nodes, liver, and spleen. The lymphocytosis varies greatly from case to case (usually ranging from $10x10^9$/l to $150x10^9$/l, but may be as high as $400x10^9$/l). Bone marrow infiltration is always present, but the extent and pattern (see section on histology below) vary and are of prognostic significance (Rozman *et al*, 1984; Mauro *et al*, 1994). Peripheral lymph nodes are frequently involved; splenic and hepatic involvement are less constant, but not uncommon.

Patients without symptoms and with minimal disease have a better prognosis than those presenting with bulky disease, anaemia, neutropenia and repeated infection (Dameshek, 1967). A number of staging systems have been proposed which consider the most important prognostic factors in CLL in order to predict the survival and therapy requirements of individual cases. The two staging systems widely used were devised by Rai *et al* (1975) and Binet *et al* (1981). In Chapter 2 of this thesis, the expression of adhesion molecules is compared with clinical features of the disease. The Binet staging system is employed as one of the criteria by which the CLL cases

are classified. In the Binet staging system, the importance of the tumour mass is emphasised by taking into account the potential enlargement of five different "lymphoid" areas: (1) cervical; (2) axillary; (3) inguinal lymph nodes; (4) spleen; (5) liver. On the basis of the number of lymphoid areas involved and the presence or absence of anaemia and/or thrombocytopenia, three prognostic groups are distinguished. Median survivals for patients in stages A, B and C are 12, 5, and 2 years respectively.

### 1.1.3 Histology

Bone marrow involvement may take on four different patterns of infiltration:- nodular, interstitial, mixed (nodular plus interstitial), and diffuse (Lipshutz *et al*, 1980; Rozman *et al*, 1984). However, for prognostic purposes, these patterns can be classified into two broad groups-diffuse and non-diffuse (Lipshutz *et al*, 1980; Rozman *et al*, 1981). Patients with diffuse bone marrow infiltration have a worse prognosis (median survival 2-4 years) than do those with non-diffuse involvement (median survival >8 years) (Montserrat and Rozman, 1987).

Within the lymph nodes, CLL cells behave like normal B cells, and occupy the B-dependent areas of the follicles, where they associate with the follicular dendritic cells. The accumulation of CLL cells in the node causes symmetrical enlargement.

The most common pattern of splenic involvement in CLL is expansion of the white pulp (Lampert *et al*, 1980). The white pulp may be infiltrated in either a nodular or a diffuse manner. T cells are displaced from their usual periarterial location, and appear more often in the red pulp (Lampert, 1983).

# 1.2 Hairy-Cell Leukaemia

## 1.2.1 Definition

HCL is a clonal proliferation of pathognomonic HCs that take on a peculiar and specific tissue distribution and alter the tissue microenvironment in a distinctive way. The HCs have a very characteristic appearance (diagnostic cytology in Romanowsky preparations and unique surface topography), specifically express an isoenzyme of acid phosphatase, and possess a distinctive immunophenotype. HCL is rare, accounting for around 2% of adult leukaemias (Gale and Foon, 1985).

It is accepted that HCs are of B cell lineage. The malignant clone shows both heavy and light chain gene rearrangements and is able to synthesise and to secrete immunoglobulin of a single light chain type (κ or λ; Gordon, 1984). The immunoglobulin is either of IgG type alone or is a combination of multiple heavy-chain isotypes, with prominent IgD (Burns *et al* 1978). The HC also expresses surface antigens considered specific for late B lymphocyte lineage (reviewed in Cawley & Burthem 1992). These antigens, characteristically CD19, CD20, CD22, and FMC7, are those associated with a mature, but pre-plasmacytoid stage of differentiation (Visser *et al* 1989).

As will be discussed later (section 1.6), the HC has a range of features indicating activation. In conclusion, then, HCL is a clonal proliferation of late B-cells with features of activation (Cawley and Burthem, 1992).

## 1.2.2 Pattern of disease

The malignant HC has an unusual and consistent predilection for certain tissue sites. The red pulp of the spleen is heavily infiltrated and its architecture distorted, while the white pulp is not involved and is frequently atrophic (Pilon *et al,* 1981; Pilon *et al,* 1982; Shi-Hua *et al,* 1992). Although marked hepatic enlargement is unusual, and liver architecture well maintained, the hepatic sinusoids are consistently infiltrated by HCs (see below). Bone marrow infiltration is always present. The HC infiltrate is accompanied by a variable degree of reticulin fibrosis (Burke, 1978). Lymph node enlargement is unusual and although HCs may be present, the infiltrate is not usually prominent (Katayama and Schneider, 1977). A variable number of circulating HCs are present in the blood.

At sites of prominent HC infiltration (especially splenic red pulp) distinctive structures known as pseudosinuses may be present (Pilon *et al*, 1982). These consist of vascular spaces lined by HCs with an associated basement membrane. Such structures require tissue remodelling by HCs (Re *al*, 1988); see below.

## 1.2.3 Histology

### Cell-cell associations

Within tissues such as spleen and liver, two major cell-cell associations are usually apparent. The first is between the HC and the endothelial cell (EC), and the second between the HC and the tissue macrophage (Nanba *et al,* 1977b; Pilon *et al,* 1982). HCs overlie and infiltrate between endothelial cells, forming close cellular contact with them. In hepatic sinusoids the HCs align with endothelial cells in a

manner resembling Kupffer cells. This association between HCs and endothelial cells appears to have metabolic consequences for the endothelial cells, which often appear atrophic and are eventually lost (Pilon *et al,* 1981).

Despite the monocytopenia of HCL, tissue macrophages are prominent and form an intimate association with surrounding HCs. This association probably has functional significance since it has been reported from this laboratory that monocytes/macrophages can stimulate HC proliferation (Griffiths and Cawley, 1991).

**Cell-matrix interactions**

Infiltrating HCs are clearly associated with existing extracellular matrix and basement membrane elements. The tendency for HCs to line existing basement membranes in a polarised manner is a well recognised histological feature of the disease (Nanba *et al,* 1977a), and may have a role in the formation of pseudosinuses. In this Department, the interactions of HCs with extracellular matrix have been characterised (Burthem *et al,* 1994a and 1994b). HCs have been shown to express the $\alpha5\beta1$ and $\alpha4\beta1$ fibronectin receptors, and the vitronectin receptor, $\alpha V\beta3$. These receptors are constitutively active, with HCs binding strongly to both vitronectin (VN) and fibronectin (FN). The adherent HCs also show cytoskeletal changes in response to this binding. On FN, HCs remain sessile and spread, attaining a flattened morphology resembling the shape of a fried egg. On VN, HCs attain a polar, motile morphology. Furthermore, immunohistochemical staining showed that FN is prominent in bone marrow (where it is at least partly responsible for the reticulin fibrosis), while VN is prominent in spleen. These observations, taken together, support the notion that integrins contribute in a major way to the peculiar and specific tissue reactions seen in HCL.

# 1.3 B-Lymphocyte Recirculation

## 1.3.1 Definition of B lymphocytes

B lymphocytes are responsible for the production of antibodies that bind to foreign organisms and facilitate their destruction. B cells are the only cells capable of synthesizing immunoglobulin molecules that, in the resting B lymphocyte, remain attached to the cell membrane. Detection of membrane Ig is the most commonly used B cell marker (Jondal *et al*, 1973). Most B cells express IgM and/or IgD. A smaller number of B cells display IgG or IgA (Preud'homme and Seligman, 1974). To efficiently protect the body from infectious organisms, lymphocytes circulate as non-adherent cells in the blood and lymph, and migrate as adherent cells in tissues when necessary (Anderson *et al*, 1982; Butcher, 1991; Springer, 1994).

## 1.3.2 Recirculation (ecotaxis)

B lymphocytes are generated in the adult in the bone marrow. When fully differentiated, B cells are released into blood (Ikuta *et al*, 1992); T cell precursors migrate to the thymus prior to differentiating (Ikuta *et al*, 1992). These B cells are described as naive, because they have not yet encountered an antigen recognised by their antigen receptors (Gray, 1992). These naive B cells are IgM+IgD+ and constitute the majority of the B cells that recirculate between blood and lymphoid tissues (Strober and Dilley, 1973). Some of the B lymphocytes migrate to the periphery and possess the ability to "recognise" special compartments within the lymphoid organs, known as T-independent or B-dependent areas. This property is

known as homing. The recirculation and homing patterns of lymphocytes were established *in vivo* using re-injection of isolated cells after fluorescence or radiolabelling (Pabst *et al*, 1993). B cells recirculate more slowly than T cells, although the reason for this is not known (Sprent, 1973). B lymphocytes prefer to home to the spleen, where they account for 50% of all lymphocytes as compared to 25% in the lymph node. The slower recirculation of the B lymphocytes is related to an even slower passage through the spleen than through the lymph nodes.

Within the lymph nodes and spleen, B lymphocytes home to the follicles. The selective localization of B lymphocytes in specific areas of the lymphoid organs may be related to their affinity for particular accessory cells called follicular dendritic cells (Burton *et al,* 1995). Follicular dendritic cells are only present in B-dependent areas; they have long cytoplasmic processes and are thought to create a microenvironment to support the differentiation and proliferation of B cells (Kosco *et al*, 1992; review by Thorbecke *et al*, 1994).

Fig.1.1 illustrates the normal pathways of B lymphocyte recirculation.

### 1.3.3 Tissue entry- the adhesion cascade.

In secondary lymphoid tissue (lymph nodes, spleen, gut associated lymphoid tissue), most B lymphocyte extravasation occurs in postcapillary venules with distinctive cuboidal histology- the high endothelial venules (HEV) (Stamper and Woodruff, 1976). Most recirculating lymphocytes selectively bind to the endothelium of HEV, (demonstrated by the Stamper-Woodruff method (1976) for allowing viable lymphocytes to adhere to frozen sections of lymphoid organs), but rarely to

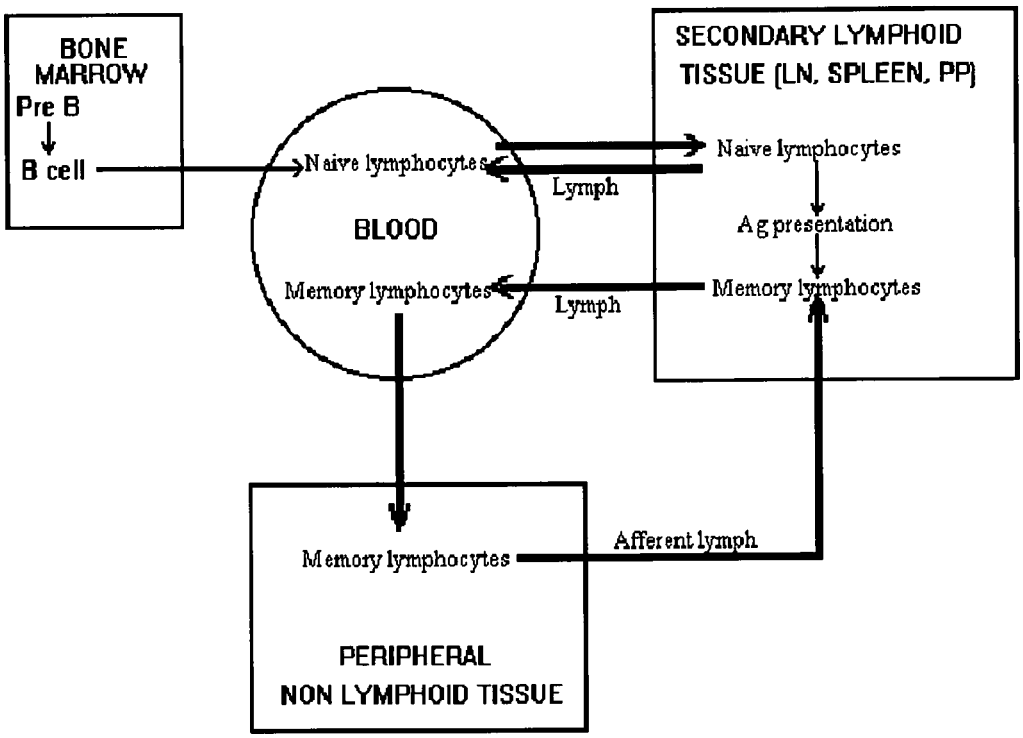



**Fig. 1.1 Normal pathways of B lymphocyte recirculation.**

normal vascular endothelium (Mackay *et al*, 1990; Hall and Morris, 1965). The exception to this is during an inflammatory response, where damaged tissue influences the adhesion properties of the adjacent endothelium, resulting in local extravasation of lymphocytes (Kapiotis *et al*, 1994). The process of tissue entry has been described as an adhesion cascade, as it involves three or more steps, each mediated by a particular set of adhesion molecules (Hynes, 1992; Sanchez-Madrid and Corbi, 1992). The steps are:-lymphocyte rolling along endothelium, triggering of tight adhesion, and transendothelial migration.

**Lymphocyte rolling**

Selectin interactions with their ligands are believed to be the main component of the first step in lymphocyte adhesion to endothelium, although recently α4

integrins have also been shown to be able to facilitate this (Berlin *et al*, 1995). These interactions are weak and transient, and because of the shear forces induced by the blood flow, the lymphocyte rolls along the endothelium (Butcher, 1991). The large number of selectin ligands suggests that some cells may simultaneously express two or more of these molecules, allowing precise control of this step (Butcher, 1991). The short-term cell-cell contact achieved by selectins brings lymphocytes into close proximity with the vascular endothelium in preparation for the next step in the adhesion cascade.

**Adhesion triggering**

During rolling the lymphocyte is exposed to chemoattractants that are displayed on or released from the endothelial lining of the vessel wall (Butcher, 1991; Rot, 1993). Chemoattractants bind to receptors that span the membrane seven times on the surface of lymphocytes. These couple to G proteins, which transduce signals that activate integrin adhesiveness (Butcher, 1991). The lymphocyte then binds tightly to the endothelium via integrin receptors on the lymphocyte and their ligands (usually members of the immunoglobulin superfamily of adhesive proteins, see below).

**Transendothelial migration**

Following directional cues from chemoattractants and using integrins for traction, lymphocytes then cross the endothelial lining of the blood vessel and enter the tissue. Transendothelial migration requires movement over the endothelial lumenal surface, and often some component of migration through subendothelial

matrix, in addition to penetration between endothelial cells (Smith, 1992). The precise adhesion receptors involved in transendothelial migration are unclear, but almost certainly include β1 integrins binding to subendothelial matrix elements as well as CD31, which is expressed on the lymphocyte surface and also on endothelial cells, where it is localised at cell-cell junctions (Elenstrom-Magnusson *et al*, 1995; Albeda *et al*, 1991). Basement membrane contains predominantly laminin, collagen, entactin, and heparan sulfate, while extracellular matrix may comprise fibronectin, vitronectin, and collagen (Li and Cheung, 1992; Chan and Aruffo, 1993). The composition of basement membrane and extracellular matrix may vary between tissues, and these differences influence the pattern of infiltration by lymphocytes (Li and Cheung, 1992).

### 1.3.4 Tissue phase of lymphocyte recirculation

Having entered the tissue, adhesive processes continue to influence the lymphocyte's behaviour. Interactions with matrix proteins and accessory cells dictate whether the lymphocyte will migrate within the tissue environment, and whether the cell will return to the circulation, or remain sequestered in an appropriate B cell area (Molossi *et al*, 1995). Soluble factors from the vascular endothelium and from accessory cells and other resident lymphocytes influence this stage of recirculation (Duijvestijn and Hamann, 1989; Randolph and Furie, 1995; Ebert, 1995).

## 1.4 Adhesion molecules

Many adhesion molecules have been identified; these have been characterised and can be grouped into four major families: the selectins, integrins, immunoglobulin superfamily, and a group of highly glycosylated molecules. Selectins, integrins and immunoglobulin-like molecules are all relevant to this thesis, and are discussed under their respective headings below. In section 1.4.1 members of the group of adhesion molecules which might have a role in CLL and HCL cell behaviour are briefly mentioned. The reasons for excluding these adhesion molecules from the experimental work are discussed.

### 1.4.1 Other adhesion molecules

Since this work started, new adhesion molecules have been described, which may have a role in normal lymphocyte recirculation, but could not be included in this study, e.g. the vascular adhesion proteins described by Jalkanen *et al* (Salmi and Jalkanen, 1992; Salmi *et al*, 1993; Airas *et al*, 1993.), and also the immunoglobulin (I)-type lectins (carbohydrate-binding proteins) such as CD22, which is expressed on the majority of mature resting B cells. CD22 mediates interactions of B cells with T cells, other B cells, activated endothelial cells, or accessory cells (Wilson *et al*, 1991; Engel *et al*, 1993).

Another adhesion molecule that may be important in CLL, is CD44. This adhesion molecule is now known to be expressed at high levels in CLL. CD44 can bind to hyaluronic acid, and to the extracellular matrix proteins collagen, laminin, and

fibronectin *in vitro* (Peach *et al*, 1993; Jalkanen and Jalkanen, 1992). CD44 may participate in lymphocyte binding to HEVs and activated endothelium, although evidence for this is contradictory (Stamenkovic *et al*, 1991; Jalkanen *et al*, 1987). However, CD44 is widely expressed in many cell types, and there are several variant isoforms of this glycoprotein with different binding activities and specificities (Dougherty *et al*, 1994; Salles *et al*, 1993). An attempt is being made by other workers to relate expression of CD44 with disease severity in CLL (De Rossi *et al*, 1994). Since, when this study began, the function of this molecule was unclear, and since there is confusion regarding the importance of this molecule in lymphocyte biology, this study does not consider the potential role of CD44 interactions to mediate CLL cell or HC distribution.

## 1.4.2 Selectins

There are three members of the selectin family of adhesion molecules. These receptors share a common mosaic structure consisting of an aminoterminal C-type lectin (sugar-binding) domain, a single epidermal growth factor (EGF)-like domain, several short consensus repeats (SCR) similar to those found in regulatory proteins that bind complement, a transmembrane domain, and a short C-terminal cytoplasmic domain (Bevilacqua *et al*, 1991). Selectins bind to carbohydrate ligands via the lectin domain, and anionic carbohydrates, such as sialyl Lewis X and sialyl Lewis A, can block *in vitro* adhesion of lymphocytes to lymph node HEVs (Springer and Lasky, 1991; Lasky, 1992). Selectin ligands are a diverse range of carbohydrates; the common feature of most is a lactosamine backbone (Larkin *et al*, 1992). Table 1.1 shows the selectin ligands described to date, which have been shown to be expressed *in vivo*, and thus may have a role in selectin-mediated interactions.

**L-selectin (CD62L)** is expressed on all leucocytes except activated, memory lymphocytes (Camerini *et al*, 1989). Its expression by leucocytes can be modulated; cell activation by chemokines or phorbol esters downregulates L-selectin expression on the plasma membrane by shedding (Kishimoto *et al*, 1990). Thus a soluble form of L-selectin can be found in plasma (Schleiffenbaum *et al*, 1992; Tamatani, 1993). L-selectin has been shown to be expressed on CLL and HCL lymphocytes (Csanaky, 1994; Knapp *et al*, 1989).

***Table 1.1. Selectin Ligands.*** The table lists ligands in the chronological order in which they were described.

| Original Name | Source | Polypeptide name | Natural ligand for | Refs. |
|---|---|---|---|---|
| Sgp50 HEV L-selectin ligand | mouse peripheral LN | GlyCAM-1 | L-selectin | Imai *et al*, 1991. Lasky *et al*, 1992 |
| 120kD myeloid P-selectin ligand | human neutrophil, HL60 cells | PSGL-1 | P-selectin | Moore *et al*, 1992. Norgard *et al*, 1993 |
| 150kD myeloid E-selectin ligand | mouse bone marrow cells | polypeptide unknown | E-selectin | Levinovitz *et al*, 1993. Lenter *et al*, 1994 |
| sgp90 sulphated L-selectin ligand | mouse peripheral LN | CD34 | L-selectin | Baumhueter *et al*, 1993. |
| MAdCAM-1 | mouse mesenteric LN | MAdCAM-1 | L-selectin | Berg *et al*, 1993. |
| 250kD E-selectin ligand | bovine γ/δ lymphocytes | polypeptide unknown | E-selectin | Walcheck *et al*, 1993 |
| Heparan sulphate | bovine and human endothelial cells | core protein not required | L-selectin | Norgard-Sumnicht *et al*, 1993 |
| 160kD myeloid P-selectin ligand | mouse bone marrow cells | polypeptide unknown | P-selectin | Levinovitz *et al*, 1993. Lenter *et al*, 1994 |

**P-selectin (CD62P)** is expressed on activated platelets and endothelium (Larsen *et al*, 1990). On the surface of endothelial cells P-selectin initiates the earliest phase of

leukocyte recruitment into inflammatory sites (McEver, 1992; Lorant, *et al*, 1993; Jones *et al*, 1993). The P-selectin gene is constitutively expressed, and P-selectin is stored in Weibel-Palade bodies; it is translocated to the plasma membrane within minutes after cell activation by histamine, thrombin, or complement factors (Geng *et al*, 1990; Smith *et al*, 1993; Sugama, *et al*, 1992).

**E-selectin (CD62E)** is expressed on endothelium after stimulation by the inflammatory cytokines IL-1 or TNFα, or by neuropeptides (Weller *et al*, 1992; Smith *et al*, 1993; Bevilacqua *et al*, 1987), as well as by lipopolysaccharide (Shimizu *et al*, 1991). Following endothelial cell activation by these agents, E-selectin is detected after 3-6 hr and decreases to basal levels after 24 hrs (Weller *et al*, 1992; Fries *et al*, 1993).

## 1.4.3 Integrins

As well as being important in lymphocyte homing, integrins are involved in many other biological processes including embryonic development and maintenance of tissue integrity (Hynes, 1992; Albeda and Buck, 1990; Sonnenberg, 1993). All integrins are heterodimers composed of an α subunit non-covalently associated with a β subunit (Hynes, 1987), illustrated in Fig.1.2.

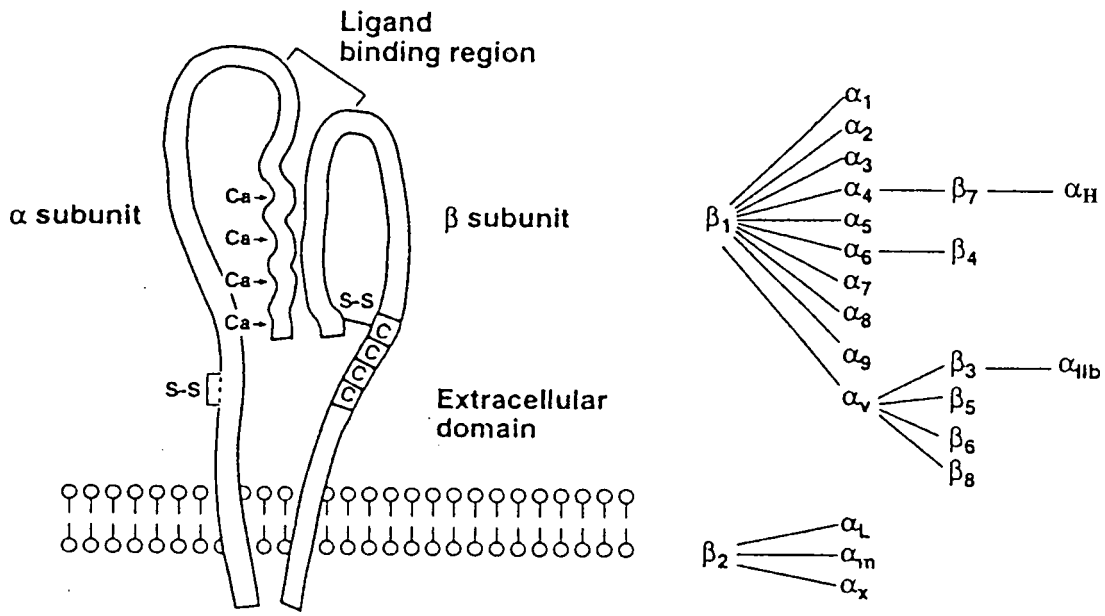



**Fig.1.2 Outline of the structure of integrins and the combinations of α-and β-chains. (S-S=disuphide bridge; Ca=cation-binding site; C=cystein-rich domain)**

At present 15 α- and 8 β-chains are known and at least 21 different heterodimers have been found (Hynes, 1992; Hemler, 1990). Lymphocyte populations can express any of thirteen integrins from the β1, β2, β3, and β7 subfamilies, and eight of these are important in the lymphocyte-endothelial

interaction; αLβ2, αMβ2, αXβ2, α4β1, α4β7, αHβ7, αVβ3, α6β1 (see below). The expression and heterodimer associations of these adhesion molecules by the abnormal cells of CLL are considered in Chapter 2. Members of the β1 family of integrins, also known as the very late activation (VLA) antigens, are involved in interactions with extracellular matrix proteins. Table 1.2 shows the diverse ligands of this family of adhesion receptors.

**Structure**

Both α and β subunits are transmembrane glycoproteins, and several cytoplasmic domains of the β1 integrin subunit interact with cytoskeletal actin filaments via the proteins talin or α-actinin. The extracellular domains contain the ligand binding region. The α chain contains areas that bind $Ca^{2+}$ or $Mg^{2+}$ and the nature of the cation can affect ligand affinity (Masumoto and Hemler, 1993; Larson *et al*, 1989). In many cells there is a retention of an intracellular pool of immature β1 integrin chains associated with the chaperone calnexin (Lenter and Vestweber, 1994). Cell-surface expression of mature heterodimer is then regulated by biosynthesis, assembly, and transportation of the α-chain. The level of α-chain expression can be regulated by the addition of inflammatory cytokines, such as TNFα (Defilippi *et al*, 1991; Defilippi *et al*, 1992). The changes in integrin expression on CLL cells after *in vitro* stimulation are investigated in Chapter 2.

**Activation of integrins**

Integrins rapidly transfer from a non-adhesive, low-affinity state to a transient high-affinity state (Hynes, 1992). This is probably due to a conformational change

caused by activation because certain mAbs react with β1 or β2 integrins only after activation and others can activate integrins after binding (van Kooyk *et al*, 1991; Sanchez-Mateos *et al*, 1993; Kovach *et al*, 1992). This controllable adhesiveness of integrins provides a versatile mechanism for the arrest and tight adhesion of circulating lymphocytes on vascular endothelium, rapidly followed by intermediate adhesion during transendothelial migration, and finally de-adhesion at extravasation (Springer, 1994; Shimizu *et al*, 1992; Mackay and Imhof, 1993). Different cell-cell interactions can induce a higher affinity; for instance, interaction of T lymphocytes with the vascular endothelium through CD31 (see Immunoglobulin Superfamily below) transduces activating signals into T cells which in turn activate integrins (Tanaka *et al*, 1992; Piali *et al*, 1993). Similar activating signals are produced from interaction of the chemokines with their receptors (Baggiolini *et al*, 1994; Durum and Oppenheim, 1993). This means that the integrin-mediated activities of any cell, including lymphocytes is influenced not only by which integrins are expressed (and what heterodimeric associations are present), but also by the functional state of these integrins. These considerations may be very important in CLL and may well explain why previous authors have had difficulty in relating simple mAb-defined expression of integrin chains with clinical behaviour. The functional studies in Chapters 3 and 4 were designed to address these issues. The homogeneous nature of HCs and their constitutive activation may explain why associations between integrin expression and clinical features of disease are more apparent.

**β2 integrins.**

There are three members of this family of integrins expressed on human leucocytes. When expressed, these adhesion receptors are important in the tight adhesion between leucocytes and endothelium (Buckley, 1993).

**αLβ2** is present on the surface of most leucocytes and interacts with the first Ig domain of the ICAMs (members of the immunoglobulin superfamily) (Kurzinger *et al*, 1981).

**αMβ2** is found predominantly on granulocytes, macrophages, and NK cells, and is also a receptor for ICAM-1 but does not bind to ICAM-2 and -3 (Miller *et al*, 1986; Keizer *et al*, 1987). Thus, αMβ2 is a receptor involved in recruiting myeloid cells to inflammatory sites. αMβ2 is also binds to, and aids phagocytosis of, complement-particles. αMβ2 has been shown to be expressed on some, but not all, HCs (Knapp *et al*, 1989).

**αXβ2** is found predominantly on macrophages, monocytes and granulocytes; also on activated T and B lymphocytes, neutrophils and dendritic cells (Larson and Springer, 1990). This molecule is expressed at high levels on HCs (Knapp *et al*, 1989) and is present in some cases of CLL (Hanson *et al*, 1990). αXβ2 can bind to fibrinogen (Postigio *et al*, 1991a) and to iC3b (Micklem and Sim, 1985). This molecule also mediates interaction with endothelium, but the endothelial ligand is unknown (Stacker and Springer, 1991).

**β1 integrins.**

These are also known as very late activation (VLA) antigens. They are expressed on a wide variety of cells, including non-haemopoietic cells (Hemler, 1990;

Hemler *et al*, 1990; Hemler, 1988). HCs have been shown to express high levels of α5β1, α4β1 and low α3β1; these cells do not express other β1 integrins (Burthem *et al*, 1994a). Moller *et al* (1991) described the expression of β1 integrins on CLL cells as α1–, α2±, α3+, α4+, α5+, α6–.

Table 1.2 Ligands of the β1 family of integrin heterodimers.

| **Heterodimer** | **Ligand(s)** |
|---|---|
| α1β1 | collagen, laminin |
| α2β1 | collagen, laminin |
| α3β1 | fibronectin, collagen, laminin |
| α4β1 | fibronectin (CS-1 domain), VCAM-1 |
| α5β1 | fibronectin (RGD domain) |
| α6β1 | laminin |
| α7β1 | laminin |
| α8β1 | ? |
| α9β1 | ? |

β1 integrins bind to extracellular matrix components (Table.1.2) such as fibronectin, laminin, and collagen, and thus play a role in lymphocyte adhesion and migration within tissues (Shimizu and Shaw, 1991; Ruoslahti and Pierschbacher, 1987).

**α3β1** is expressed at higher levels on CLL cells than on normal peripheral blood B-cells or other B-lymphoproliferative disorders, although the functional significance of this is unknown (Baldini *et al*, 1992).

**α4β1** is the only member of the β1 integrins which recognises, apart from the extracellular matrix component fibronectin, a transmembrane molecule which is a member of the immunoglobulin superfamily. VCAM-1, the ligand of α4β1, is expressed on activated endothelium (Osborn *et al*, 1989; Elices *et al*, 1990). Therefore, α4β1 is also involved in lymphocyte-endothelial cell interactions. The α4-chain can be expressed on cell surfaces as a 180 or a 150 kD form; the latter can also appear as a cleaved configuration with 80 and 70 kD chain fragments. These forms may result in differential activation of this integrin by cellular signals. α4β1 and α5β1 are important in lymphocyte adhesion and migration on fibronectin and mediate lymphocyte movement underneath bone marrow stromal cells (Miyake *et al*, 1991; Miyake *et al*, 1992). α4β1 also plays a role in leukocyte spreading on endothelium, and also in transendothelial migration since blocking of this integrin blocks extravasation (Butcher, 1991; McEver, 1992).

**α5β1** expression contributes to the disseminative phenotype of malignant B cells-presence of α5β1 correlates with ability of various lymphomas to spread (Blase *et al*, 1995).

**β7 integrins.**

**α4β7** is expressed on a subset of lymphocytes which colonise the gut and gut-associated lymphoid tissues (Holzmann *et al*, 1989; Schweighoffer *et al*, 1993). Integrin α4β7 recognises the mucosal endothelial ligand MAdCAM-1 (see Table 1.1) and mediates lymphocyte homing to Peyer's patches. This integrin can also bind to VCAM-1 and fibronectin (Berlin *et al*, 1993; Strauch *et al*, 1994; Schweighoffer *et al*,

1993); while MAdCAM-1 is involved in lymphocyte recirculation under normal conditions, interactions with VCAM-1 may only become important in inflammatory reactions. α4β7 can also mediate lymphocyte tethering under conditions of high shear stress, using loose reversible interactions prior to activation and firm sticking (Berlin *et al*, 1995). α4β7 does not appear to have a role in transendothelial migration (Chan and Aruffo, 1993).

**αHβ7** is involved in the interaction of lymphocytes with the intestinal epithelium (Cerf-Bensussan *et al*, 1992; Shaw *et al*, 1994). Recently it has been described that the heterotypic adhesive interactions between epithelial cells and intraepithelial lymphocytes are mediated by E-cadherin, the ligand of αHβ7 (Cepek *et al*, 1994). E-cadherin is one of a member of a family of $Ca^{2+}$-dependent cell adhesion molecules with a large extracellular domain with four repeated $Ca^{2+}$ binding regions, a single transmembrane sequence, and a 15kD cytoplasmic domain (Nagafuchi *et al*, 1987; Ringwald *et al*, 1987). The presence of αHβ7 has been described on HCs, and is used as a marker to identify these abnormal B lymphocytes (Knapp *et al,* 1989).

**β3 integrins.**

**αVβ3** is the classical vitronectin receptor (Smith and Cheresh, 1988). This heterodimer is expressed on HCs and mediates HC adhesion and migration in the vitronectin-rich environment of splenic red pulp. Thus αVβ3 has a role in directing the particular distribution of these cells *in vivo* (Burthem *et al*, 1994a). The expression of this integrin on CLL cells has been described (De Rossi *et al*, 1993), although expression was confined to a small number of cases, most of which were

atypical (i.e. CD5-negative). The expression of the heterodimer in these cases of CLL correlated with poor prognosis. A recent publication (Piali *et al*, 1995) showed that αVβ3 can also bind to endothelial CD31, and may therefore have a role in transendothelial migration of αVβ3-expressing cell types.

## 1.4.4 Immunoglobulin superfamily.

The immunoglobulin (Ig) superfamily encompasses a large group of molecules with multiple Ig-like domains (Fig.1.3). This family includes a number of adhesion molecules, the majority of which are expressed on endothelium, although some (ICAM-1, ICAM-3, and recently VCAM-1) have been described on lymphocyte populations (Dustin et *al,* 1986; de Fougerolles et *al*, 1991; Reuss-Borst et *al*, 1995). Hence these receptors are important in the adhesion of lymphocytes to endothelial cells as a prerequisite for tissue entry.

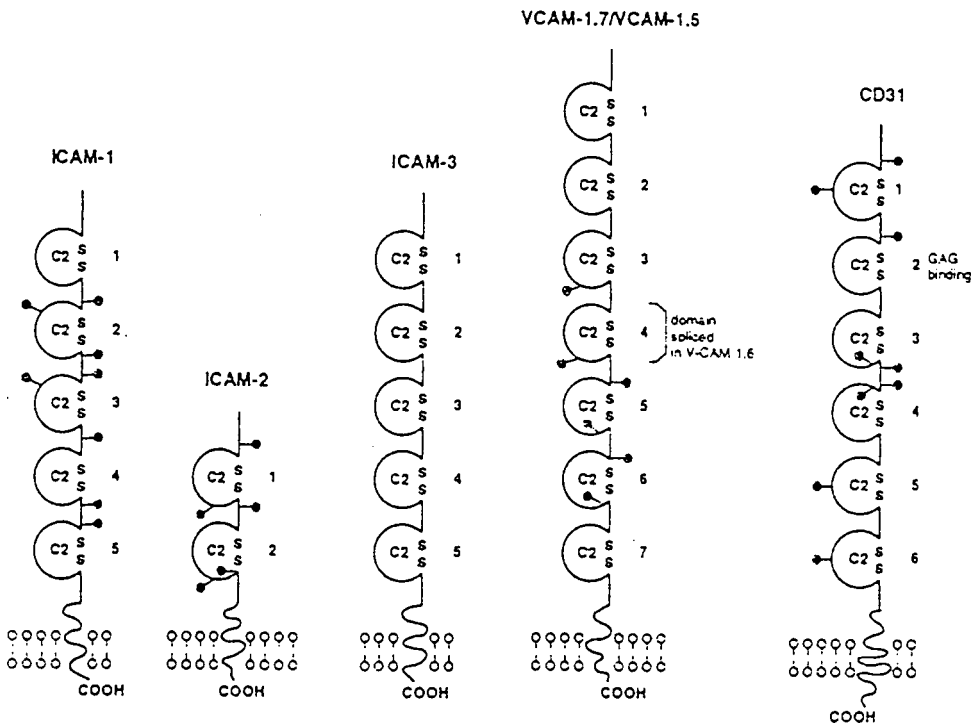



**Fig.1.3 The general structure of the immunoglobulin superfamily adhesion molecules. (COOH=C-terminal cytoplasmic part; =sugar side-chain; C2=constant immunglobulin domain; S-S=disulphide bridge.**

Each Ig-like domain is usually encoded by a discrete exon, and consists of a primary sequence of 60-70 amino acids with a disulphide bridge spanning 50-70 residues; several other conserved residues are involved in establishing a tertiary structure referred to as an antibody fold (Simmons *et al*, 1988; Staunton *et al*, 1989; Newman *et al*, 1990; Osborn *et al*, 1989; de Fougerolles *et al*, 1993).

Five members of this family are involved in lymphocyte-endothelial cell interaction: ICAM-1, ICAM-2, ICAM-3, VCAM-1, PECAM-1 (CD31). Except for ICAM-3, these all serve as endothelial ligands for leucocytes.

**ICAMs.** **I**ntercellular **a**dhesion **m**olecules (ICAMs) -1 and -2 were both initially identified by their ability to bind to αLβ2 integrin (Staunton *et al*, 1989; Marlin and Springer, 1987). All three ICAMs are involved in antigen-specific interactions (de Fougerolles *et al*, 1994).

**ICAM-1** binds αLβ2 by the first domain and has also been found to bind αMβ2 by a distinct site in the third domain (Diamond *et al*, 1990; Staunton *et al*, 1990). ICAM-1 also binds CD43, which is widely expressed on leucocytes, although the significance of this interaction has not been established (Rosenstein *et al*, 1991; Cyster and Williams, 1992). ICAM-1 is weakly expressed on resting endothelium, but is strongly induced after several hours of stimulation by IL-1, TNFα, or interferon-γ (Dustin *et al*, 1986; Pohlman *et al*, 1987; Rohlein *et al*, 1988). ICAM-1 is widely expressed in an inducible manner on all leucocytes (de Fougerolles *et al*, 1993).

**ICAM-2** is constitutively expressed at a high level on resting endothelial cells and its expression is not increased by activation (Nortamo *et al*, 1991; de Fougerolles *et al*, 1991); the affinity of ICAM-2 for αLβ2 is weaker than that of ICAM-1 (de Fougerolles *et al*, 1994), and is probably responsible for constitutive low

transendothelial leukocyte traffic. ICAM-2 is not expressed on leucocytes (de Fougerolles *et al*, 1991)

**ICAM-3** is not expressed on endothelium (Elgabalawy *et al*, 1994), and is strongly expressed on resting lymphocytes and monocytes (de Fougerolles *et al*, 1994).

**VCAM-1.** The vascular cell adhesion molecule VCAM-1 was originally identified as a cytokine-inducible adhesion molecule on human endothelial cells, mediating the binding to leucocytes and melanoma cells (Osborn *et al*, 1989; Hession *et al*, 1992; Hession *et al*, 1991). VCAM-1 is a ligand for the $\alpha 4\beta 1$ integrin and binds weakly to $\alpha 4\beta 7$ (Strauch *et al*, 1994; Elices *et al*, 1990; Ruegg *et al*, 1992). VCAM-1 is characterised by the presence of a variable number of Ig domains resulting from alternative splicing of the VCAM-1 gene (Osborn *et al*, 1989; Polte *et al*, 1990). The predominant form of VCAM-1 on endothelium contains 7 Ig domains (Hession *et al*, 1991; Cybulski *et al*, 1991). Additional forms with 6 and 8 Ig domains have been described (Osborn *et al*, 1989; Cybulski *et al*, 1991) (Fig.1.4). In murine stromal and endothelial cells glycophosphatidylinositol-anchored and -non-anchored isoforms have also been described (Kinashi *et al*, 1995). This PI-linked form contains only the three N-terminal Ig domains followed by a unique 21-amino acid C-terminus tail (Moy *et al*, 1993). In addition, a soluble 95-110kD form of VCAM-1 has been purified from the supernatant of human cultured endothelial cells, as well as from blood of patients suffering from rheumatoid arthritis and SLE (Pigott *et al*, 1992; Wellicome *et al*, 1993).

VCAM-1 is absent on resting endothelial cells, but is induced by IL-1 and TNF, with maximal activity reached by 6-12 hr (Masinovsky, et *al*, 1990; Swerlick, et *al*, 1992). IL-4 also increases the expression of VCAM-1, but not E-selectin or

ICAM-1 (Masinovsky, et *al*, 1990). Thus VCAM-1 and ICAM-1 are regulators of lymphocyte extravasation at sites of inflammation. VCAM-1 is also expressed in several non-vascular cell types, including populations of dendritic cells found in lymph nodes and skin, bone marrow stromal cells, splenic red pulp fibroblasts, and synovial cells in inflamed joints (Miyake *et al*, 1991; Freedman *et al*, 1990). At these locations it is involved in the adhesion of lymphocyte precursors to bone marrow stromal cells, the binding of B cells to lymph node follicular dendritic cells, and VCAM-1/$\alpha 4\beta 1$ interaction acts as a costimulator for T cell proliferation (Miyake *et al*, 1991; Ryan *et al*, 1991).

**CD31.** This is a six domain molecule which mediates both leukocyte and platelet/endothelial cell adhesion and transendothelial migration (Newman *et al*, 1992; Xie and Muller, 1993; Muller *et al*, 1993; Schimmenti *et al*, 1992). CD31 is expressed on platelets and on most leucocytes and is constitutively present on endothelial linings *in vivo*. CD31 mediates adhesion through homophilic interaction; however, a heparin-binding consensus sequence on domain 2 may mediate heterophilic interaction with cell surface or extracellular matrix proteoglycans (DeLisser *et al*, 1993; Newman *et al*, 1992; Muller *et al*, 1992). As stated above in the section on $\beta 3$ integrins, CD31 can bind leukocyte $\alpha V\beta 3$ (Piali *et al*, 1995). CD31 is able to activate $\beta 1$ and $\beta 2$ integrins by ligand-induced signalling (Tanaka *et al*, 1992; Piali *et al*, 1993). When endothelial cells come into contact with each other to form a cobblestone-like monolayer, CD31 redistributes to the cell border and is thought to participate in the endothelial cell-endothelial cell interaction that limits vascular permeability (Albeda *et al*, 1991).

## 1.5 Lymphocyte Movement

The previous studies of HCL in this department have identified specific properties of HCs which influence their *in vivo* behaviour. This thesis applies the same theory to the *in vitro* investigation of CLL cell migration (Chapter 4). This section of the General Introduction is included to explain the morphological analysis of cells in order to identify their motility responses to adherence and to stimulation.

Motile cells of the immune system adopt a common basic morphology (Berneman 1990). A motile lymphocyte is illustrated in Fig. 1.4 below.

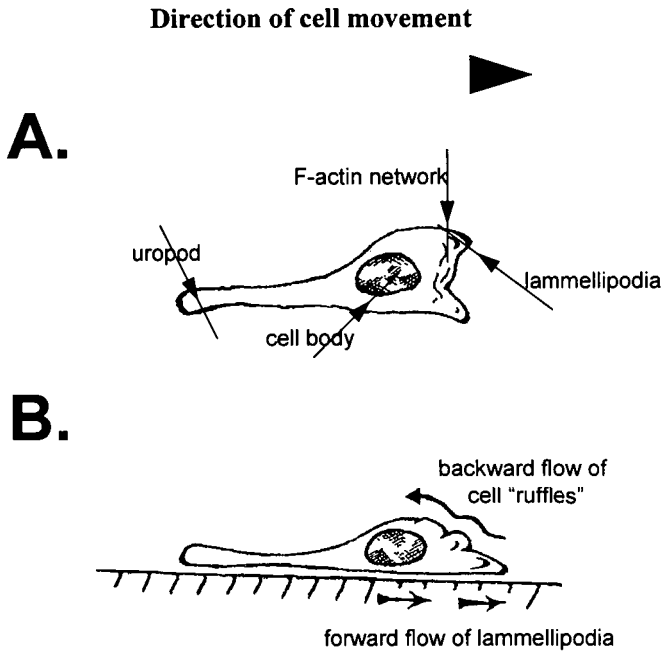



**Fig. 1.4 Features of a typical motile lymphocyte.**

A. from above

B. side view

The distinctive morphological feature of motility is that of front-tail polarity (Keller *et al* 1989). Non-motile cells, in contrast, appear circular from above, resembling a fried egg shape.

Simple adhesion, mediated by glass-bound non-stimulating lectins, does not induce the motile behaviour of B lymphocytes. However if the bound lectin has stimulatory properties, cell motility ensues (Alexander *et al* 1976). This implies that simple cell adhesion requires an additional "activating" signal to induce cell motility. Evidence is now accumulating to suggest that different adhesive proteins can provide such a signal. Various workers in the field have employed different cell types and different experimental systems. However, in lymphocyte biology, three proteins in particular have been implicated: collagen, laminin and fibronectin. The factors which influence B cell migration are not fully defined, but in some studies it has been shown that B-lymphocyte migration can be induced by fibronectin substrata (Davies 1990). The capacity of different adhesive proteins to promote, or maintain, motile behaviour of lymphocytes strongly suggests that signals received from integrin receptors may be responsible for the motile behaviour of these cells. Engagement of $\alpha 5\beta 1$ and/or $\alpha V\beta 3$ can induce motility in various cell types (Akiyama *et al* 1989; Bauer *et al* 1992; Albelda *et al* 1990; Seftor *et al* 1992). Adherence of HCs to vitronectin via $\alpha V\beta 3$ induces a motile response (Burthem *et al*, 1994a).

Whilst a role for integrin receptors and their adhesive protein ligands in cell motility is increasingly recognised, it remains clear that soluble factors such as the chemotactic peptides and cytokines are more potent and probably more flexible mediators of cell movement. Moreover in some cases adhesion-induced migration appears to be dependent on prior stimulation by soluble mediators (Wilkinson, 1986,

Ratner *et al* 1992). The migration response of CLL cells to adhesion to extracellular matrix proteins, as well as the modulation of this response by *in vitro* stimulators are investigated in Chapter 4 of this thesis.

# 1.6 Activation

## 1.6.1 B-cell activation

The concept of activation is not easy to define. The term is usually used to refer to the dynamic sequence of changes that normally follow exposure of the cell to antigen, although less specific stimulators can clearly induce some of these changes. Immature B cells that escape negative selection develop into quiescent naive B cells that circulate in the blood. As these cells pass through the secondary lymphoid organs such as the spleen, they scan for the presence of trapped antigens that their sIgM and sIgD recognise. The binding of antigen to the B-cell antigen receptor initiates signals that activate the B cell to undergo maturation to become memory and antibody-secreting cells. The process of going from a resting B cell to an antibody-secreting cell can be divided into three stages: early activation, proliferation, and differentiation (reviewed in Gold and DeFranco, 1994). Early activation is characterised by (1) entry into the $G_1$ phase of the cell cycle; (2) increased synthesis of biosynthetic/secretory machinery (such as ribosomes and endoplasmic reticulum) that is needed for high-rate antibody secretion; (3) increased expression of surface proteins involved in the activation of T cells such as MHC class II molecules and CD80, the ligand for CD28; (4) increased expression of receptors for T cell-derived cytokines that promote B cell proliferation and antibody secretion, and (5) migration of a B cell into specific functional microenvironments (Clark and Lane, 1991; Wortis *et al*, 1995; Jumper *et al*, 1995; Jutila, 1994; Turunen, 1994). Such activation involves a range of stimuli in addition to crosslinking of the antigen receptor, and leads to proliferation, differentiation. Stimulation through the antigen receptor may also induce the cell to

undergo apoptosis if the cell is immature or if other signals (via CD40 or cytokines) are absent.

### 1.6.2 Activation in HCL

The HC is now widely thought to be a highly activated cell. Many features of HCs support this conclusion. The heavy-chain isotype-switching referred to earlier (section 1.2.1) may be regarded as an activation feature, as might strong expression of sIg. However, a range of other activation features is also present. Immunophenotypic studies show that markers normally lost after B-cell activation, such as CD21 and CD24, are expressed only at low levels on HCs, while B-cell activation markers CD22, CD25 and CD72 are strongly represented (Knapp *et al*, 1989; Visser *et al,* 1989). "HC-restricted" antigens such as CD11c, B-Ly7 and HC2 are activation antigens on other cell types (Mulligan *et al* 1990; Chadburn *et al,* 1990; Posnett *et al,* 1990). Furthermore, the adhesion receptor profile of HCs resembles that of normal activated B cells (Burthem *et al*, 1994a).

Perhaps the most obvious and unique feature of activation on HCs is the appearance of the cell. The HC is large, with an open chromatin pattern when compared with normal mature circulating lymphocytes indicating an activated cell (Katayama *et al,* 1977; Braylan *et al,* 1978). The characteristic and fluid pattern of microvilli and ruffles that characterise the surface of the HC reflect ongoing cytoskeletal and signalling activity. The cortical actin cytoskeleton and actin-binding structures are highly active (Caligaris-Cappio *et al* 1986; Zauli *et al* 1988), and there is strong evidence of ongoing signalling activity involving a number of pathways. Pronounced PKC activity in HCL has been long suggested by the induction of an

"HC-like" phenotype in normal or malignant B-cells stimulated *in vitro* by phorbol ester (Caligaris-Cappio *et al* 1986; al Katib *et al*, 1992). More recently, high levels of activity involving the tyrosine protein kinase/phosphatase system have been identified (Zauli *et al* 1988), as has enhanced activity of certain serine/threonine kinases (Tamaki *et al*, 1992). Having emphasised the activation features of HCs, it must be acknowledged that the cells lack other characteristics (e.g. CD23) associated with normal B-cell activation. This presumably means that the activation of HCs is more specific than the activation that occurs during polyclonal B-cell stimulation.

Although the cell is known to be activated, this state is not rigid, and the HC retains the capacity to respond to further stimuli. For example, the HCs possess a rich cortical network of polymerised (F) actin, indicative of an "activated" cytoskeleton, however the cell may achieve further actin polymerisation and morphological change in response to pharmacological stimulation of protein kinase C (Caligaris-Cappio *et al,* 1986; Zauli *et al,* 1988).

Since most of the features described above are determinants of cell migration and adhesion, it is likely that the characteristic tissue distribution of the malignant cell reflects this activation.

### 1.6.3 Activation in CLL

Since CLL cells are small and morphologically appear inactive, the concept of activation less obviously applies to this cell type. Nevertheless CLL cells frequently do express certain surface antigens normally expressed by activated B cells, and such expression varies from case-to-case. For example, CLL cells frequently express the following activation antigens:- B5, CD25, CD38, and CD71 (Merle-Beral, 1990).

Furthermore, all cases of CLL express the activation antigen, CD23, which is upregulated on B cells stimulated with phorbol ester (Fournier *et al*, 1992; Fournier *et al*, 1994). However, the position of the CLL cell within the cell cycle is fully resting as documented by classic kinetic studies (Andreef *et al*, 1908) and by lack of c-*myc* protein expression (Larsson *et al*, 1987).

Since activation influences expression and function of cell surface adhesion receptors, the activation state of CLL lymphocytes is relevant to this thesis. In the experimental work described in the following chapters, a number of activation markers were selected to determine the state of activation of different cases of CLL. In order to avoid giving special emphasis to a particular activation antigen, a large panel of markers was used to assess activation (CD23, CD25, CD39, CD71, CD80, $\alpha$5, and $\alpha$X). All of these activation markers have been shown to be expressed following B-cell early activation, either by phorbol esters, CD40-crosslinking, or by T-cell derived cytokines. CD23 is upregulated on normal B-cells which have been treated with phorbol ester and have progressed from $G_0$ to $G_1$, (Delespesse *et al*, 1992). CD25 is the $\alpha$-chain of the high-affinity IL-2 receptor, and is upregulated on normal B-cells which require further stimulation from accessory cells (Waldmann, 1989). CD39 is widely reported to only be expressed on activated B-cells; it may have a role in homotypic adhesion but its major function is uncertain (Knapp *et al*, 1989). CD71 identifies cells which have been stimulated to proliferate (CD71 is the transferrin receptor and iron is essential for proliferation; Schwarting and Stein, 1989). CD80 was selected since this molecule is required for interaction with T cells in co-stimulation responses during B cell differentiation (Freeman *et al*, 1989). Since activation may induce an adhesive/motile response, two integrin sub-units, $\alpha$X and

α5, are recognised activation markers on B-cells (Sanchez-Madrid and Corbi, 1992; Ballard et al, 1991); these are included the assignment of activated status in this thesis. Certain activation markers which are always expressed in CLL (CD23, CD25) are included in the panel of antigens, and high or low expression of these was determined. Other surface antigens (CD39, CD71, CD80, α5, and αX) which are not always expressed in CLL are also determined. Because each of the antigens selected highlights a different aspect of early activation (i.e. CD80 is involved in T-cell co-stimulation, CD71 is essential for proliferation, CD25 is required to transduce signals to promote proliferation and antibody secretion, etc.), expression of more of these antigens should indicate that the cell is more activated. Thus cells expressing five or more of these antigens in a given case (>50% positive for CD23 and CD25) are designated, in this study, as activated. Such activation is then related to clinical features and immunophenotype (Chapter 2) and to various functional capabilities (Chapters 3 and 4).

# Chapter 2

## The expression of adhesion molecules and activation markers in CLL

### Introduction

When this thesis was begun there was little information concerning adhesion molecule expression in CLL. During the period the present work was being performed a number of publications have appeared which examined the integrin expression of CLL cells and have related the findings to the clinical behaviour of the disease (De Rossi *et al*, 1993; Baldini and Cro, 1994; Takeuchi and Katayama, 1993). No consistent conclusions have emerged, although individual studies have suggested that the presence of certain chains may have prognostic significance and may influence tissue distribution (Woessner *et al*, 1994; Baldini *et al*, 1992). These other studies of CLL have not, however, determined detailed integrin heterodimer associations and have not examined *in vitro* cell responses to ligand binding.

This chapter examines adhesion molecule expression in CLL. For reasons discussed in the General Introduction (Chapter 1), the particular adhesion molecules studied were the integrins, CD31, and L-selectin. Since different α- and β-chain associations influence ligand specificity, immunoprecipitation studies were employed to demonstrate particular heterodimeric associations- something not previously done in CLL.

Because previous studies of HCL in this Department have emphasised the importance of B-cell activation in integrin-mediated responses, and because CLL cells are known to display different degrees of activation, the observations in the present chapter are related to the activation phenotype of the CLL cases (see the discussion of CLL cell activation in the General Introduction, section 1.6.3).

Chapters 3 and 4 will examine how these adhesion molecules and activation markers influence the response of the CLL cell both to endothelium and to extracellular matrix.

## Methods

### Antibodies and Immunologic Techniques

#### *Monoclonal antibodies (mAbs)*

All the mAbs used in this chapter are listed in Table 2.1. The mAbs were against a range of adhesion receptors (Table 2.1) and activation antigens, or were used for purification (mAbs to CD3, CD16, and CD41).

#### *Detection of mAb staining*

All antigens for adhesion molecule expression were detected by APAAP. APAAP reagents (Dakopatts) were used according to standard protocols (Appendix 1.1). Positive controls (usually peripheral blood mononuclear cells or hairy cells) were included in all staining series. Antigens which were positive in some or all cases of CLL were then analysed by triple-layer FACS.

#### *Triple-layer flow cytometry*

Such methods have been shown to enhance sensitivity, and lower the detection limit for surface molecules in low copy number (Zola *et al*, 1990). Briefly (see also Appendix 1.3), primary antibodies at saturating concentrations were followed by biotin-conjugated horse-anti-mouse mAb (Vector Laboratories, Peterborough, UK). The third layer was streptavidin conjugated to phycoerythrin (Becton Dickinson, Ltd. used at recommended working dilution).

**Table 2.1 mAbs used in this Chapter**

| Specificity (common name) | CD | Clone (Isotype) | Source |
|---|---|---|---|
| α2 | 49b | 5E8 | Dr. R. Bankert Roswell Park Cancer Inst, NY |
| α3 | 49c | M-KID 2 (IgG1) | Immunotech, SA |
| α4 | 49d | 44H6 (IgG1) | Serotec |
| α5 | 49e | SAM1 (IgG2b) | Immunotech, SA |
| α6 | 49f | 4F10 (IgG2b) | Cymbus Bioscience Ltd. |
| αL | 11a | 25.3.1 (IgG1) | Immunotech, SA |
| αM | 11b | BEAR 1 (IgG1) | Immunotech, SA |
| αX | 11c | S-HCL-3 (IgG2b) | Becton Dickinson |
| αV | 51 | 13C1 (IgG1) | Prof. G. Burns, Newcastle, NSW, Australia |
| αH | 103 | 265.1 (IgG1) | Immunotech, SA |
| β1 | 29 | K20 (IgG2a) | Immunotech, SA |
| β2 | 18 | BL5 (IgG1) | Immunotech, SA |
| β3 | 61 | 1076 (IgG1) | Prof. G. Burns, Newcastle, NSW, Australia |
| PECAM-1 | 31 | 5.6E (IgG1) | Immunotech, SA |
| L-selectin | 62L | Dreg56 (IgG1) | Immunotech, SA |
| Fcε receptor (R) | 23 | EBVCS5 (IgG1) | Becton Dickinson |
| IL-2 R | 25 | 2A3 (IgG1) | Becton Dickinson |
| | 39 | AC2 (IgG1) | Immunotech, SA |
| transferrin R | 71 | LO1.1 (IgG2a) | Becton Dickinson |
| BB1 or B7 | 80 | L307.4 (IgG1) | Becton Dickinson |
| | 3 | SK7 (IgG1) | Becton Dickinson |
| | 16 | GO22 (IgG1) | Becton Dickinson |
| | 41 | M148 (IgG1) | Serotec |

The intensity of fluorescence detected by FACS varied greatly for the different antigens tested. When large amounts of antigen were expressed, the positive peak was completely distinct from the isotypic control. However, when the antigen was weakly expressed, the positive peak overlapped that of the isotypic control (Fig.2.1c overleaf). Since pure populations of cells were being used and because APAAP staining had already indicated that all the cells were positive for the antigens in question, all the cells in the right-shifted overlapping peak were reactive. Nevertheless, the positivity was expressed as a percentage of cells to the right of the control marker (set to exclude 98% of the cells stained with the isotypic control, Fig2.1a). Positive staining was expressed in this way to indicate the intensity of such staining.

### *Immunoprecipitation*

This technique was performed using published protocols, and is described in full in Appendix 1.2.

## Patient Material

### *Selection of CLL patients*

Ten morphologically typical cases of CLL were used in this study. A tight immunophenotypic definition of CLL was employed. In all cases the cells had the following phenotype as detected by direct fluorescence in the diagnostic laboratory:- weak light-chain-restricted surface Ig+, CD5+, CD22+, CD23+ (intensity of CD22 not greater than CD23). Four entirely typical cases of HCL were included for comparison (Cawley and Burthem, 1992). In all cases informed consent was given.

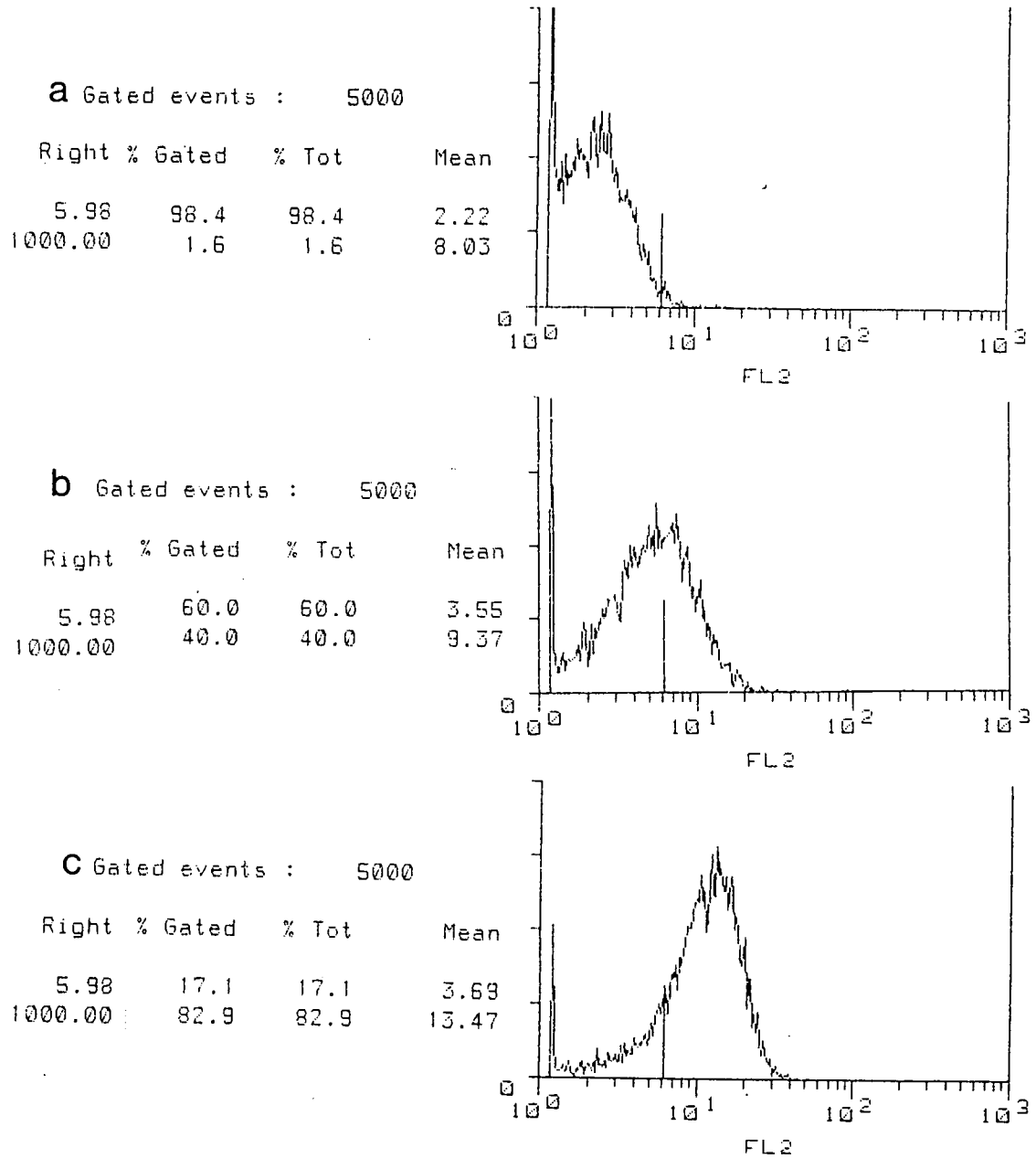



**Fig.2.1 Level of expression of cell surface antigens by triple-layer FACS.**

**a) Isotypic control mAb used to set a gate to indicate approx. 2% positivity (%Tot on table).**

**b) anti-α5 staining. Moderate expression in this case, giving 40% positive (although Gaussian distribution indicates 100% of the cells actually express the antigen).**

**c) anti-α3 staining. High expression here moves the peak to the right, and percent positive is approaching 100%.**

***Isolation, purification, and storage of leukaemic cells***

Peripheral blood samples were collected into heparin (20U/ml final concentration). Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque (Lymphoprep; Nycomed, Oslo, Norway) density gradient centrifugation. Cells were stored in liquid nitrogen in complete medium containing 10% dimethyl sulphoxide. T cells, monocytes and platelets were removed using magnetic beads (DYNA-BEAD; Dynal A.S., Oslo. Norway) coated with antibodies to CD3, CD16, and CD41 respectively; after this depletion contaminating platelets, T lymphocytes or monocytes were all less than 1% as judged by alkaline-phosphatase anti-alkaline phosphatase (APAAP) staining of cytospin preparations.

***The clinical features of the CLL patients***

Clinical details of the ten cases of CLL were collated in order to compare these data with adhesion molecule expression, although the limitations of this exercise were acknowledged, given the small number of patients. The following parameters of the disease were tabulated:- stage, major organ involvement, peripheral white cell count, lymphocyte doubling time, haemoglobin level/platelet count and activated vs. non-activated phenotype (see below).

**Activation state of CLL lymphocytes**

Case-to-case variation in adhesion molecule expression in CLL may be due to differences in the activation states of the cells (Nakamura *et al*, 1994). In order to test this hypothesis, an arbitrary definition of activation state was employed. The expression of a panel of seven known B-cell activation antigens was determined by triple-layer FACS for the ten CLL cell populations, and cases expressing five or more

of these antigens were said to be activated. The justification for the use of these antigens has already been given in detail in Chapter 1. The activation markers were as follows: CD23, the Fcε receptor, (Defrance *et al*, 1987, Flores-Romo *et al*, 1989); CD25, the high affinity IL-2 receptor, (Paloczi *et al*, 1990); CD39, (Flores-Romo *et al*, 1989); CD71, the transferrin receptor, (Trowbridge and Omary, 1981); CD80, also known as BB1 or B7 (Yellin *et al*, 1994); α5 integrin subunit (Sanchez-Madrid and Corbi, 1992); and αX integrin subunit (Ballard et al, 1991).

**In-vitro activation of CLL cells**

Various known B-cell activators were used to stimulate CLL cells *in vitro*. These were TNFα (100ng/ml), TNFα (10 ng/ml) combined with 1ng/ml PMA, IL-4 (3ng/ml, Sigma), IL-10 (10ng/ml, Genzyme), or SAC (0.001%, purchased from Calbiochem), each added to CLL cells ($2x10^6$/ml) for 2 or 24hrs. Changes in adhesion molecule expression were then analysed using triple-layer FACS.

# Results

## 2.1 Expression of adhesion molecules

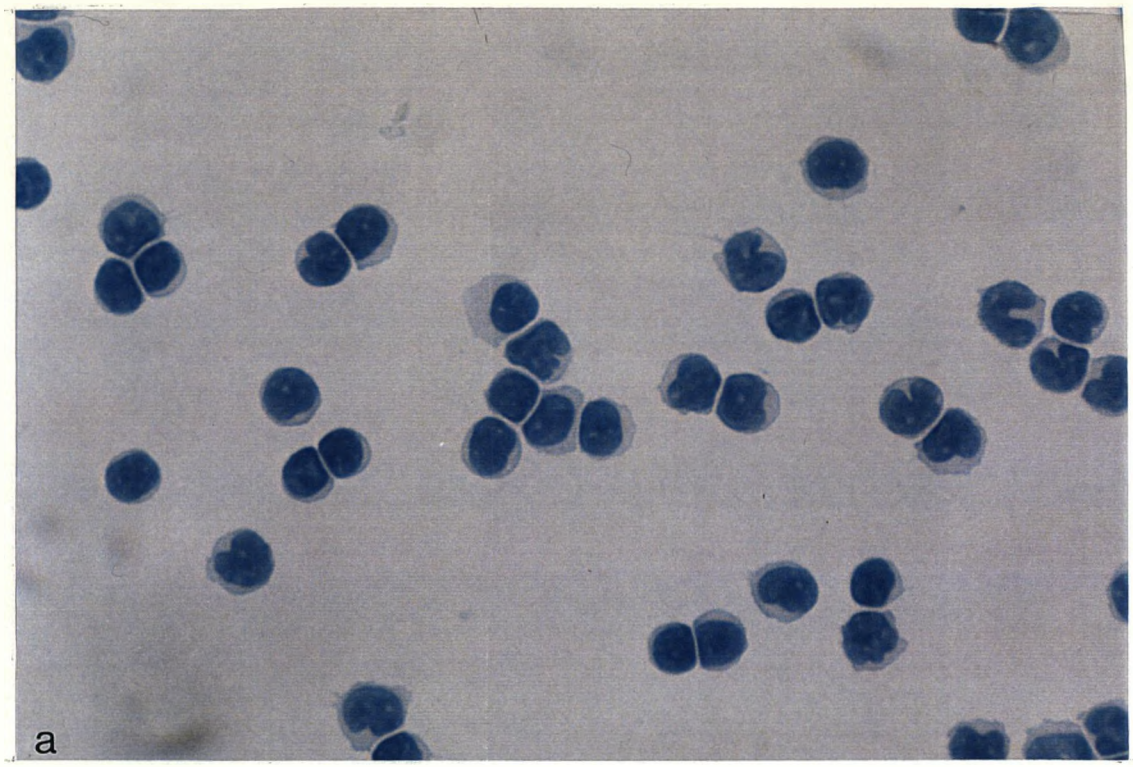
### 2.1.1 APAAP

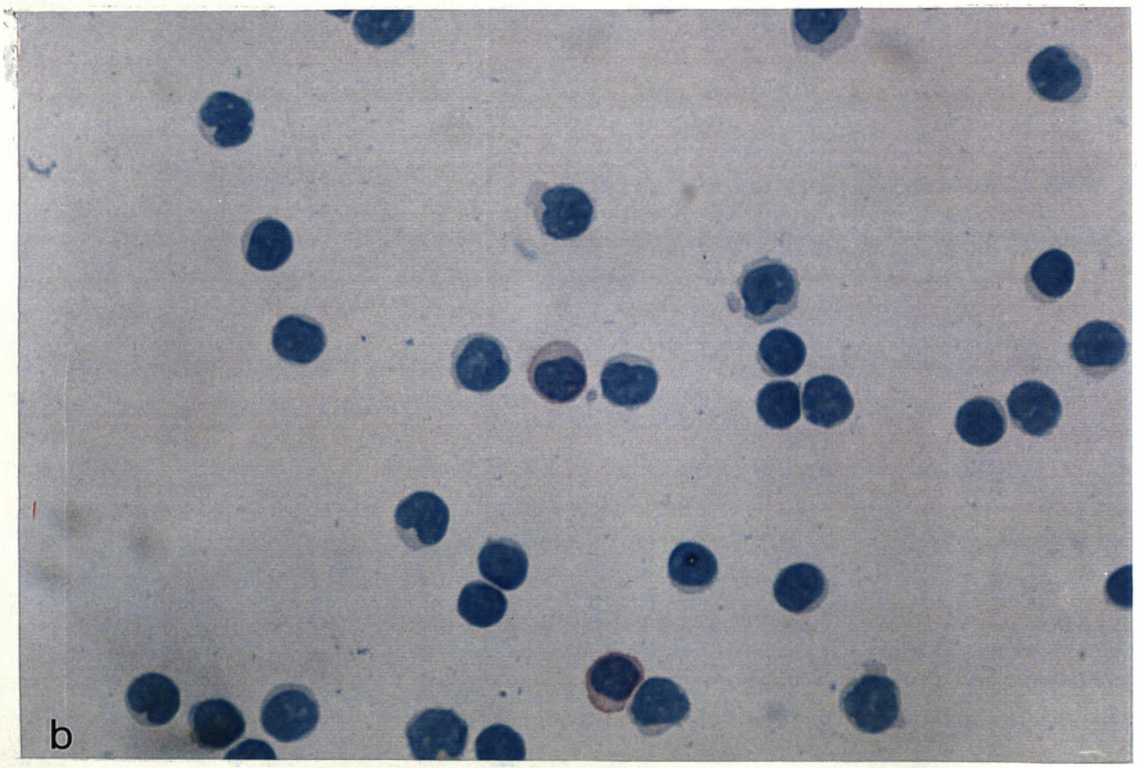
Although APAAP detects both surface and cytoplasmic antigens, for the antigens examined in this study, APAAP positivity was always associated with positivity by FACS, indicating that the antigen in question was, at least partly, at the cell surface.

Adhesion molecules were variably expressed by CLL cells. Some of the adhesion molecules investigated, i.e. $\alpha 2$, $\alpha V$, and $\beta 3$ integrin chains were not expressed in any case, and were therefore not examined by FACS.

When positivity was weak, judgments were difficult owing to CLL cells having very little cytoplasm; potentially positive antigens were re-examined by FACS. Moreover, due to both day-to-day variations in the substrate, and to the subjective nature of the assessment, determination of the level of antigen expression between samples is not possible by APAAP.

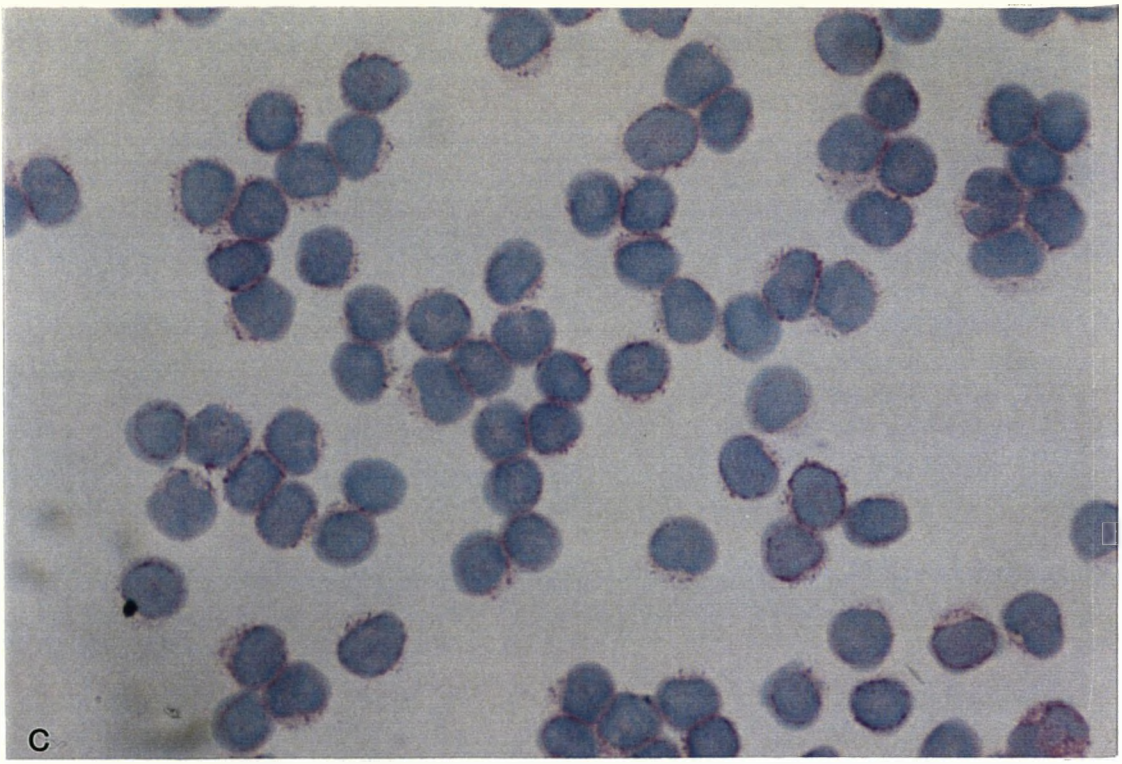
Fig.2.2, on the following pages, shows several APAAP preparations. Fast Red alkaline phosphatase substrate staining gives a red positivity, the counter-stain is haematoxylin (blue). The slides show a negative control stain, a test using anti-CD3 antibody (CD3 is a T-cell marker, and was incorporated in the staining runs to confirm the efficiency of the purification process), two APAAPs with anti-$\alpha 5$ identifying a positive and a negative CLL cell clone, and a positive anti-L-selectin stain.

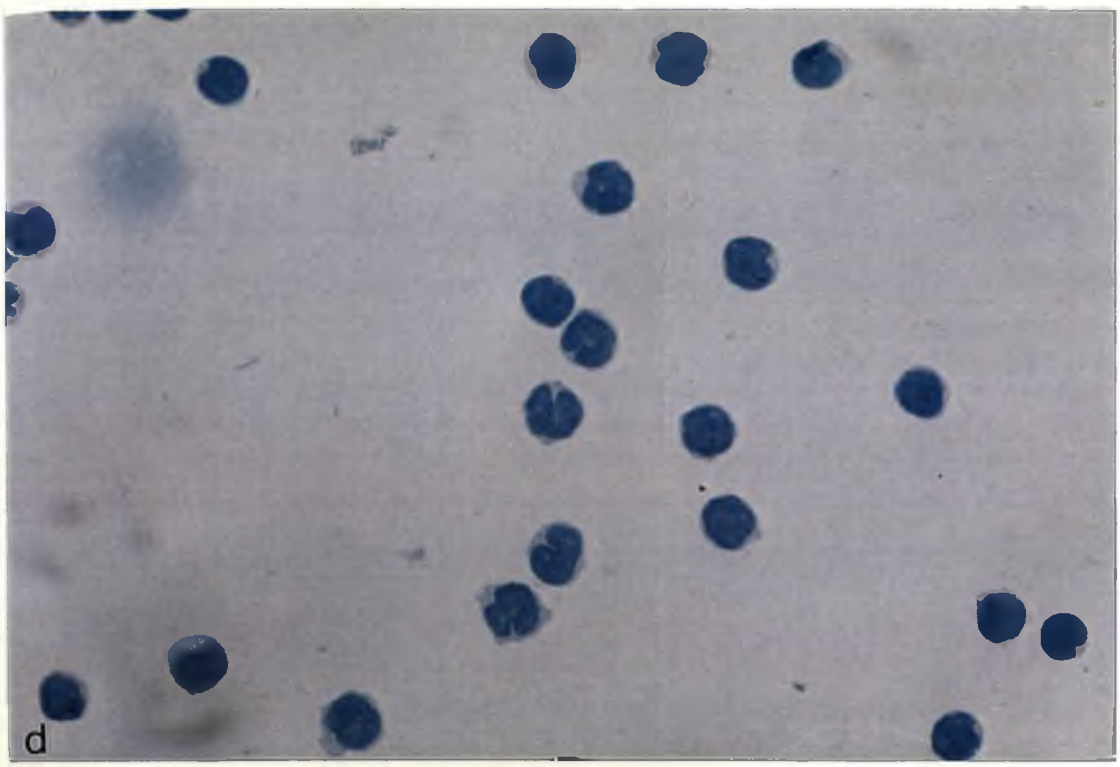



**2.2a** Isotypic control cytospin non-specific or Fc mediated staining is absent.

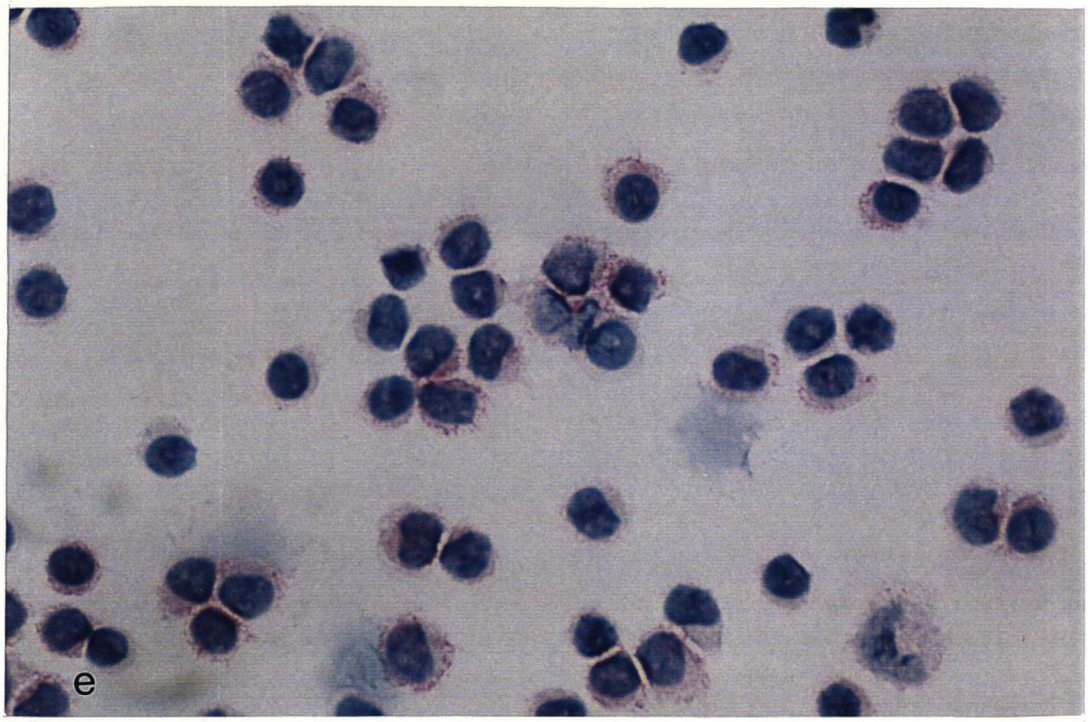
**2.2b** Anti-CD3 staining to demonstrate residual T cells in the CLL cell preparation.




**2.2c** Anti-α5 staining to demonstrate strong α5 positivity in a particular case.

**2.2d** Anti-α5 staining to demonstrate an α5 negative case.




**2.2e** Anti-L-selectin staining of CLL cells showing variable strong positivity. This slide also shows the presence of "ghost" or "smeared" cells, which are common in CLL cell preparations. These are believed to be ruptured cells, and appear because the CLL cells are particularly fragile.

### 2.1.2 Flow Cytometry

***β1 integrins***. The shared β1 chain that defines the VLA family was demonstrable by FACS in all cases. However, the intensity of staining varied from case to case, giving positivity of 23-92%. In CLL, β1 could be co-expressed with any of α3, α4, α5, and α6. Although these α-chains could all be expressed, the relative proportions of the four chains differed considerably between cases (Fig.2.3). In contrast, HCs consistently possessed high levels of α4 and α5; α3 expression was low or absent, and α6 was absent (Fig.2.4).

***β2 integrins***. In CLL, the β2 chain was consistently present. Regarding associated α chains, a typical profile could be identified, namely αL+, αX-, with variable αM expression (Fig.2.3). In HCL, a reciprocal pattern is seen (αL-, αX+) (Knapp *et al*, 1989), and this was confirmed here (Fig.2.4).

***Other (non-integrin) adhesion molecules***

CD31 was expressed at a high level in most cases, although 2 of the 10 were negative. L-selectin was consistently high. Similar results were obtained for HCs.

### 2.1.3 Immunoprecipitation

Previous studies in this Department identified specific associations of certain heterodimers on HCs which have implications on the homing properties of these cells. In particular, HCs express α4β1 and αHβ7 integrins but, unlike many B cell types, do not express the mucosa-homing-associated molecule α4β7 (Burthem *et al*, 1994a).

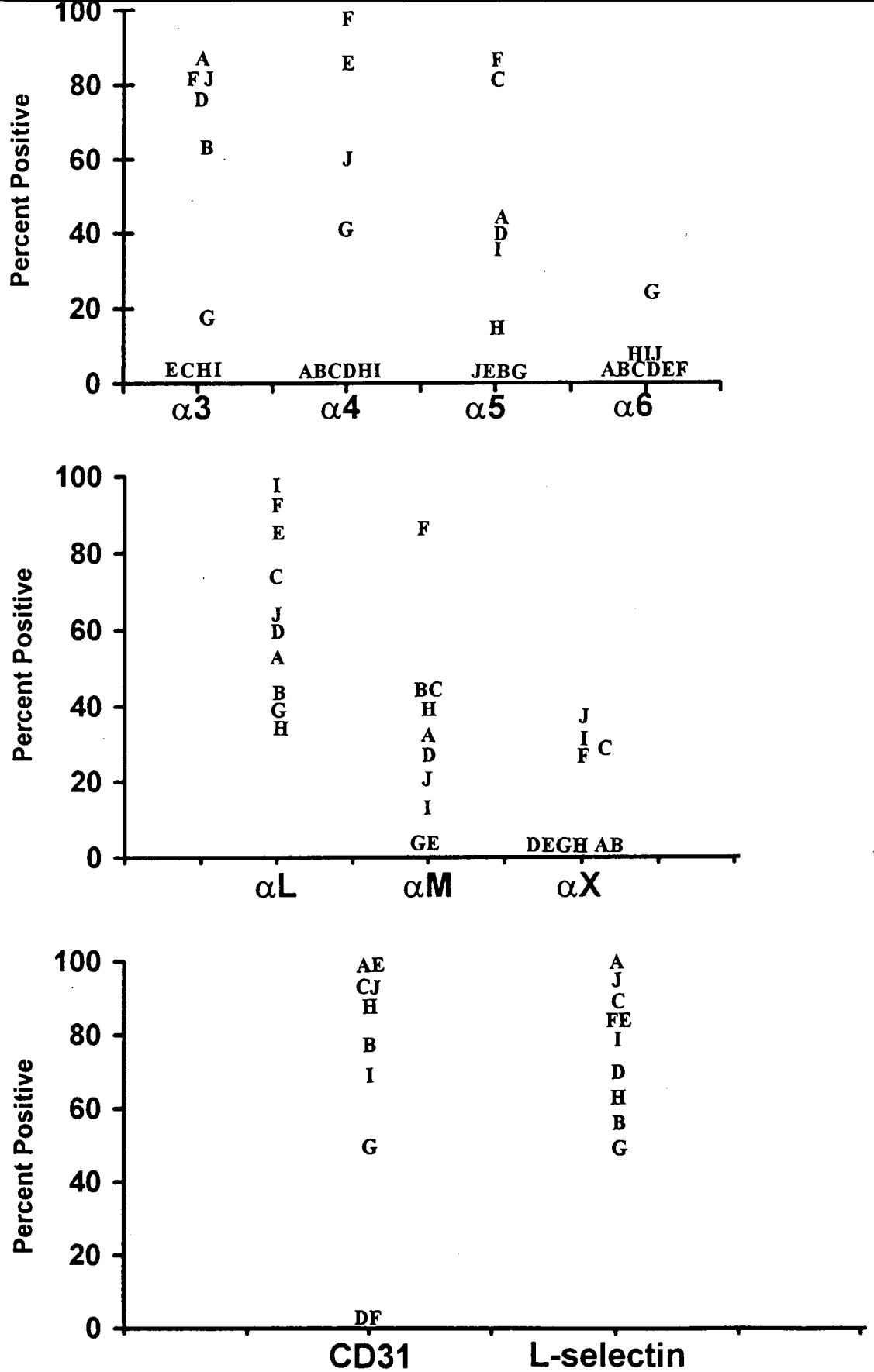



**Fig.2.3 Expression of integrin α-chains and other adhesion molecules in ten cases of CLL by triple-layer FACS.** Each letter represents an individual patient. For explanation of "Percent Positive" see Fig.2.1. Top panel=α-chains associated with β1. Middle panel=α-chains associated with β2. Bottom panel=other adhesion molecules.

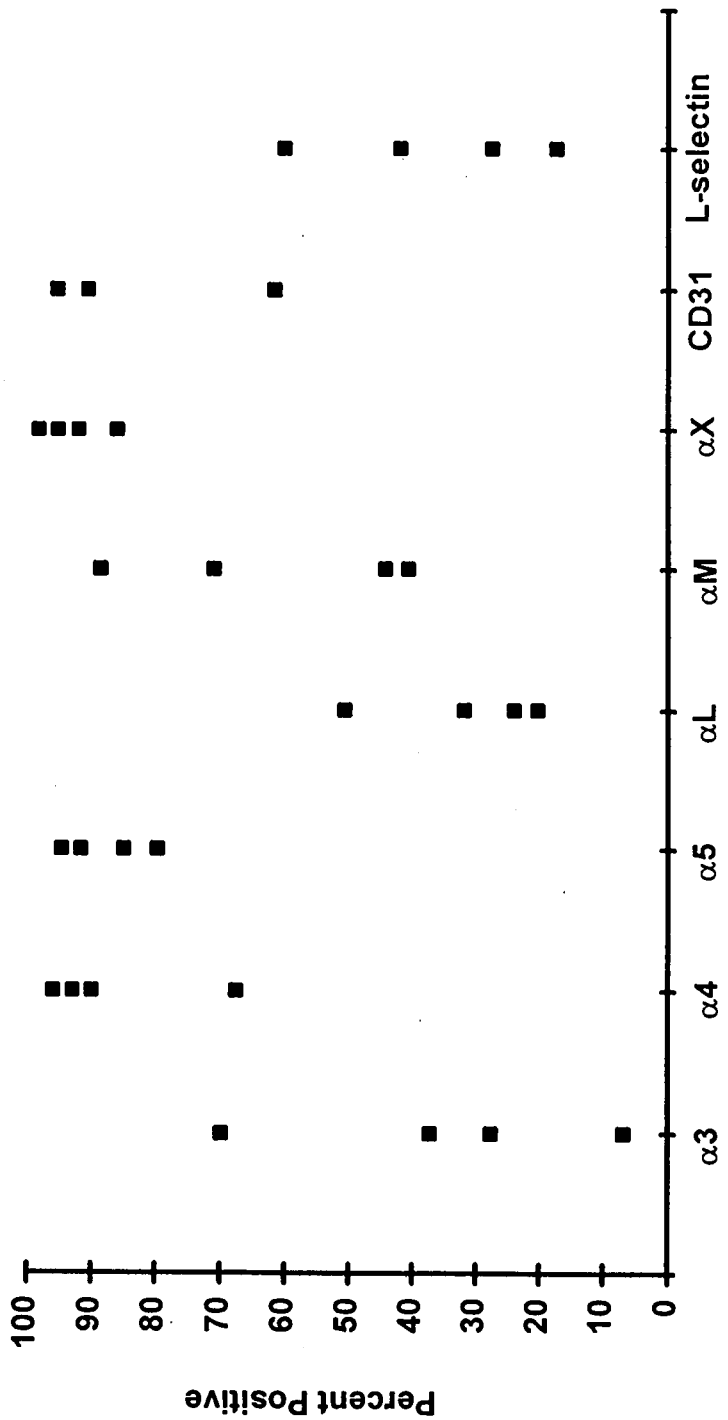



**Fig.2.4 Expression of adhesion molecules in four cases of HCL by triple-layer FACS.** (only three cases tested for CD31 due to lack of reagents when HCs were available)

Flow cytometry showed that some (4/10) cases of CLL expressed α4 (none expressed αH), and thus it was important to determine the β-chain(s) associated with the α4 in these cases.

Immunoprecipitation, in addition to demonstrating heterodimeric associations, allows detection of certain β-chains for which there is not yet an antibody available (e.g. β7, which is potentially important here). Fig.2.5 is an immunoprecipitation of β1 and α4, and shows that α4, when expressed in CLL, can associate with both β1 and β7. α4 can exist in three isoforms (Hemler *et al*, 1990), one of which (the cleaved 70/80-kD form) is associated with activation of certain lymphocyte types. Fig.2.5 shows that $\alpha4_{160}$ is the predominant form in these cells, but all three isoforms are present. The figure includes data for hairy-cell immunoprecipitations produced in the same way; these HC data demonstrate the position of the β7 band (Lane 6).

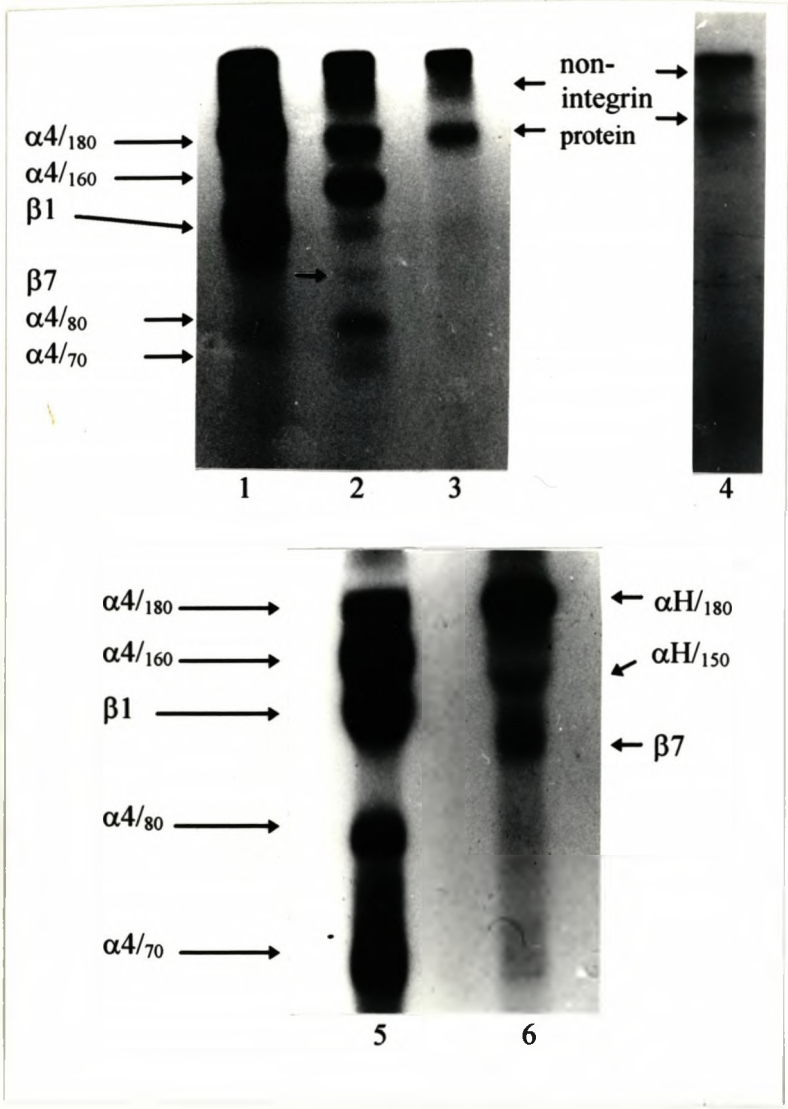


**Fig.2.5 Upper panel: Non-reduced immunoprecipitation of β1 and α4 integrins.** In lanes 1-3 complexes were precipitated from surface $^{125}$I-labelled CLL cell lysates (8% SDS-PAGE). Lane 4 is derived from similarly prepared HCL lysates.

Lane 1. This β1 I.P. reveals 5 specific bands corresponding to β1 itself and to co-precipitated α-chains ($\alpha4_{180,\ 160,\ 80,\ 70kD}$); small amounts of α3/α5 were also expressed, but are obscured by the larger amounts of $\alpha4_{160}$.

Lane 2. α4 I.P. Six specific bands are present. A single different band (indicated by the arrow immediately adjacent to Lane 2) not present in Lane 1 is demonstrated; this band lies between β1 and $\alpha4_{80}$ in the expected electrophoretic position of β7.

Lane 3. Isotypic control mAb I.P. At least two high molecular weight bands are shown which are also present in Lanes 1, 2 and 4; their identity is not clear, but they are not integrin chains, and they also appear in lysates from other B-cells (Lane 4).

Lane 4. Isotypic control mAb I.P. from identically prepared HCs. As compared with Lane 3, similar high molecular weight, non-integrin bands are present, confirming that such bands have no specific significance for CLL.

**Lower panel: Non-reduced I.P. from HC lysates** (10% SDS-PAGE) included as a control for β-chain associations in CLL. Since the acrylamide concentration differed from that in the upper panel, the mobility of the bands is slightly different.

Lane 5 α4 I.P. Five specific bands are shown ($\alpha4_{180,\ 160,\ 80,\ 70,}$ plus β1); in contrast to the same I.P. in CLL (Lane 2), there is no band between β1 and $\alpha4_{80}$ (HCs do not express α4β7).

Lane 6. αHβ7 I.P. Three specific bands are shown ($\alpha H_{180,}$ and $_{150}$ and β7). This illustrates the position of β7, with a mobility between that of β1 and $\alpha4_{80}$ (compare Lanes 6, 5, and 2).

## 2.2 Activation

### 2.2.1 Determination of activation state of CLL cells

The results, including a case of HCL for comparison, are shown in Table 2.2. Thus, four of the CLL cases, as well as the HCL case, could be said to be activated. Detection was performed by triple-layer flow cytometry. Both CD23 and CD25 were expressed to some extent in all cases, hence positivity was assigned only to cases with ≥ 50% positivity.

**Table 2.2 Expression of activation antigens on the abnormal cells of the panel of CLL patients, and one HCL patient.**

| | CD23 | CD25 | CD39 | CD71 | CD80 | α5 | αX | Total | Activated |
|---|---|---|---|---|---|---|---|---|---|
| Patient A | + | 0 | + | + | 0 | + | 0 | 4 | × |
| Patient B | + | 0 | + | + | 0 | 0 | 0 | 3 | × |
| Patient C | + | + | + | + | 0 | 0 | 0 | 4 | × |
| Patient D | + | + | + | + | + | + | 0 | 6 | ✓ |
| Patient E | + | + | + | 0 | 0 | 0 | 0 | 3 | × |
| Patient F | + | 0 | + | 0 | 0 | + | + | 4 | × |
| Patient G | 0 | 0 | + | 0 | 0 | 0 | 0 | 1 | × |
| Patient H | + | + | + | + | 0 | + | 0 | 5 | ✓ |
| Patient I | + | + | + | 0 | + | + | + | 6 | ✓ |
| Patient J | + | + | + | + | + | + | + | 7 | ✓ |
| **HCL** | 0 | + | + | + | 0 | + | + | 5 | ✓ |

### 2.2.2 Relationship of activation state to expression of adhesion molecules

Fig.2.6 (overleaf) shows the same adhesion molecule expression data as before (Fig.2.3), but now the activated (o) and non-activated (•) cases are indicated. Regarding *ex vivo* expression of adhesion molecules, there was no correlation between levels of expression and activation as determined by expression of the panel of monoclonal antibodies against B-cell activation markers.

### 2.2.3 Effect of *in-vitro* stimulation on adhesion molecule expression

A number of known stimulators of B cells were used to activate the CLL cells (see Methods). Adhesion molecule expression remained largely unchanged or was variably decreased (Table 2.3). However, stimulation with IL-10 produced variable, modest increases in the levels of many of the adhesion molecules. The various forms of stimulation, however, increased the expression of activation markers, thus validating the methods of stimulation, as well as the use of these particular antigens to assign activated status to cases of CLL.

**Table 2.3. Change in expression of adhesion molecules and activation markers in a case of CLL following 24hrs incubation in the presence of various B-cell stimulators.** Numbers represent the percent positive on the FACS histogram, which indicates the level of antigen expression (see Fig.2.1). This case of CLL was designated as activated before stimulation (patient J). Results from other cases show similar patterns of changes in antigen expression.

| **Adhesion molecule** | **no stimulus** | **PMA+TNF** | **TNF** | **SAC** | **IL-4** | **IL-10** |
|---|---|---|---|---|---|---|
| α3 | 61.4 | 53.7 | 63.1 | 38.8 | 46.0 | 64.1 |
| α4 | 27.9 | 20.6 | 22.4 | 13.0 | 12.5 | 32.5 |
| α5 | 49.2 | 21.4 | 25.2 | 26.7 | 36.6 | 59.2 |
| α6 | 10.6 | 6.4 | 3.7 | 7.0 | 3.4 | 11.3 |
| αL | 21.3 | 12.8 | 7.1 | 10.0 | 6.6 | 20.0 |
| αM | 15.7 | 6.0 | 4.4 | 7.0 | 5.5 | 19.2 |
| αX | 41.3 | 18.7 | 30.8 | 18.0 | 31.7 | 48.8 |
| L-selectin | 1.2 | 0.6 | 1.4 | 5.0 | 1.0 | 7.3 |
| CD31 | 2.1 | 0.6 | 0.8 | 5.5 | 1.1 | 11.9 |
| **Activation marker** | | | | | | |
| CD23 | 10.7 | 4.1 | 8.1 | 8.2 | 8.0 | 16.9 |
| CD25 | 80.9 | 72.7 | 88.6 | 80.7 | 83.4 | 94.4 |
| CD39 | 94.6 | 81.8 | 89.6 | 88.9 | 86.6 | 98.5 |
| CD71 | 0.9 | 2.0 | 5.7 | 6.9 | 1.3 | 5.9 |
| CD80 | 0.7 | 3.9 | 4.0 | 4.9 | 0.7 | 5.7 |

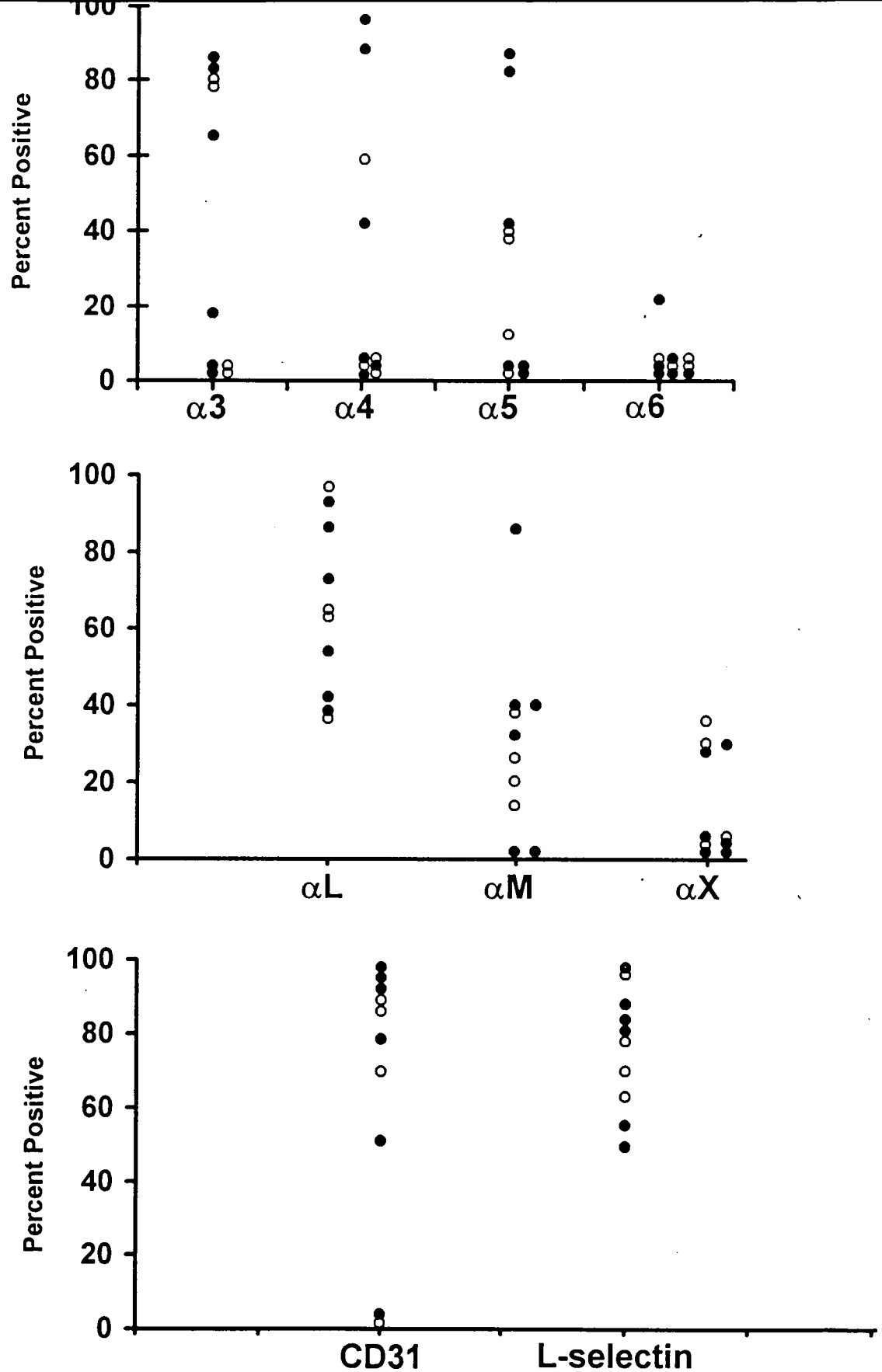



**Fig.2.6 Adhesion molecule expression data showing no correlation with activation state of CLL cells.** Activated (o) and non-activated (•) cases are indicated; for explanation of "Percent Positive", see Fig.2.1.

## 2.3 Relationship between the clinical features of CLL and the phenotype of the abnormal cells

Table 2.4 shows the clinical features of the ten cases of CLL. There was no obvious link between any of these disease manifestations and the adhesion molecule expression. Neither did there appear to be any correlation between clinical features and intrinsic activation as defined by the panel of mAbs.

**Table 2.4 Clinical parameters of the ten cases of CLL**

| Patient | Stage (Binet) | Major organ enlargement | WCC | LDT >12 months | Hb/platelets | Phenotype |
|---|---|---|---|---|---|---|
| A | B | S, LN | 25 | + | 11.4/178 | $\beta1^{++}$ α4– • |
| B | B | S, LN | 56 | – | 12.8/284 | $\beta1^{++}$ α4– • |
| C | A | S | 16.1 | NA | 13.6/172 | $\beta1^{+++}$ α4– • |
| D | C | S, LN | 21.8 | – | 8.9/21.8 | $\beta1^{+++}$ α4– o |
| E | B | S, LN | 147 | NA | 15.1/149 | $\beta1^{+++}$ α4+ • |
| F | C | LN | 56 | – | 9.5/230 | $\beta1^{+++}$ α4+ • |
| G | C | none | 228 | NA | 8.8/221 | $\beta1^{+}$ α4+ • |
| H | B | S, LN, K | 33 | – | 10.1/161 | $\beta1^{+}$ α4– o |
| I | A | none | 16 | + | 13.4/151 | $\beta1^{+++}$ α4– o |
| J | A | S | 38 | – | 15.3/143 | $\beta1^{+}$ α4+ o |

S=spleen, LN=lymph nodes, K=kidney, WCC=peripheral white cell count, LDT=lymphocyte doubling time, NA=not assessable, • and o refer to non-activated and activated state respectively (see above). $\beta1^{+}$, $\beta1^{++}$, $\beta1^{+++}$= low, moderate, and high level of expression of β1 integrins respectively.

## Discussion

The studies presented in this chapter have defined the integrin expression of CLL lymphocytes in more detail than has previous work. Thus the cells were shown consistently to express β1 in association with any combination of α3, α4, α5, and α6. However, there was considerable variation in the expression of these α chains from case to case, and the level of a particular chain did not necessarily correlate with that of the other three. This variable pattern of expression was different from the consistent pattern observed in HCL, where α4 and α5 were always present at high levels, while α3 was very low or absent.

The present findings in CLL are broadly in keeping with those of Moller *et al* (1992) and Baldini and Cro (1994), but differ from those of De Rossi *et al* (1993) who found that a substantial proportion of cases lacked β1. The reason for this latter discrepancy is not clear, but it may reflect the highly sensitive triple-layer immunophenotyping techniques used and the tightly defined immunophenotypic definition of CLL employed in this study. The present investigations have added to previous work in showing that, in addition to possessing α4β1, CLL cells can express α4β7. This latter heterodimer shares the same ligands as α4β1 but, in addition, is concerned with gut and possibly peripheral node homing (Berlin *et al,* 1993; Streeter *et al,* 1988). Furthermore, most nodal lymphomas express α4β7 (Andrew *et al,* 1994), while in the non-nodal malignancies, HCL and myeloma, the abnormal cells consistently lack the heterodimer (Burthem *et al*, 1994a; Erle *et al*, 1994). It is tempting, therefore, to speculate that α4β7 may be important in the localisation of malignant lymphoid cells to the lymph node. However, the data presented here

indicate that other mechanisms must also be involved in localisation to the node, as four of the six α4-negative cases of CLL showed lymph node enlargement (Table 2.4).

The present immunophenotypic data on β2 integrins are largely in keeping with previous studies. Thus, CLL cells expressed αLβ2, but generally lacked αXβ2 (Woessner *et al*, 1994; Kimby *et al*, 1994); the converse is true in HCL (Cawley and Burthem, 1992). Both CLL cells and HCs displayed variable levels of αMβ2. Regarding β3 expression, the integrin was not present on the lymphocytes of any of the cases of CLL studied. This was in marked distinction to HCL where αVβ3 is consistently demonstrable and mediates cell motility (Burthem *et al*, 1994a). The findings concerning β3 differ slightly from those of a study by De Rossi *et al* where a minority (around 15%) of patients expressed this chain at low levels (De Rossi *et al*, 1993). However, many of De Rossi's β3-positive cases were noted to be CD5 negative. Since CD5-negative cases were specifically excluded from this study, it is perhaps not surprising that the group of patients studied here did not include an αVβ3-expressing case.

The consistently high expression of L-selectin observed here is typical of recirculating cells and has previously been reported in CLL (Csanaky *et al*, 1994). CD31 (PECAM-1) expression in CLL has not previously been reported, and the present study found that expression could be high or negative. The functional significance of this variable CD31 expression is not clear.

The consistent expression and activity of HC integrins is probably related to the uniformly activated nature of the malignant cells (Cawley and Burthem, 1992). Therefore, the present study examined the hypothesis that the variable degrees of

activation observed in different cases of CLL might underlie the variability of adhesion molecule expression observed in the present study. To do this, a panel of seven recognised B-cell activation antigens was employed to divide the CLL patients according to their degree of activation. Arbitrarily, activated cases were defined as those expressing five or more of the panel of antigens. There was no correlation between integrin chain expression and activation as defined by simple mAb staining. Furthermore, in-vitro stimulation of CLL cells had no consistent effect on the integrin expression as determined by mAb staining. Following *in vitro* stimulation, however, expression of activation markers often increased, indicating that activation can be adequately examined using the selected panel of activation markers. Later in the thesis (Chapters 3 and 4) a clear correlation between activation state and adhesive behaviour will be demonstrated. As will be discussed later, these results taken together indicate that the functional state, rather than simple level of expression, of integrins is important in determining certain aspects of the adhesive behaviour of CLL cells.

There was no apparent relationship between known prognostic features in CLL and the simple expression of adhesion molecules. Although it is possible that examination of a very large number of patients might have helped to identify a trend which was not apparent from the small number of cases in this study, this seems unlikely and the labour-intensive nature of such a study made it impossible here. The finding that adhesion molecule expression in CLL does not directly correlate with clinical manifestations of the disease contrasts with HCL, where colonisation of particular tissues may be attributable to specific integrin-mediated functions of HCs (Burthem et al, 1994a and 1994b).

In conclusion, then, the present study has demonstrated that there are clear qualitative and quantitative differences in adhesion molecule expression between different cases of CLL, but there were no obvious correlations with activation state or with patterns of clinical disease. This may partly explain why previous studies have failed to establish a clear relationship in CLL between simple integrin expression and clinical behaviour.

It seemed important to extend the present work to functional responses, and this is done in Chapters 3 and 4. In these chapters it will be shown that there is a relationship between activation and functional responses to adhesion.

# CHAPTER 3

## Interaction of CLL cells with endothelial ligands

### Introduction

Chapter 2 identified the adhesion molecule expression of a group of CLL cases and found no obvious correlation between such expression and clinical disease features. However, since adhesive behaviour is not simply determined by adhesion molecule expression but also by the functional state of these molecules, it seemed important to examine the functional adhesive properties of the cells of these same cases.

Lymphocyte interaction with endothelium is the first step in the process of tissue entry (see General Introduction, section 1.3) and is therefore potentially an important determinant of patterns of tissue involvement in the different lymphoproliferative disorders. The purpose of this chapter is to examine the interaction of CLL cells with endothelium and relate these functional observations to the phenotypic findings presented in Chapter 2.

To do this, the interaction between CLL cells and stimulated human umbilical vein endothelial cells (HUVEC) was examined. The differential expression of endothelial adhesion molecules by the HUVEC after various periods of TNFα stimulation, together with use of blocking mAbs, enabled an analysis of the potentially important receptor-ligand interactions. Furthermore, while activation (either intrinsic or following *in-vitro* stimulation) did not correlate with simple

adhesion molecule expression in CLL (Chapter 2), this chapter identifies a clear correlation between activation and adhesive behaviour.

At the time the present work was started, the interaction between CLL cells and endothelium had not been investigated. Subsequently, two groups have published studies on CLL/EC adhesion. Csanaky *et al* (1993) looked at HEV-binding and found that binding was highest in cases with Binet Stage A disease and, in a subsequent study (1994), that the level of L-selectin on CLL cells directly correlated with adherence. A second group (Takeuchi and Katayama, 1993) examined adhesion to HUVEC after prolonged co-culture and showed a correlation between binding and CLL-cell-expression of αMβ2 and ICAM-1.

The present studies add significantly to these investigations by directly establishing the receptors and ligands involved in strong binding of CLL cells to endothelial ligands, and by determining the importance of CLL-cell activation in this process.

## Methods

### Antibodies and Immunologic Techniques

The mAbs used in this chapter are listed in Table 3.1. The mAbs were against a range of endothelial and CLL cell adhesion receptors:- some for phenotypic analysis and others for function blocking studies.

**Table 3.1 mAbs used in Chapter 3**

| Specificity (common name) | CD | Clone (Isotype) | Source |
|---|---|---|---|
| VCAM-1 (blocking) | 106 | 1G11 (IgG1) | Immunotech S.A. |
| E-selectin | 62E | 1.2B6 (IgG1) | Serotec |
| ICAM-1 | 102 | 6.5B5 (IgG1) | Dako |
| cFN | - | CBL 181 (IgG1) | Cymbus Bioscience Ltd |
| PECAM-1 | 31 | 5.6E (IgG1) | Immunotech, SA |
| P-selectin | 62P | WGA-1 (IgG1) | TaKaRa Biomedicals |
| Factor VIII-related Ag | - | F8/86 (IgG1) | Dako |
| ICAM-1 (blocking) | 102 | LB-2 | Becton Dickinson |
| E-selectin (blocking) | 62E | BBIG-E4 (IgG1) | R&D Systems |

### Cell ELISA

A cell ELISA was employed to determine the expression of adhesion molecules (listed in Table 3.1) by confluent endothelial cells, both before and after stimulation with TNFα. The method is described in full in Appendix 1.6.

**HUVEC Culture**

HUVEC were cultured according to published protocols (Till and Cawley, 1994; described in full in Appendix 1.4). Cells were grown to confluence in flat-bottomed 96-well flexible assay plates coated with basement membrane proteins. All assays were performed at first passage as soon as HUVEC reached confluence.

**Culture of endothelial cell line**

These cells were grown in the same way as the HUVEC, except that the serum concentration was reduced to 10%.

**Adhesion assay**

A reproducible adhesion assay was required which provided a consistent means of removing weakly- or non-adherent cells. Therefore a reversed centrifugation method was developed. $^{35}$S-labelled CLL cells were allowed to adhere for a fixed time, then plates were sealed, inverted, and centrifuged at 250g for 5 minutes. This removed weakly- and non-adherent cells. Adherent cells were then fixed and the proportion of cells bound were determined using a microbeta plate counter. The method is described in full in Appendix 1.7.

**Blocking Assay**

Blocking was performed by pre-incubation for 1hr with mAbs as indicated. Saturating concentrations of antibody (> 4μg/ml) were included in the culture media.

**Activation studies and clinical parameters of the CLL cases**

The activation states of the CLL cases were determined in Chapter 2, and were compared with functional behaviour in this chapter. CLL cells were stimulated using a panel of B-cell mitogens in the same way as in Chapter 2 to determine the effects of this stimulation on the function of adhesion molecules. The adhesive behaviour was also compared with clinical disease features, and the table of clinical details of the CLL cases can be found in Chapter 2 (Table 2.3).

# Results

## 3.1 Endothelial adhesion molecules

### *3.1.1 HUVEC*

Of the adhesion molecules examined, only ICAM-1 was constitutively present at high levels on the cultured HUVEC; there were low levels of CD31 and cellular fibronectin (cFN), and very low levels of VCAM-1. Stimulation of the endothelium with TNFα induced expression of E-selectin within 1 hour; the level of expression peaked at around 6 hours, and then returned to the baseline by 24 hours (Fig.3.1a). VCAM-1 was induced more slowly, increasing from 3-4 hours; expression peaked at 6-7 hours and was still at this level after 24 hours (Fig.3.1a). ICAM-1, cFN, and CD31 expression were not increased after TNFα stimulation (Fig.3.1b). P-selectin could not be detected.

### *3.1.2 Endothelial cell line*

For convenience, an attempt was made to replace HUVEC in the adhesion assay with this cell line. However, these ECs expressed only ICAM-1 constitutively and did not upregulate any adhesion molecules after TNFα stimulation (data not shown). Thus the endothelial cell line was only used in the assays of adherence of *in vitro*-stimulated CLL cells to unstimulated endothelium.

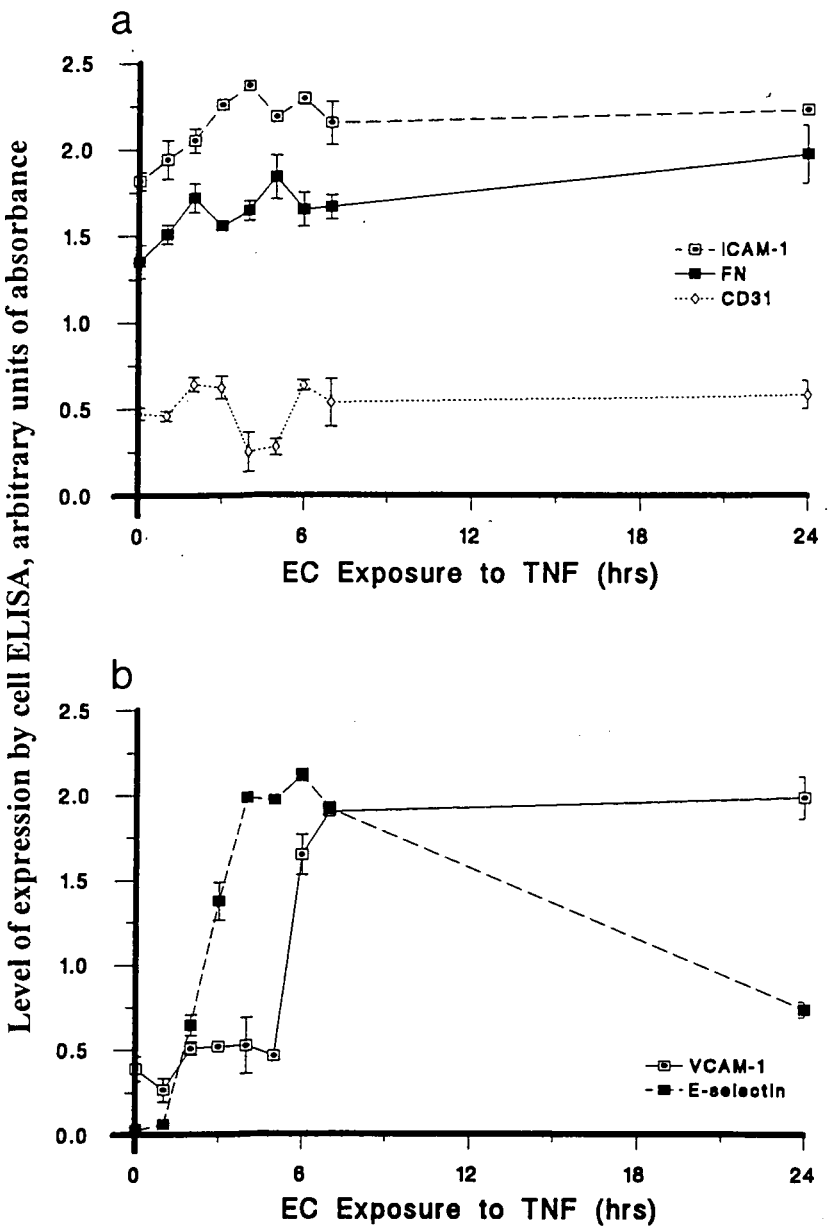



**Fig.3.1 Expression of adhesion molecules on TNFα-stimulated HUVEC. 3.1a shows those molecules that are induced by TNFα (VCAM-1 and E-selectin). 3.1b shows molecules not affected by TNFα (CD31, ICAM-1, cFN).** Detection was performed by cell-ELISA on confluent monolayers of cells, stimulated for various times with 10U/ml TNFα, then fixed in iso-osmoitc fixative.

## 3.2 Adhesion to unstimulated HUVEC

The ability of the CLL cells to bind to unstimulated endothelium varied from case to case (7-27% of cells bound; Fig.3.2).

### *3.2.1 Binding to unstimulated HUVEC involves more than one receptor-ligand pair*

Monoclonal blocking antibodies to endothelial adhesion molecules could reduce CLL cells' binding to the endothelium. Table 3.2 shows a typical example of blocking of endothelial ligands. Approximately 20% of binding was inhibited by blocking ICAM-1, and a further 20% of binding was inhibited by anti-E-selectin. The blocking of these two components was additive. However, this blocking only accounts for 40% of the total adhesion of CLL cells to endothelium, and while this may be attributable to incomplete blocking by the antibodies, other adhesion molecules may also be involved in this process.

**Table 3.2 Blocking of CLL cell adhesion to unstimulated endothelium**

| Blocking antibody | Percentage of Control (%) |
|---|---|
| Control (Factor VIII-related Ag) | 100 |
| VCAM-1 | 104 ± 6.1 |
| ICAM-1 | 79.6 ± 9.2 |
| E-selectin | 81.5 ± 4.6 |
| VCAM-1+ICAM-1+E-selectin | 60.3 ± 5.8 |

### *3.2.2 Adhesion to unstimulated HUVEC is not related to quantitative expression of any adhesion molecule*

The ability to bind to unstimulated HUVEC was compared with the expression of adhesion molecules by the CLL cells as described in Chapter 2. There was no correlation between qualitative or quantitative expression of any of the adhesion molecules and the ability to bind to HUVEC. For example, the blocking studies (3.2.1) show that a component of constitutive binding was due to endothelial ICAM-1; the CLL cell ligand must be a member of the β2 family of integrins. All cases of CLL express some αLβ2, but there was no correlation between the percentage of CLL cells bound to unstimulated HUVEC and the percent αL positivity (Fig.3.2). This lack of a correlation could be shown for all adhesion molecules on the CLL cells. The ligand on CLL cells for E-selectin has not been described.

### *3.2.3 Adhesion to unstimulated HUVEC correlates with intrinsic activation state of the CLL cell*

Using the criteria to determine activation state described in Chapter 2 (2.2.1; Determination of activation state of CLL cells), there was a significant correlation between activation state and adherence to unstimulated HUVEC (Fig.3.3).

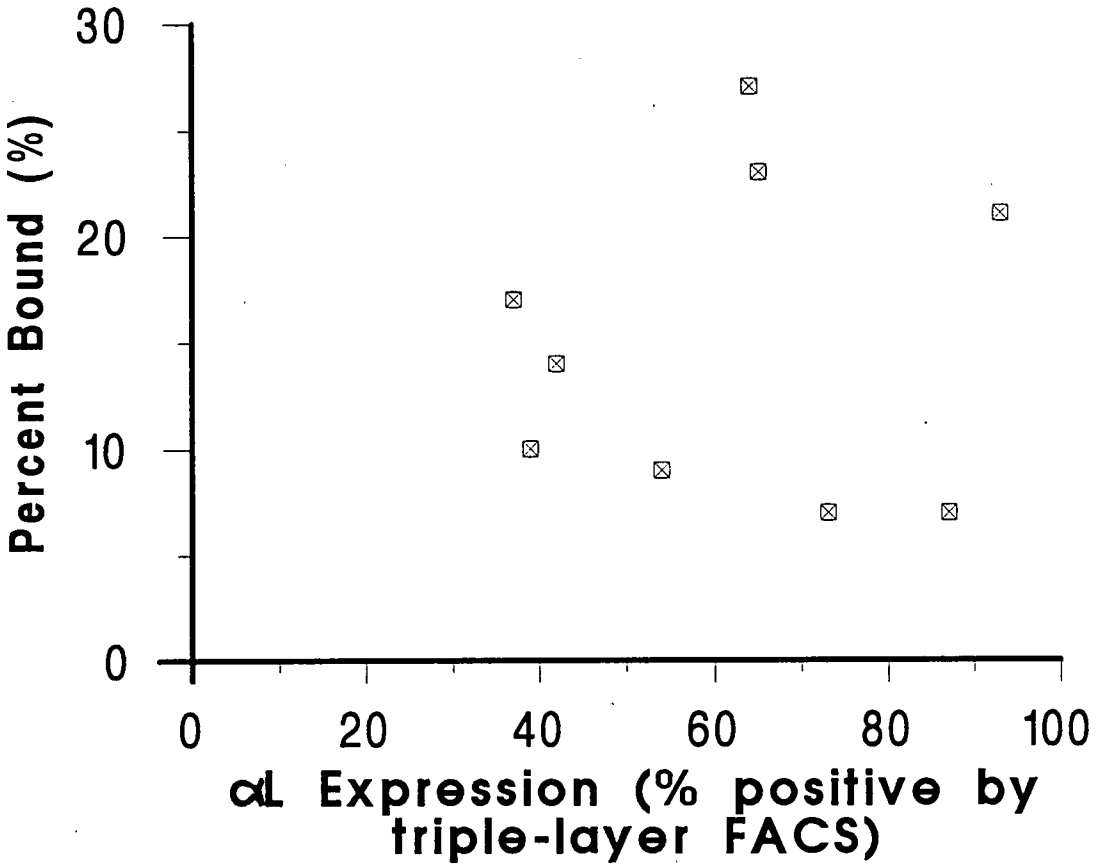
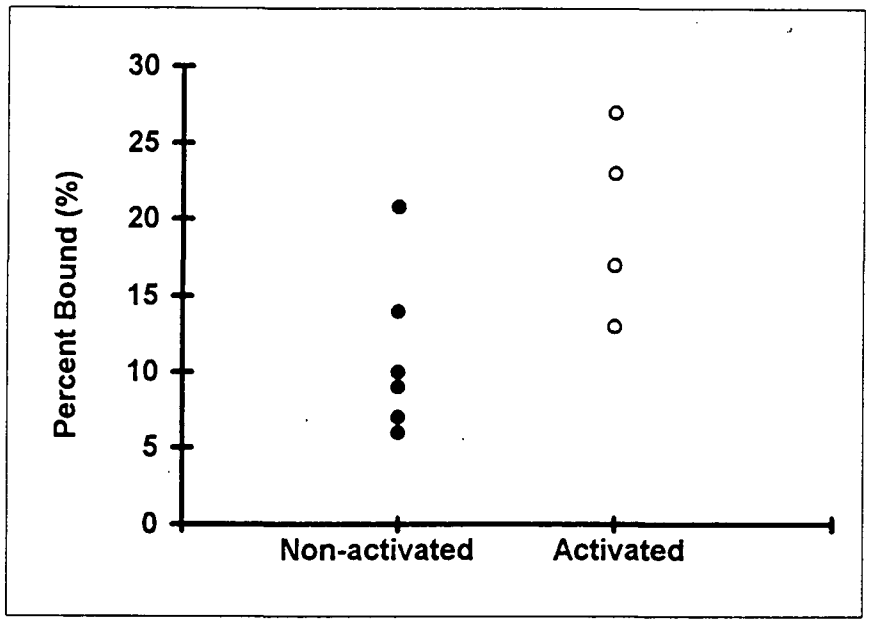



**Fig.3.2 Comparison of expression of αL on CLL cells with adherence to unstimulated HUVEC.** Adherence is expressed as the percentage of cells bound after 10 minutes when a monolayer of CLL cells are placed on the HUVEC. Adherence is calculated from the mean of triplicate assays. The correlation coefficient, $r^2$=0.00427.




t-Test: Two-Sample Assuming Unequal Variances

| | *Non-activated* | *Activated* |
|---|---|---|
| Mean | 11.3 | 20.75 |
| Variance | 29.42 | 26.91666667 |
| Observations | 6 | 4 |
| Hypothesized Mean Difference | 0 | |
| df | 7 | |
| t Stat | -2.770736832 | |
| P(T<=t) two-tail | 0.027664731 | |
| t Critical two-tail | 2.36462256 | |

**Fig.3.3 Comparison of intrinsic activation state with adherence to unstimulated HUVEC.** Intrinsic activation state is defined in section 2.2.1; adherence is expressed as the percentage of cells which adhere after 10 minutes when a monolayer of CLL cells are placed on the HUVEC. Lower panel shows statistical analysis of the mean adherence for the activated (o) and non-activated cases (•); $p<0.05$.

### *3.2.4 Adhesion of in-vitro stimulated CLL cells to unstimulated endothelium*

CLL cells were stimulated with TNFα, TNFα plus PMA, IL-4, IL-10, or SAC (concentrations as in Chapter 2). After all these stimuli, CLL cells showed a significant increase in adherence to the vascular endothelial cell line. SAC was the most efficient stimulator of adhesion, and these data are shown in Table 3.3. Since the test statistic is less than the critical values, and $p<0.05$, the difference between the mean adherence before and after stimulation is significant. This was not caused by changes in adhesion molecule expression (see Chapter 2), and increased adherence was due to activation of the adhesion molecules.

**Table 3.3 Adhesion of in-vitro stimulated CLL cells to unstimulated endothelial cells.** The binding was calculated from the proportion of cells bound after 10 minutes (mean of triplicate assays).

| **Endothelial adhesion** | **untreated** (% bound) | **SAC, 24hrs** (% bound) |
|---|---|---|
| **Patient 1** (N, Table 2.2) | 68.7 | 102 |
| **Patient 2** (G, Table 2.2) | 85.4 | 122.6 |
| **Patient 3** (C, Table 2.2) | 35 | 48.5 |
| **Patient 4** (M, Table 2.2) | 56.6 | 68.8 |
| **t-Test: Paired Two Sample for Means** | | |
| | **Variable 1** | **Variable 2** |
| **Mean** | 61.425 | 85.475 |
| **Variance** | 449.7625 | 1098.849 |
| **Observations** | 4 | 4 |
| **Pearson Correlation** | 0.980459 | |
| **Hypothesized Mean Difference** | 0 | |
| **df** | 3 | |
| **t Stat** | -3.68834 | |
| **P(T<=t) two-tail** | 0.034556 | |
| **t Critical two-tail** | 3.182449 | |

## 3.3 Adhesion to stimulated HUVEC

The adherence of CLL cells following stimulation of the endothelium was not uniform between cases. In 6/10 cases the binding of CLL cells did not change after induction of endothelial adhesion molecules. In other cases the adherence of CLL cells increased with the duration of endothelial stimulation.

### *3.3.1 Enhanced binding to stimulated HUVEC is due to expression of α4 integrin*

The time-course of the enhanced adherence of some cases of CLL was compared with the induction of VCAM-1 and E-selectin on the endothelium. The increased adherence mirrored the pattern of VCAM-1 expression on the HUVEC (Fig.3.4). The importance of binding via VCAM-1 was confirmed by the observation that only the four CLL cell populations which expressed α4 integrin (the receptor for VCAM-1) demonstrated enhanced binding to stimulated HUVEC. Further evidence was provided by blocking of the adhesion by anti-VCAM-1 mAb (Table 3.4). At four hours VCAM-1, E-selectin, and ICAM-1 all appeared to have a role in the adherence of CLL cells, however, at 7 and 24 hrs only the VCAM-1 antibody blocked the increased adhesion to stimulated HUVEC.

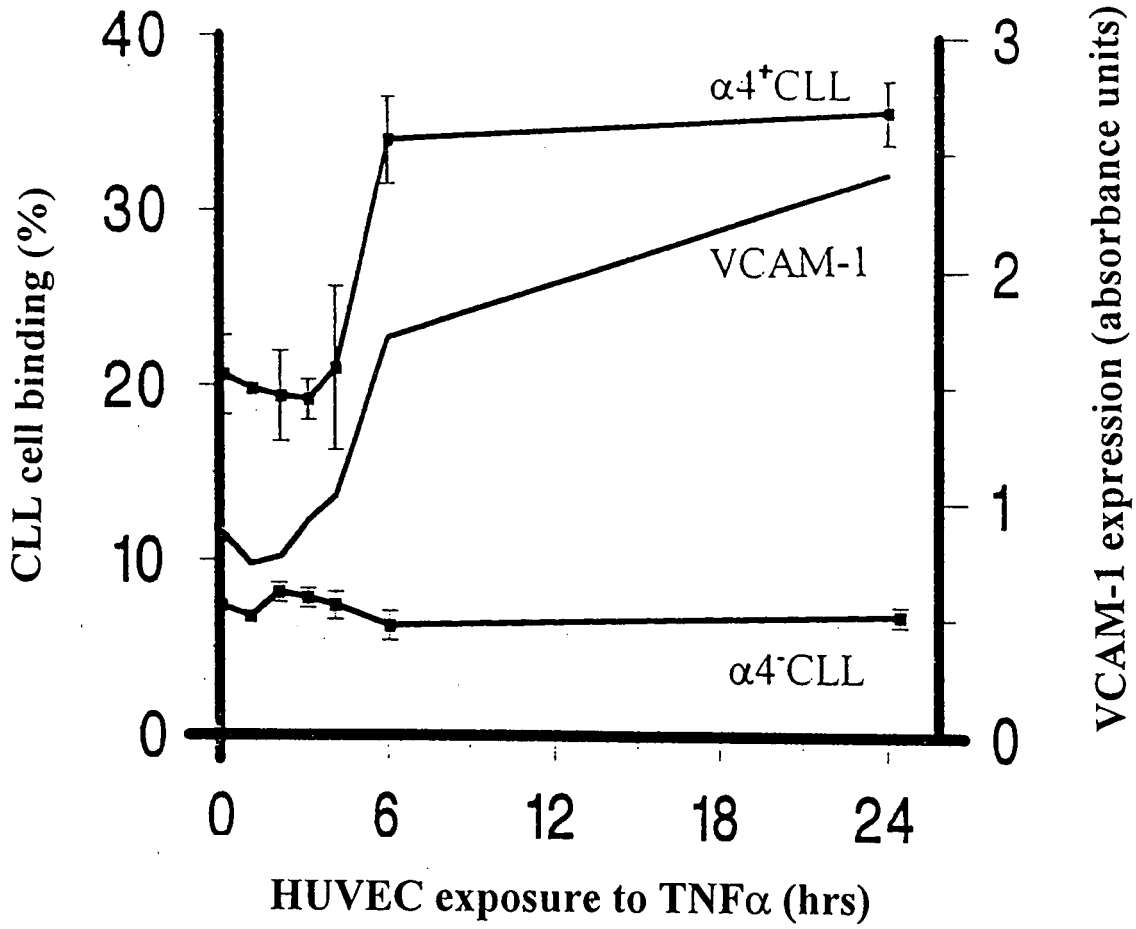



**Fig.3.4 CLL cell binding to HUVEC stimulated with TNFα.** Cases not expressing α4 (example shown, α4- CLL) did not increase following stimulation of HUVEC with TNFα. In cases expressing α4, markedly enhanced binding was observed when the endothelium was stimulated (representative example shown, α4+ CLL). This increased binding closely paralleled the increased VCAM-1 expression induced by TNFα; other relevant adhesion molecules (ICAM-1, P-selectin, E-selectin) did not parallel the CLL binding. Both cases represent the mean of triplicate assays ± one standard deviation.

**Table 3.4. The effect of function-blocking antibodies on the adherence of α4+ CLL cells to TNFα-stimulated endothelium.**

| | Percent binding * | | |
|---|---|---|---|
| Duration of HUVEC exposure to TNFα | 4 hrs | 7 hrs | 24 hrs |
| control | 100 | 100 | 100 |
| VCAM-1 | 38.1±8.0 | 30.7±10.7 | 20.0±10.6 |
| ICAM-1 | 54.3±17.2 | 91.5±31.4 | 110±26.3 |
| E-selectin | 45.8±8.6 | 115±10.5 | 120±6.2 |

* Results expressed as a percentage of cpm bound in the presence of a control mAb (factor VIII-related antigen).

## Discussion

Chapter 2 established the detailed adhesion molecule expression by CLL cells, and showed that there was no correlation between simple expression and *in vivo* disease features. Therefore, this chapter investigated the function of the CLL adhesion molecules to determine how they might influence the different *in-vivo* behaviour of CLL cells between cases.

In order to determine the adhesion processes involved in tight binding to cell-expressed ligands (a pre-requisite for tissue entry), a model of stimulated HUVEC was employed. Unstimulated HUVEC expressed high levels of ICAM-1, as well as very low levels of VCAM-1. After stimulation of the endothelium using TNFα, VCAM-1 and E-selectin were induced, but with different kinetics (ICAM-1 expression was unchanged). Thus the endothelial ligand responsible for some CLL cells binding more strongly after endothelial stimulation was identified by comparing the pattern of endothelial adhesion molecule induction with the time-course of enhanced CLL cell binding.

The intrinsic ability of the CLL cells to bind strongly to unstimulated endothelium was variable, but generally low. This binding was shown to be mediated via αLβ2/ICAM interactions and possibly by endothelial E-selectin binding to an unknown ligand on the CLL cell. However, when the endothelium was stimulated using TNFα, two patterns of adhesion were observed. In 4 of the 10 cases studied, lymphocyte adhesion was markedly enhanced; this enhanced binding was shown to be mediated by α4 integrin/VCAM-1 interaction (the subunit associated with α4 could be β1 or β7, see Chapter 2). In the six cases where no enhanced adhesion to stimulated endothelium was observed, the CLL cells lacked α4. Overall, these results

indicate not only that CLL cells from individual cases differ in their integrin expression, but also that these differences can affect the functional capabilities of the cell. This may have important clinical consequences. For example, in a given case of CLL, the presence of $\alpha 4\beta 1$ may confer on the malignant population a propensity to localise at major sites of VCAM-1 expression; these include bone marrow, liver, and spleen (Schweitzer *et al*, 1996; Simmons *et al*, 1992; Steinhoff *et al*, 1993).

As simple expression of adhesion molecules did not correlate with the ability to bind to unstimulated endothelium, such adhesion was compared with cell activation state as determined by a panel of mAbs. The CLL cases with an activated phenotype bound significantly better than non-activated cases. The concept that activation is an important determinant of CLL adhesion was further examined by stimulating CLL cells *in vitro*. These experiments showed that such stimulation increased the adhesion of cells from all cases studied, without affecting the level of adhesion molecule expression. These studies of activation, taken together, indicate that although qualitative adhesion molecule expression can influence the adhesion of CLL cells (e.g. $\alpha 4$ expression facilitates adhesion to VCAM-1) adhesive behaviour is also dependent on the activation state of the cells from any particular case. This conclusion may partly explain why the previous chapter (and other studies of CLL adhesion molecules) have not found firm correlations between simple expression and *in vivo* disease behaviour. The variable intrinsic activation state of the abnormal cells, the effect of local cytokines, and the interaction with accessory cells will all influence the activity of adhesion molecules which direct the tissue distribution of the cell.

Following binding to the endothelium, the CLL cell must then transfer adherence from endothelial ligands to the extracellular matrix in order to transmigrate.

The following chapter continues functional investigation of CLL adhesion molecules to show how they may be involved in migration and adhesion within tissues.

# Chapter 4

## Response of CLL cells to extracellular matrix ligands

### Introduction

The previous chapter described how CLL cells can interact with endothelium as a pre-requisite for tissue entry. During transendothelial migration the CLL cells must transfer adhesion from endothelial ligands to extracellular matrix ligands (Smith, 1992). The extracellular matrix composition encountered by the lymphocyte varies between tissues (Li and Cheung, 1992). Thus interaction with matrix ligands within tissues effects different patterns of migration into and within different organs. Both the transduction of signals and the ability to adhere are facilitated by integrins on the CLL cells. Therefore this chapter is concerned with integrin-mediated interactions with purified extracellular matrix elements. The ability to adhere is investigated, and the integrins responsible are examined; the influence of cell activation (both intrinsic and after *in vitro* stimulation) is explored.

There have been no published studies of CLL cell binding to purified extracellular matrix proteins, so the data presented here is new. The study reflects previous work in this Department, discussed in the General Introduction to this thesis (Section 1.2.3 on histology in HCL; Burthem *et al*, 1994a and 1994b), where the consistent expression of integrins and the activated state of HCs confer particular properties regarding interaction with extracellular matrix elements, and these correlate

with the specific tissue localisation of HCs. Since the interactions of HCs with extracellular matrix proteins are well defined, four cases of HCL were included in this study as a comparison.

## Materials and Methods

### Adhesion Studies

#### *Adhesive proteins*

Wells of Falcon microtest II flexible 96-well plates were coated with 40μg/ml purified extracellular matrix proteins overnight at 4°C. The efficiency of this coating method has been established previously by workers in this Department (Burthem *et al*, 1994a). The purity of proteins used in this study was determined to be greater than 95% using sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining of 8% polyacrylamide gels.

*Fibronectin (FN).* FN was purified by gelatin-sepharose affinity chromatography according to the method of Engvall and Ruoslahti (1977) (fresh frozen plasma unsuitable for human therapeutic use was provided by BTS Liverpool, UK).

*Vitronectin (VN) and collagen (Coll).* Human VN and type I Coll were purchased from Sigma Chemical Company Ltd..

*Thrombospondin.* Human thrombospondin was purchased from BDH Ltd..

*Extract of mouse sarcoma basement membrane.* ECL attachment matrix containing entactin, collagen, and laminin was purchased from Promega Ltd..

#### ***Relation of adhesion to phenotype***

The ability of CLL cells to adhere to the various substrata was compared with expression of adhesion molecules and activation markers, as described previously in Chapter 2 (section 2.2.1). The CLL cells were also stimulated using known B-cell mitogens as before (2.2.3), and compared with unstimulated cells in the adhesion assays.

**Morphological analysis**

The size and shape of cells adherent on FN and VN were analysed using a computer image analysis system, which eliminated subjective human judgement. The methods are described in full in the Appendix (section 1.10).

**Trans-cell migration**

Splenic red pulp fibroblasts were kindly donated by K. Till (Department of Haematology, Royal Liverpool University Hospital) and were from splenic outgrowth following splenectomy of a hairy-cell leukaemia patient. Bone marrow stromal cells were grown out from long term bone marrow culture after seven weeks, kindly donated by R. Harris (Department of Haematology, Royal Liverpool University Hospital).

HUVEC, splenic red pulp fibroblasts, or bone marrow stromal cells were grown on ECL-coated glass coverslips for 48hrs; they did not reach confluence before the assay. Purified CLL or HCL cells were added to the coverslips and allowed to adhere for a few minutes; then they were rinsed in warm medium and placed in 24-well plates. These were cultured for 24hrs in RPMI 1640 medium containing 10% foetal calf serum. Coverslips were then washed and fixed by adding 4% glutaraldehyde to the medium for 60 minutes. Cells were stained using rhodamine phalloidin, and visualised using fluorescence microscopy at an excitation of 545 nm.

# Results

## 4.1 Adhesion of CLL cells to purified extracellular matrix elements

A standardised assay of adhesion strength as described in Chapter 3 and Appendix 1.7 was applied to purified CLL cells from the ten cases already characterised in terms of their adhesion receptors in Chapter 2. Fig.4.1 shows the adherence of cells of different CLL cases to fibronectin (FN), extract of basement membrane proteins (ECL), collagen (Coll), and vitronectin (VN), and includes four cases of HCL for comparison. None of the cells bound to collagen or thrombospondin in this system (thrombospondin not included in Fig.4.1). Most CLL cells did not bind the entactin and laminin of ECL, and binding was always low; HCs bound rather better. CLL cells displayed a wide range of abilities to adhere to fibronectin, whilst HCs bound consistently and strongly. CLL cell binding to vitronectin tended to be lower than for FN, although variable, and was also lower for CLL cells than HCs; HCs again bound consistently at a high level.

### *4.1.1 Relationship of adhesive activity to adhesion molecule expression*

Each case of CLL expressed at least one of the known integrin adhesion receptors for fibronectin ($\alpha 3\beta 1$, $\alpha 4\beta 1$, $\alpha 5\beta 1$). There was no correlation between the ability to bind fibronectin and expression of a particular integrin, or the level of expression of any of the subunits. CLL cells do not express the classic vitronectin receptor, $\alpha V\beta 3$, but some cases bound relatively well to VN.

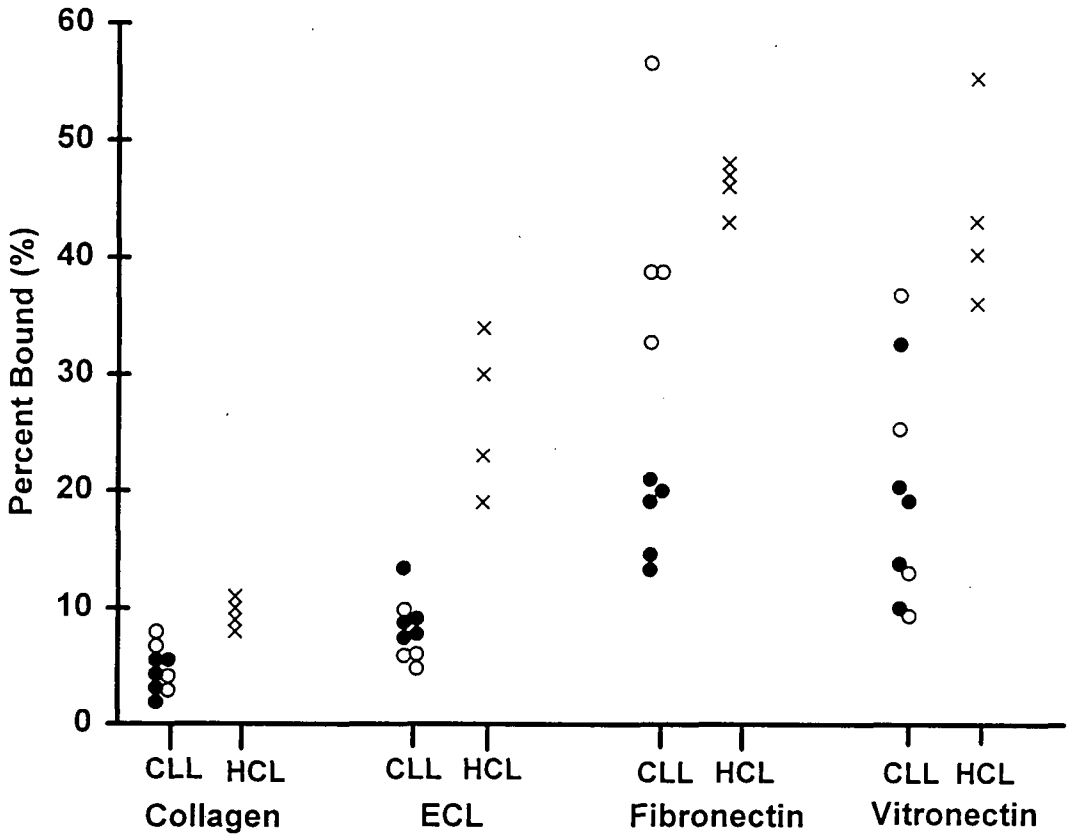



**Fig.4.1 Adherence of cells from nine cases of CLL and four cases of HCL to collagen (Coll), extract of basement membrane proteins (ECL), fibronectin (FN), and vitronectin (VN).** Binding is expressed as the percentage of cells which adhere after 10 minutes when a monolayer of CLL cells are placed in the protein-coated well. The mean of triplicate assays are shown; error bars indicating one standard deviation from the mean are too small to show on this scale. Activated (o) and non-activated (•) cases of CLL are indicated.

### *4.1.2 Relationship of adhesive activity to activation*

Activated cases of CLL (o, Fig.4.1) adhered much more strongly to fibronectin than non-activated (•). This difference was highly significant. However, binding to vitronectin did not correlate to the activation state of the CLL cells. Binding of HCs was always consistent between cases.

### *4.1.3 Effect of in vitro activation on adhesion*

In vitro stimulation of CLL cells as described previously (Chapter 2, Section 2.2.3) always increased adherence of CLL cells to FN, and the increase was significant. SAC was the most efficient stimulator of adhesion. As stated in Chapter 2, this stimulation did not consistently alter the adhesion molecule expression of the CLL cells. Table 4.1 shows the increase in adhesion in four cases due to stimulation of the CLL cells using SAC.

**Table 4.1 Adhesion of in-vitro stimulated CLL cells to fibronectin**

| **Fibronectin adherence** | **untreated (% bound)** | **SAC, 24hrs (% bound)** |
|---|---|---|
| **Patient 1** (N, Table 2.2) | 7.9 | 21.7 |
| **Patient 2** (G, Table 2.2) | 37.2 | 92.8 |
| **Patient 3** (C, Table 2.2) | 45.8 | 89.8 |
| **Patient 4** (M, Table 2.2) | 38 | 68.8 |
| **t-Test: Paired Two Sample for Means** | | |
| | **Variable 1** | **Variable 2** |
| **Mean** | 32.225 | 68.275 |
| **Variance** | 278.0292 | 1078.103 |
| **Observations** | 4 | 4 |
| **Pearson Correlation** | 0.943814 | |
| **Hypothesized Mean Difference** | 0 | |
| **degrees of freedom** | 3 | |
| **Test Statistic** | -4.01376 | |
| **P(T<=t) two-tail** | 0.027758 | |
| **t Critical two-tail** | 3.182449 | |

## 4.2 Morphology of adherent cells on FN and VN

Interaction of lymphocytes with their environment may lead to a variety of responses, i.e. they may adhere firmly and become sessile, in which case they spread; or they may quickly detach. Alternatively, lymphocytes may migrate on a substrate, which causes them to attain a polar shape. Thus computer image analysis (see Methods) of cells on a substrate allows determination of the response of a cell to that substrate. The morphology of HCs on fibronectin and vitronectin has been examined previously (see General Introduction). These studies are now also applied to CLL cells, and HCs are included for comparison under exactly the same conditions. The results (Fig.4.2) show that while HCs achieve a polar morphology on VN and become spread on FN, CLL cells remain round and unspread.

### *4.2.1 Effect of in vitro activation on morphology of adherent cells*

In Section 4.1.3 above, the *in vitro* stimulation of CLL cells was shown to increase their adherence to extracellular matrix proteins. The shape-change of these adherent cells was then assessed by image analysis. With TNFα-treatment of CLL cells there were minor differences in cell size and circularity, but after IL-10 treatment, the changes were striking, with a significant reduction in circularity, (i.e. the cells became motile, and an increase in size (i.e. the cells were spread). Fig.4.3 shows the difference in shape before and after stimulation, and Table 4.2 shows a statistical analysis of the shape-change in three cases of CLL.

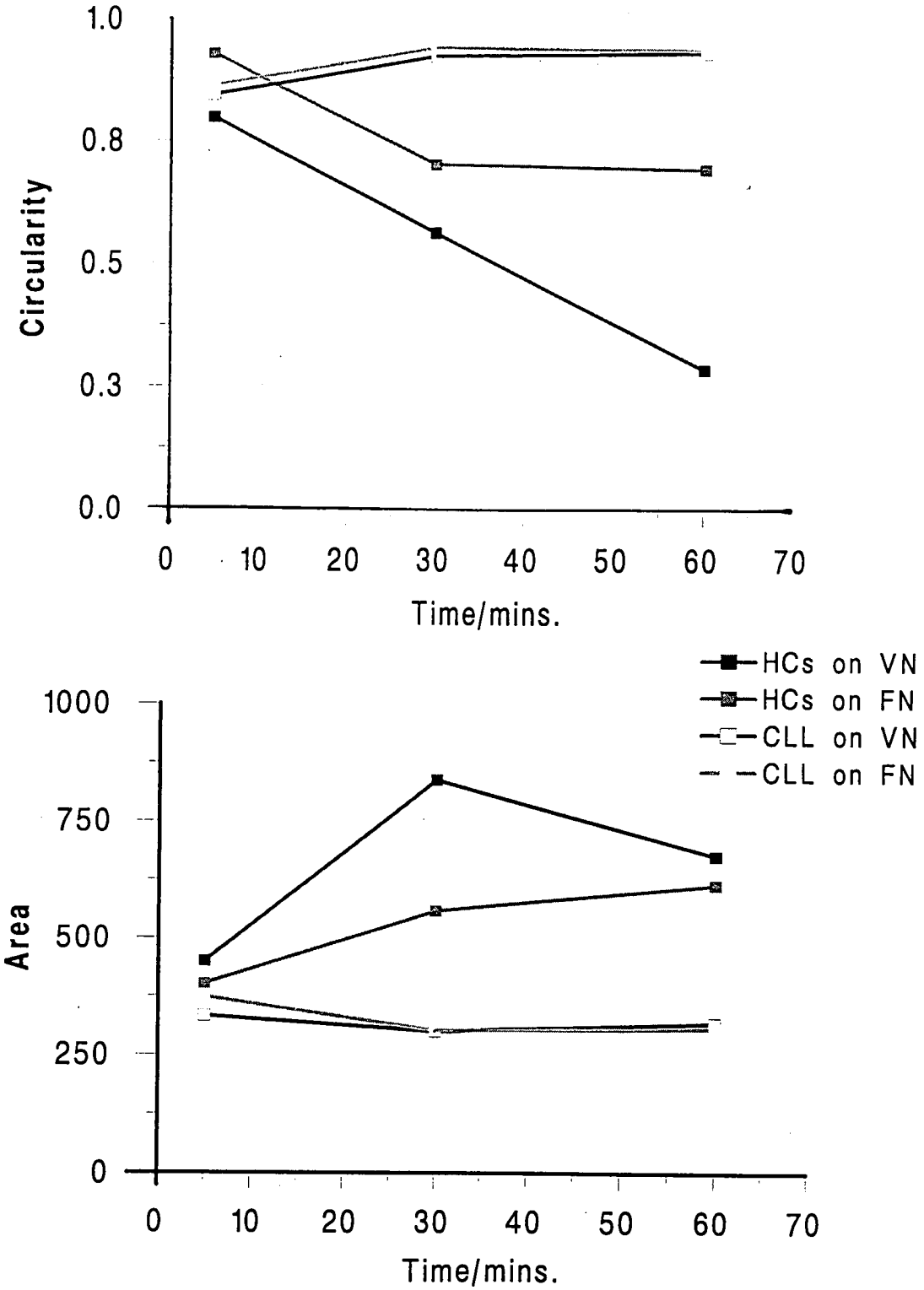



**Fig.4.2 Morphological change of CLL cells (open squares) and HCs (filled squares) adherent on VN and FN.** Circularity and area are expressed in arbitrary units from the computer image analyser. The results show increased area and loss of circularity (VN>FN) in HCs, while CLL cells show little or no change. Error bars indicating one standard deviation from the mean are too small to show on this scale.

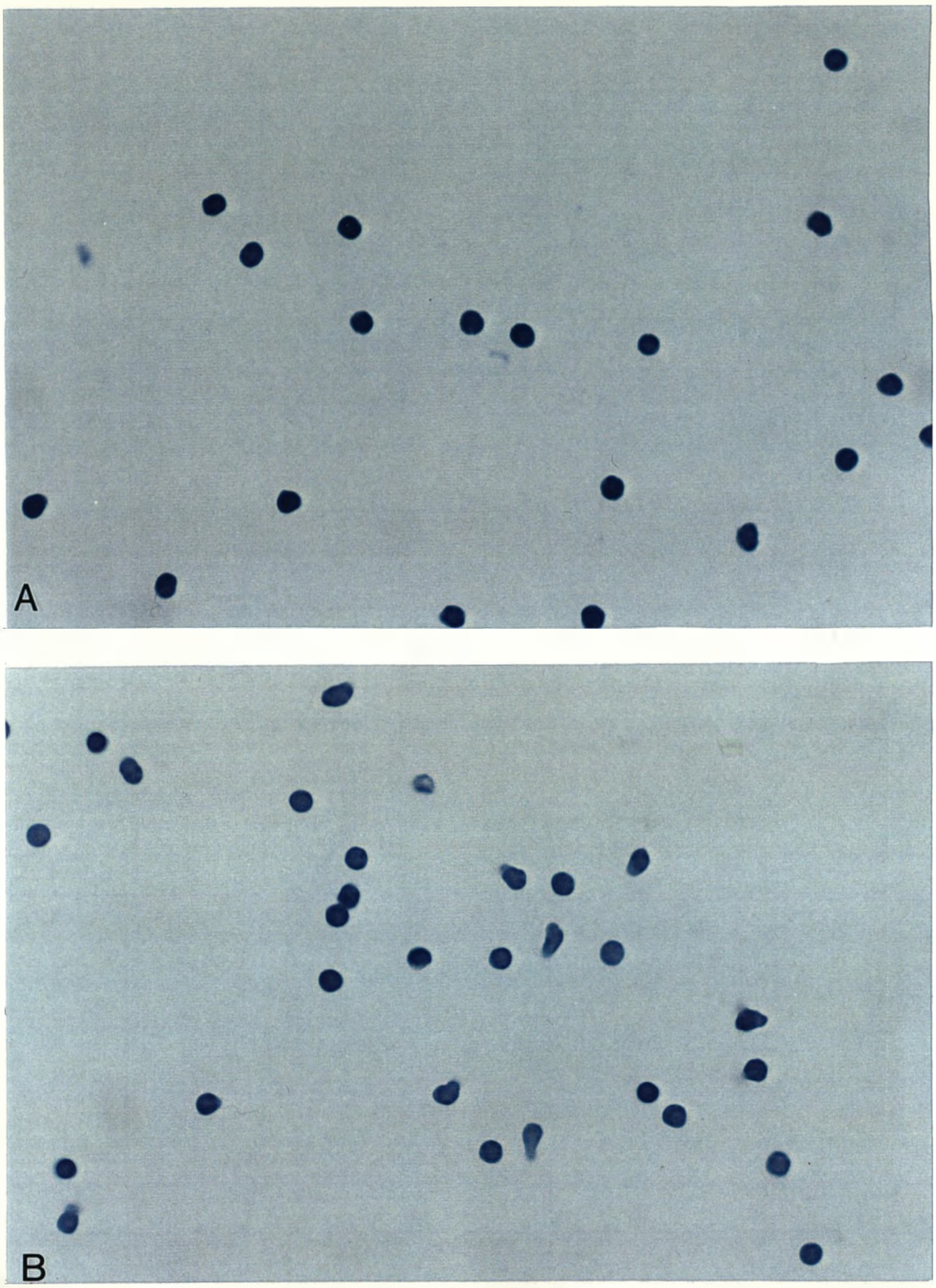



**Fig.4.3 Shape-change of CLL cells following stimulation with IL-10. (A) shows CLL cells before treatment. (B) shows cells from the same patient after culture for 24hrs with 100ng/ml IL-10. Cells are stained with Harris Haematoxylin.**

These experiments were performed on intrinsically activated (n=2) and non-activated (n=2) cases of CLL, and results were similar.

**Table 4.2 Analysis of shape-change of CLL cells following IL-10-stimulation**

Circularity and area are expressed in arbitrary units from the computer image analyser. * Letters refer to patients as in Table 2.2.

| | AREA | | CIRCULARITY | |
|---|---|---|---|---|
| | **untreated** | **IL-10, 24hrs** | **untreated** | **IL-10, 24hrs** |
| **Patient 1** (N*) | 1098 | 1165 | 0.918 | 0.849 |
| **Patient 2** (G*) | 991 | 1179 | 0.906 | 0.849 |
| **Patient 3** (C*) | 1217 | 1340 | 0.925 | 0.848 |
| **Patient 4** (M*) | 210 | 302 | 0.946 | 0.871 |

**t-Test: Paired Two Sample for Means**

| | **Variable 1** | **Variable 2** | **Variable 1** | **Variable 2** |
|---|---|---|---|---|
| **Mean** | 879 | 996.5 | 0.92375 | 0.85425 |
| **Variance** | 207436.7 | 220673.7 | 0.000282 | 0.000125 |
| **Observations** | 4 | 4 | 4 | 4 |
| **Pearson Correlation** | 0.99409 | | 0.867775 | |
| **Hypothesized Mean Difference** | 0 | | 0 | |
| **df** | 3 | | 3 | |
| **t Stat** | -4.49464 | | 15.44444 | |
| **P(T<=t) two-tail** | 0.020556 | | 0.00059 | |
| **t Critical two-tail** | 3.182449 | | 3.182449 | |

### 4.3 Trans-cell migration of CLL cells

In order to enter a tissue the adherent cells must be able to migrate to endothelial cell junctions and crawl between the cells. Having shown that CLL cells can be made to become motile, this study attempted to demonstrate the conditions necessary for trans-cell migration. The assay is described in the methods above, and typical results are shown in Fig.4.4. In no case did CLL cells constitutively crawl under endothelium although cells could remain adherent on the endothelial surface for the duration of the assay (24hrs). Stimulation of the CLL cells with IL-10 caused significant migration under endothelium (Fig.4.4). CLL cells did not constitutively migrate under bone marrow stromal cells or splenic red pulp fibroblasts, and due to

lack of material, the ability of stimulated CLL cells to migrate under these cell types could not be tested. HCs appeared under endothelium after 24hrs incubation without exogenous stimulation; this property of HCs is further examined in Chapter 5.

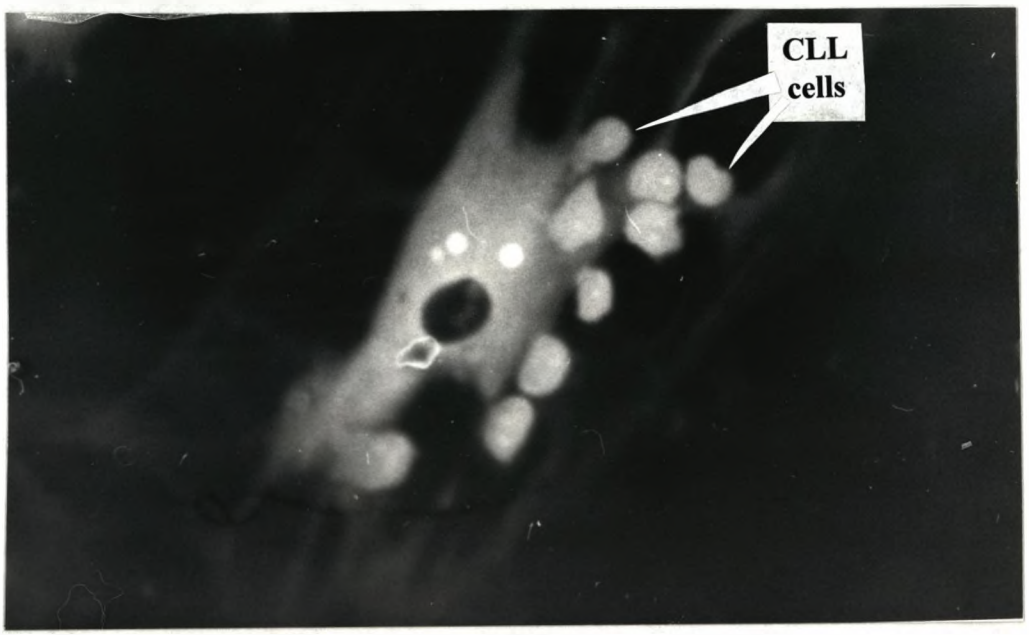



**Fig.4.4 Migration of IL-10-stimulated CLL cells under endothelial cells after 24hrs co-culture. Cells are stained with rhodamine phalloidin and visualised under a fluorescent microscope.** All cases tested (n=4) showed this migratory response. Migration was difficult to quantitate; the ratio of CLL to HUVEC was 8:1 at the start, and after 24hrs the modal number of CLL cells underneath HUVEC was 4, while the range was 0 to 11.

## Discussion

The results in this chapter have identified specific patterns of behaviour on various extracellular matrix substrates. Generally, CLL cells adhered poorly in comparison with HCs. CLL lymphocytes did not adhere to basement membrane proteins (purified collagen, and basement membrane extract containing entactin, collagen and laminin), but were able to bind the stromal adhesive proteins fibronectin and vitronectin. Such binding was, however, very variable and was not correlated with the level of expression of any particular FN-binding integrin ($\alpha3\beta1$, $\alpha4\beta1$, $\alpha5\beta1$), or VN-binding integrin. In contrast, HCs were able to bind to laminin, and adherence to FN and VN was consistently high. These findings reflect the different *in-vivo* behaviour of CLL and HCL. Thus, HCs show a strong propensity to localise in stroma (e.g. the splenic red pulp) and along basement membrane (Cawley and Burthem, 1992). In contrast, CLL cells do not associate with basement membrane, and tissue involvement (e.g. splenic or hepatic infiltration) varies greatly from case to case (Rai, 1993). Comparison of the variable binding of CLL cells to FN and VN with their activation state as defined previously (section 2.2.1) revealed that binding to FN correlated highly significantly with activated phenotype, whilst binding to VN did not. This difference may be due to CLL cells binding to VN via an unidentified (possibly non-integrin) adhesion molecule, with binding activity which is less dependent on the degree of cell activation than the known integrin FN receptors. *In-vitro* stimulation of the CLL cells further increased their adherence to FN; this increase was more marked than the stimulated endothelial binding shown in chapter 3 (3.2.3).

Having bound to a substrate, the lymphocyte may then respond by spreading or becoming motile. Image analysis of adherent cells revealed that CLL cells do not make either of these responses, and often detach after prolonged incubation. HCs, on the other hand, spread on FN and become motile on VN. Stimulation of the CLL cells could alter this behaviour, and the greatest response was due to IL-10; the CLL cells on FN showed a significant, but small, increase in size, and a significant loss of circularity.

Finally, the ability to migrate under stromal cells was investigated. Unlike HCs, no case of CLL had the ability to intrinsically crawl under endothelial cells, although some could sustain adhesion to the endothelial surface for long periods. After stimulation of the CLL cells with IL-10, the CLL cells could be found under the endothelial cells.

Thus, regarding adhesion to extracellular matrix proteins, the expression of particular adhesion molecules by CLL cells can facilitate binding to appropriate substrates. However, expression alone does not predict the actual adhesive behaviour of CLL cells. This conclusion was true for endothelial binding shown in the previous chapter; intrinsic activation state is important in determining the function of integrins, and local activating factors such as cytokines will play a major role in influencing the *in vivo* behaviour of the cells. These studies may explain why simple expression data in other investigations (discussed in Chapter 2) does not correlate well with clinical features of the disease.

The techniques employed in this and the previous chapter have provided an insight into the adhesive behaviour of CLL cells. Whilst the idea for this study originated from investigations into HCL, some of the assays developed for this study

had not been performed on HCs. The following chapter, therefore, returns to HCL and applies the new experiments to HCs to further explore the interactions of HCs with endothelium and stromal cells.

# Chapter 5

## The interactions of HCs with endothelial ligands; the role of α4β1 in HC function.

## Introduction

In this chapter, the approaches used successfully in Chapter 3 to examine CLL cell-endothelial interaction are applied to HCs. This seemed important not only because HCs, like other lymphoid cell types, have to attach to and penetrate endothelium before gaining access to the tissues, but also because HC-endothelial interactions are prominent in HCL. Thus, in HCL, it has long been known that HCs form a close association with endothelial cells lining the red pulp of the spleen and the hepatic sinusoids; the malignant cells in these locations then come to replace endothelium and align themselves along the basement membrane of the sinusoids (Pilon *et al*, 1982). This process results in the so-called pseudosinuses which are diagnostic of HCL (Nanba *et al*, 1977a). Despite the diagnostic importance of pseudosinus formation, its pathophysiological basis has never been explained.

Here, adhesion and transmigration assays are used to examine the HC-EC associations, and blocking mAbs are employed to establish the receptors/ligands involved in this process. It is shown that, unlike CLL cells, HCs adhere to, and migrate under endothelium without any prior stimulation; this, it will be argued, is a

reflection of their intrinsic state of activation. Furthermore, these functions are shown to be dependent on the interaction between α4β1 on HCs and VCAM-1 on ECs. Finally, the likely pathophysiological importance of α4β1/VCAM-1 interactions is indicated by demonstration of VCAM-1 on splenic red pulp fibroblasts and bone marrow stromal cells, by adhesion assays involving these cell types, and by binding assays on splenic sections.

## Materials and Methods

Most of the methods have been described previously, and will not be repeated here. Full experimental protocols are in Appendix 1 or in appropriate chapters. Relevant methods are:- isolation and purification of B-cells (Ch.2); HUVEC culture (A1.4); splenic red pulp fibroblast and bone marrow stromal cell culture (Ch.4); triple-layer FACS (A.1.3); cell ELISA (A.1.6); adhesion assay (A1.7); blocking adhesion assay (A1.7); co-culture (Ch.4). Three techniques not used previously are immunohistochemistry on splenic sections, the Stamper and Woodruff frozen section assay, and a simple aggregation assay.

### *Immunohistochemistry*

For this assay, paraffin-embedded tissue sections were employed. Paraffin was removed by heating to 56°C for 15 minutes followed by sequential washes in xylene, then 100%, 95%, then 80% ethanol. Endogenous peroxidase was then blocked using 3% $H_2O_2$. Primary antibody (VCAM-1 or matched class control antibody) was added in PBS/1%BSA for 1 hr at room temperature. Second and third layers were the peroxidase staining kit (ExtrAvidin EXTRA-2, Sigma Immunochemicals). In this case the substrate was diaminobenzidine (Sigma) with Mayer's counterstain (Sigma).

***Frozen section assay***

This method has been adapted from that first published by Stamper and Woodruff in 1976, which demonstrated the role of HEV in lymphocyte homing into lymph nodes.

HCL spleen, normal spleen, and tonsil tissue were obtained and frozen in liquid nitrogen before sectioning. Cryostat sections (5μm) were prepared at −15°C in a microtome, mounted on microslides, and air dried for 1 hour. These sections were either used immediately or stored at −20°C. All subsequent steps were performed at 5°C. HCs were prelabelled with the red fluorescent dye PKH26 (Sigma, staining according to manufacturer's instructions). Aliquots of $2x10^5$ cells in 200μL RPMI containing 10% serum were layered onto each section, and the slides immediately placed on a rotating platform for 30 minutes. After incubation, the sections were washed in PBS, then fixed in 1% glutaraldehyde for 1hr, and mounted in PBS/glycerol. Slides could then be examined and photographed using phase contrast fluorescence microscopy (adherent HCs stained red).

***Aggregation assay***

HCs were pre-incubated with saturating concentrations (>4μg/ml) of blocking or control antibodies, then cells were washed and placed in RPMI without serum, also containing antibody, and incubated at room temperature. Aggregation was assessed visually and the time for aggregates to form in serum-free medium noted.

# Results

## 5.1 HCs interact strongly with endothelium

Initially, the HUVEC model of Chapter 3 was used to examine the capacity of HCs to adhere to endothelial cells. In this experiment the HUVEC were stimulated with TNFα for various periods of time in order to induce expression of VCAM-1 and E-selectin. The adhesion assay identified the strong binding to HUVEC which is required for initial tissue localisation and subsequent extravasation. The capacity of HCs to adhere to the HUVEC (either unstimulated or exposed to TNFα) was determined by a reverse centrifugation method that reproducibly removed HCs that were not strongly bound.

When HCs were plated with "unstimulated" HUVEC at concentrations that potentially allowed all HCs to adhere, 20-60% (mean 35%) of HCs became adherent. However, when the endothelial layers were stimulated using TNFα, the adhesion of HCs was increased in all cases up to a maximum of 85% (mean 58%). These results suggest that HCs have a strong capacity to interact with endothelial ligands (see Fig.5.2).

## 15.2 Analysis of receptors potentially mediating the HC/HUVEC interaction

At least five endothelium-binding receptors are reportedly expressed by HCs, namely αXβ2, α4β1, αMβ2, CD31, and L-selectin (Moller *et al*, 1992; Knapp *et al*, 1989.). To gain some further insight into their potential importance in the highly consistent adhesive behaviour of HCs, the level of expression of each receptor was examined on the cells from different cases of HCL. The results confirmed that each of the receptors was present in all cases, but that not all of the adhesion molecules

were expressed strongly, or at consistent levels. Thus, αXβ2, α4β1 and CD31 were each expressed at consistent and high levels; αMβ2 was present at intermediate levels, while the level of L-selectin was more variable and generally lower (Fig.5.1).

## 5.3 Identification of the adhesive role of these different receptors/ligands in the HC interaction with HUVEC

Earlier chapters have highlighted the concept that the simple expression of a given receptor or ligand does not necessarily indicate its functional importance. Therefore, in this Chapter, three complementary approaches to probe the adhesive functions of the different HC receptors were employed. First, the temporal relationship between the number of HCs bound to the endothelium and the appearance/disappearance of HUVEC adhesion molecules following stimulation was determined. This identified a potential importance for VCAM-1 and E-selectin. Next, the ability of HCs to adhere to surfaces coated with these two proteins was examined. Thirdly, the importance of VCAM-1 and other adhesion molecules was further tested using adhesion-blocking mAbs.

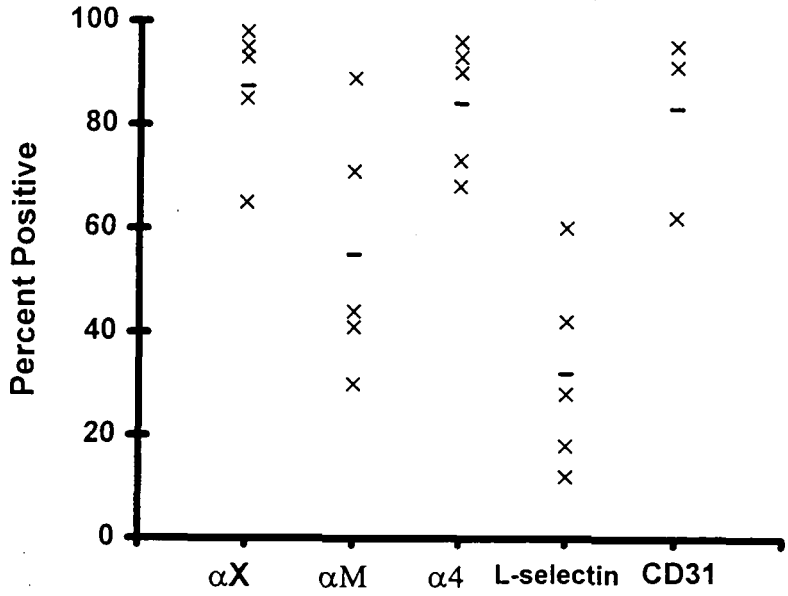



**Fig.5.1 Expression of endothelium-binding adhesion molecules by HCs.**

Determined by triple-layer FACS; for explanation of "Percent Positive", which indicates the level of antigen expression on the HCs, see Fig.2.1.

The level of expression in each case is marked by an **x**. The dash (-) indicates the mean level of expression of each antigen. Five cases of HCL are represented, but only three are shown for CD31, due to shortage of reagents when patient material was available.

### 5.3.1 HC adhesion mirrors the expression of particular ligands on HUVEC.

The clear enhancement in the adhesion of HCs that followed TNF stimulation of HUVEC implied that changes in ligand expression were occurring that were important to HC adhesion.

The altered expression of adhesion ligands seen in the ELISA assay at various times after TNF stimulation (Chapter 3, Fig.3.1) was therefore compared to the capacity of HCs to adhere to the endothelial cells. These assays suggested that the "baseline adhesion" of HCs to unstimulated HUVEC could be determined by any of a number of different ligands, since ICAM-1, CD31, and cFN, and even low levels of VCAM-1 were all expressed (Fig. 3.1). In contrast, the significantly enhanced binding that followed TNFα stimulation appeared to parallel the changes in expression of VCAM-1, although early involvement of E-selectin remained possible (Fig.5.2).

### 5.3.2 Purified endothelial ligands E-selectin and VCAM-1 both strongly support HC adhesion.

Adhesion assays similar to those used in Chapter 4 for binding to extracellular matrix proteins were employed. Affinity purified VCAM-1 and E-selectin were purchased from R&D Systems Ltd., Abingdon, UK). Binding of HCs to E-selectin and VCAM-1 was compared with their binding to FN (a strong ligand for HCs) or to artificial basement membrane (a weak ligand). The results showed that both VCAM-1 and E-selectin very strongly supported the adhesion of HCs, in fact allowing greater levels of adhesion than were supported by FN (Fig.5.3).

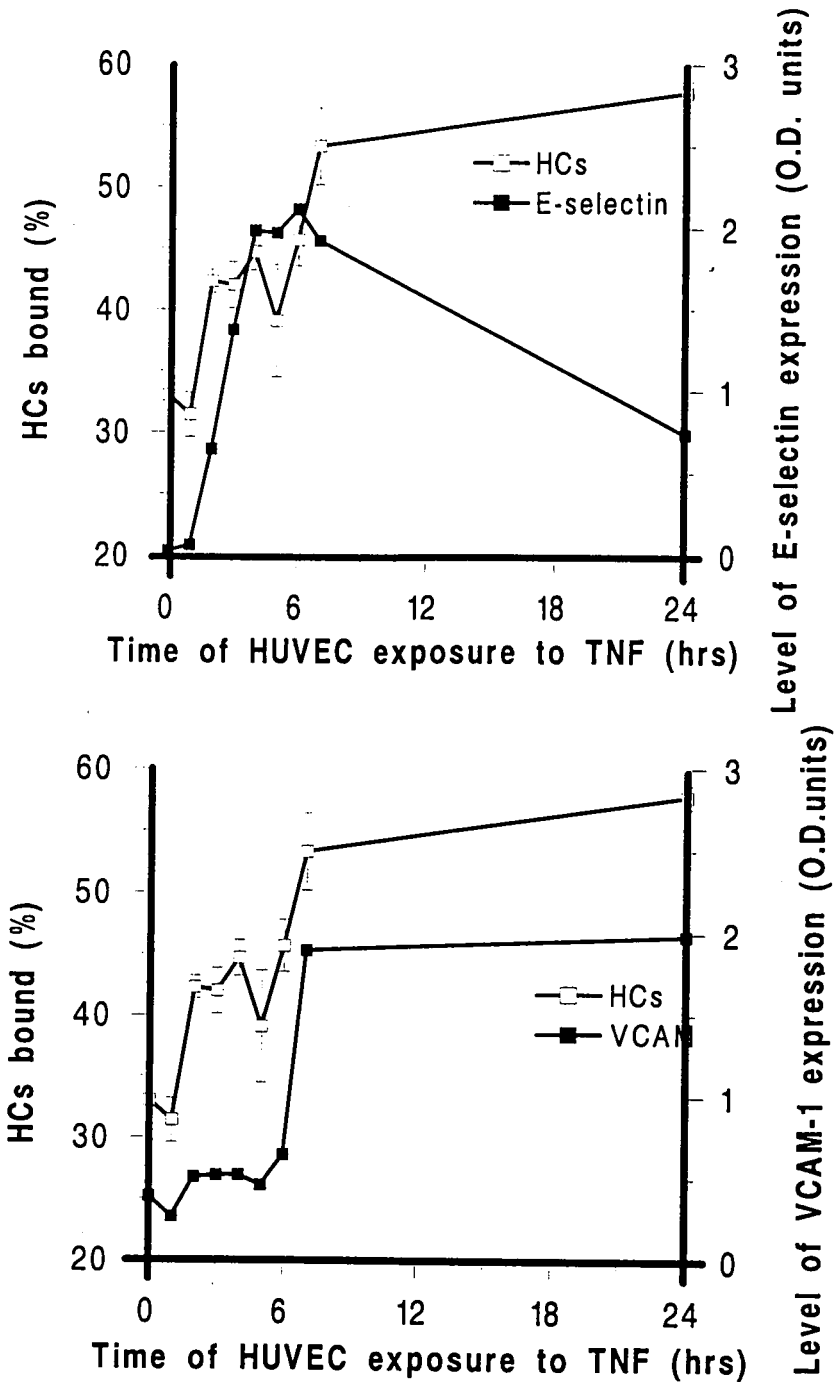



**Fig.5.2 Comparison of HC binding to TNFα-stimulated HUVEC with endothelial expression of VCAM-1 and E-selectin.** Adherence is expressed as the percentage of cells which adhere after 10 minutes when a monolayer of CLL cells are placed on the HUVEC. The values are calculated from the mean of triplicate assays, and error bars represent one standard deviation from the mean. Expression of adhesion molecules was determined by cell-ELISA; duplicate assays were performed, and error bars showing standard deviation are too small to be seen on this scale.

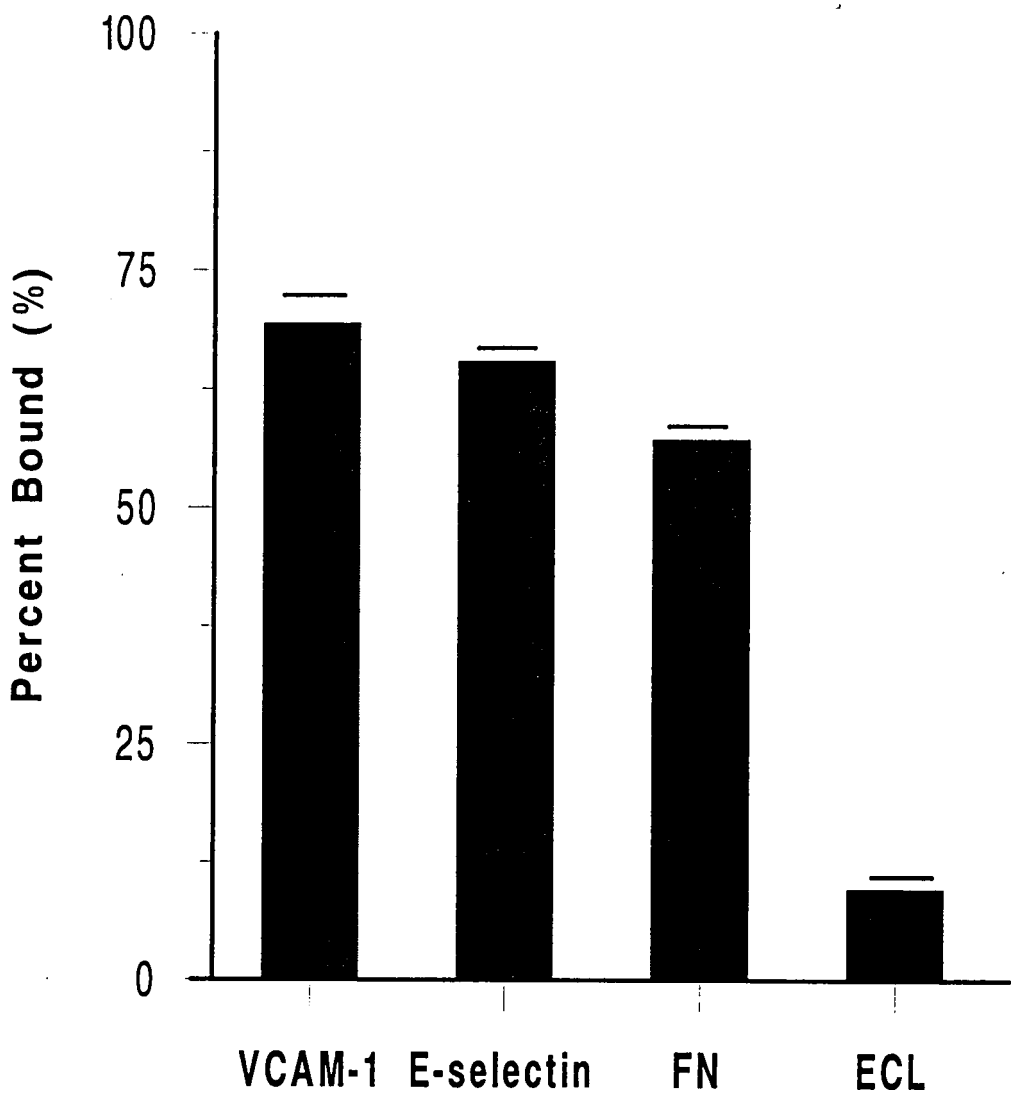



**Fig.5.3 Adhesion of HCs to purified VCAM-1 and E-selectin; comparison with FN and ECL.** The percentage of cells adherent are the mean of triplicate samples in two separate typical cases of HCL. Horizontal (error) bars represent one standard deviation from the mean.

### 5.3.3 Blocking studies identify an important role for VCAM-1.

Although the preceding section indicates that HCs can adhere to both VCAM-1 and E-selectin, the stimulated HUVEC experiments indicated a predominant role for VCAM-1. Therefore a blocking mAb to β1 integrin (the β subunit of α4β1, the ligand of VCAM-1) was used to establish whether or not α4β1/VCAM-1 interaction was the major determinant of HC adhesion in the HUVEC model. A further mAb that blocked the adhesion of β2 integrins (the β subunit of the two endothelial-binding integrins, αMβ2 and αXβ2) was used both as a control, and to probe the interaction of HCs with β2 ligands such as ICAM-1.

With anti-β2 mAbs, no blocking of adhesion, either to stimulated or non-stimulated HUVEC, was seen. In contrast, although anti-β1 did not affect the binding of HCs to unstimulated endothelium, it strongly inhibited (to baseline levels) the binding of HCs to stimulated endothelium. This was true both after 6 hours stimulation (when VCAM-1 and E-selectin were expressed on the endothelium), and after 24 hours stimulation (when only VCAM-1 was expressed) (Fig. 5.4).

Taken together, all the above results suggest that a number of different adhesion molecules can potentially mediate the binding of HCs to endothelia. The molecules that mediate the "baseline" binding of HCs were not identified. Thus, the failure of specific anti-β2 or anti-β1 antibodies to inhibit such binding suggests that the mechanism of HC adhesion is likely to be integrin-independent or, perhaps, to involve several different molecules acting together. In contrast, the cause of the specific additional binding of HCs to stimulated endothelium was clearly attributable to VCAM-1 on HUVEC binding to its specific α4β1 receptor on HCs.

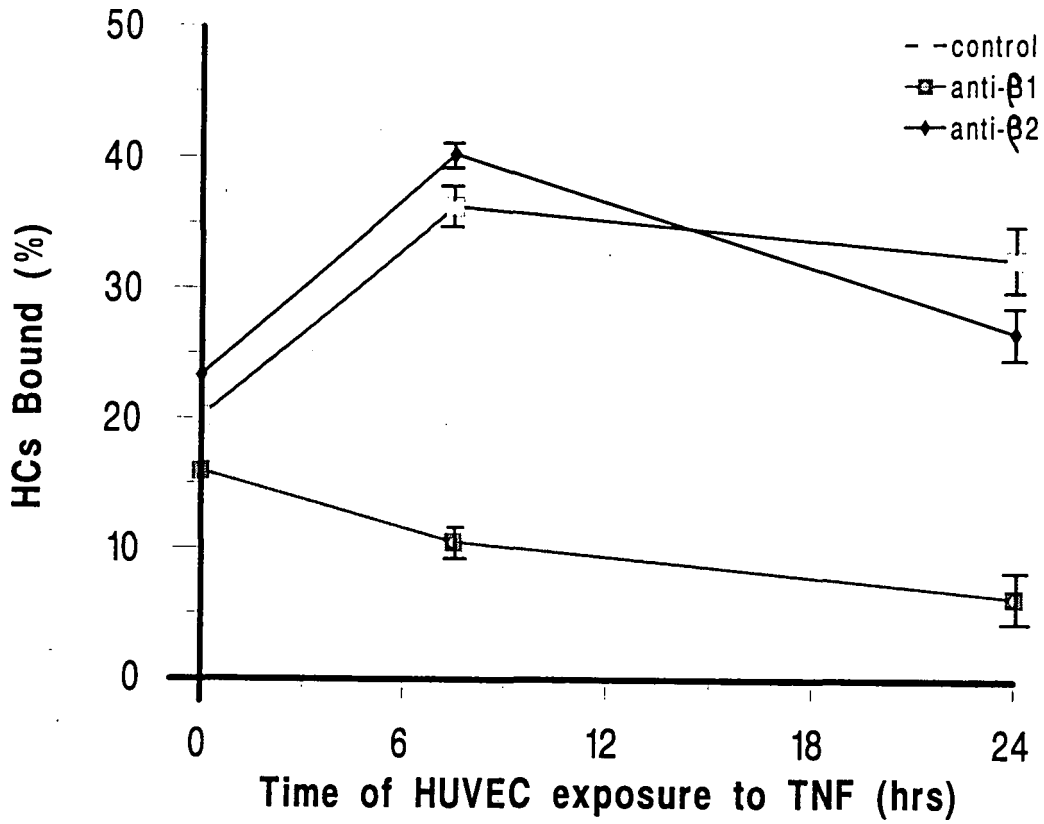



**Fig.5.4 Blocking of HC adhesion to stimulated HUVEC using mAbs against HC-expressed integrins.** Results are expressed as a percentage of cells which adhered in the presence of a control (isotype-matched) mAb. Triplicate assays were performed for one case of HCL, and indicated as the mean ± one standard deviation.

## 5.4 The α4β1/VCAM-1 interaction has specific functional importance in HC transmigration

Although adhesion alone is clearly important in lymphocyte homing, the ability of the cell subsequently to traverse the endothelium is also important; VCAM-1/α4β1 interaction has been implicated in this process (Chuluyan *et al*, 1995; Sano *et al*, 1995.). It was therefore necessary to establish the functional consequences of HCs binding to VCAM-1.

### 5.4.1 VCAM-1 induces HC motility.

Previous studies in this Department have shown that HCs respond to adhesion by undergoing specific morphological changes. The different morphological forms clearly correlate with specific functional behaviour, and these were discussed in Chapter 1 (section 1.5):- polarised cells are actively motile; non-polar cells are non-motile, and may undergo either spreading or sessile adhesion. The different forms may readily be distinguished by microscopy, and can be quantified and statistically evaluated by image analysis.

HCs were allowed to adhere to glass cover slips coated with VCAM-1, E-selectin, or FN. Adherent cell preparations were then fixed at various time points and examined by image analysis. These experiments clearly showed that VCAM-1 induced a polarised morphology suggestive of motility (Fig. 5.5). In contrast, HCs strongly adhered to E-selectin and FN, but far less change in shape was observed with little elongation. Fig.5.5 shows the results obtained for FN; HCs tended to have slightly larger area on E-selectin than on FN, although the circularity was similar; this implies that the cells were more spread, not elongated (not shown on Fig.5.5). These

findings suggest the potential involvement of VCAM-1 in the transendothelial migration of the neoplastic HC.

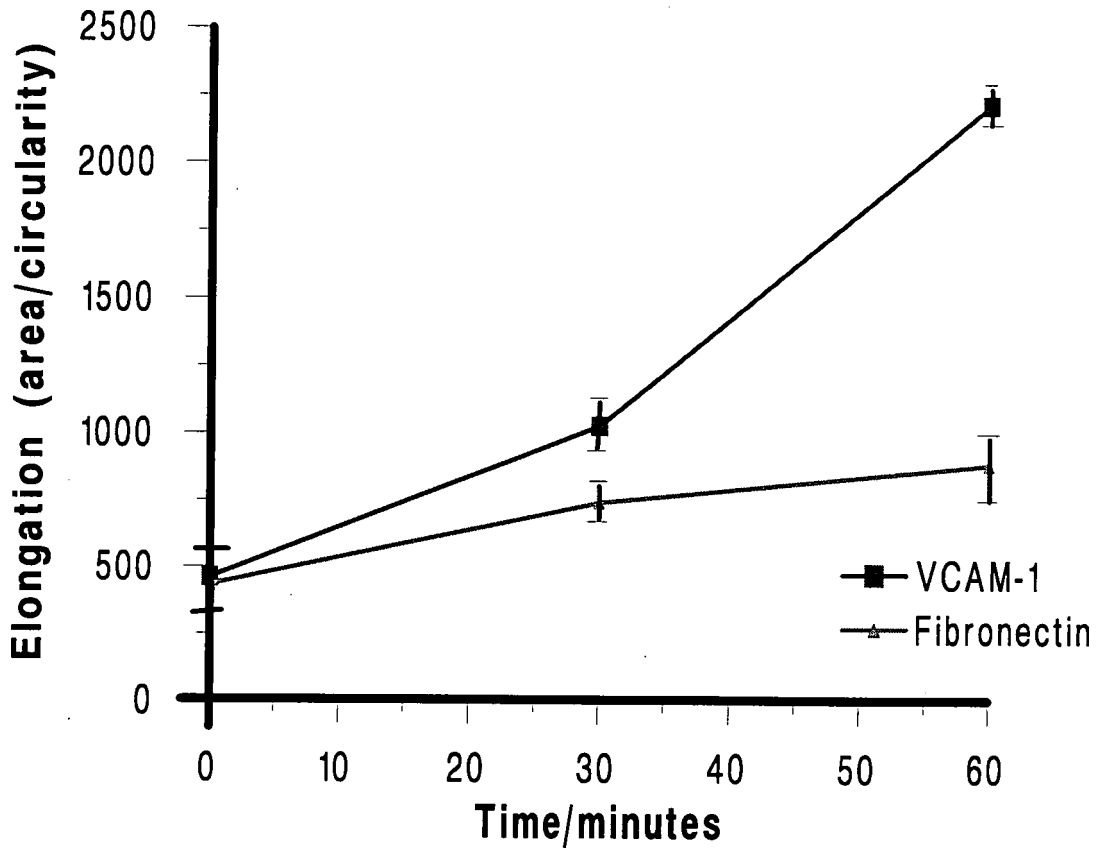



**Fig.5.5 Morphology of HCs adherent on VCAM-1 compared with FN (E-selectin not shown).** Elongation (area/circularity; see Methods) indicates the degree of polarisation of the cell. Results represent the mean of 200 cells from a single case; similar results were seen in other experiments. Error bars indicate one standard deviation from the mean.

### 5.4.2 Binding of HCs to VCAM-1 is essential for their transmigration.

The co-culture experiments described in Chapter 4 were used to demonstrate interaction with HUVEC after prolonged incubation. After 24 hrs in culture, most of the HCs had migrated underneath the HUVEC (Fig.5.6). When HCs were co-cultured with HUVEC in the presence of blocking anti-VCAM-1 mAb, such transmigration was abolished.

## 5.5 The α4β1/VCAM-1 interaction is important in the tissue-accumulation of HCs *in vivo*

Normal vascular endothelia express few adhesion receptors on their luminal surface. However, there is accumulating evidence that the endothelia of certain organs may, constitutively or in disease states, express particular adhesion molecules. With regard to the present study, it has recently been shown that VCAM-1 is expressed constitutively on the endothelia of bone marrow, and on spleen and liver sinusoids in certain haemopoetic disorders, but not on lymph node or splenic white pulp (Schweitzer *et al*, 1996; Rice *et al*, 1990). In addition, however, VCAM-1 is also expressed on certain stromal cell types; in particular it is expressed on hepatic Kuppfer cells and on splenic red pulp macrophages - two cell types with which HCs are recognised to associate. Such stromal expression of adhesion ligands can play a further role in the localisation of migrating cells within tissues.

In order to determine more closely the *in vivo* relevance of the α4β1/VCAM-1 interaction to the tissue distribution of HCs, the interaction of HCs with a number of relevant stromal cell types, and with relevant tissue sections, were examined.

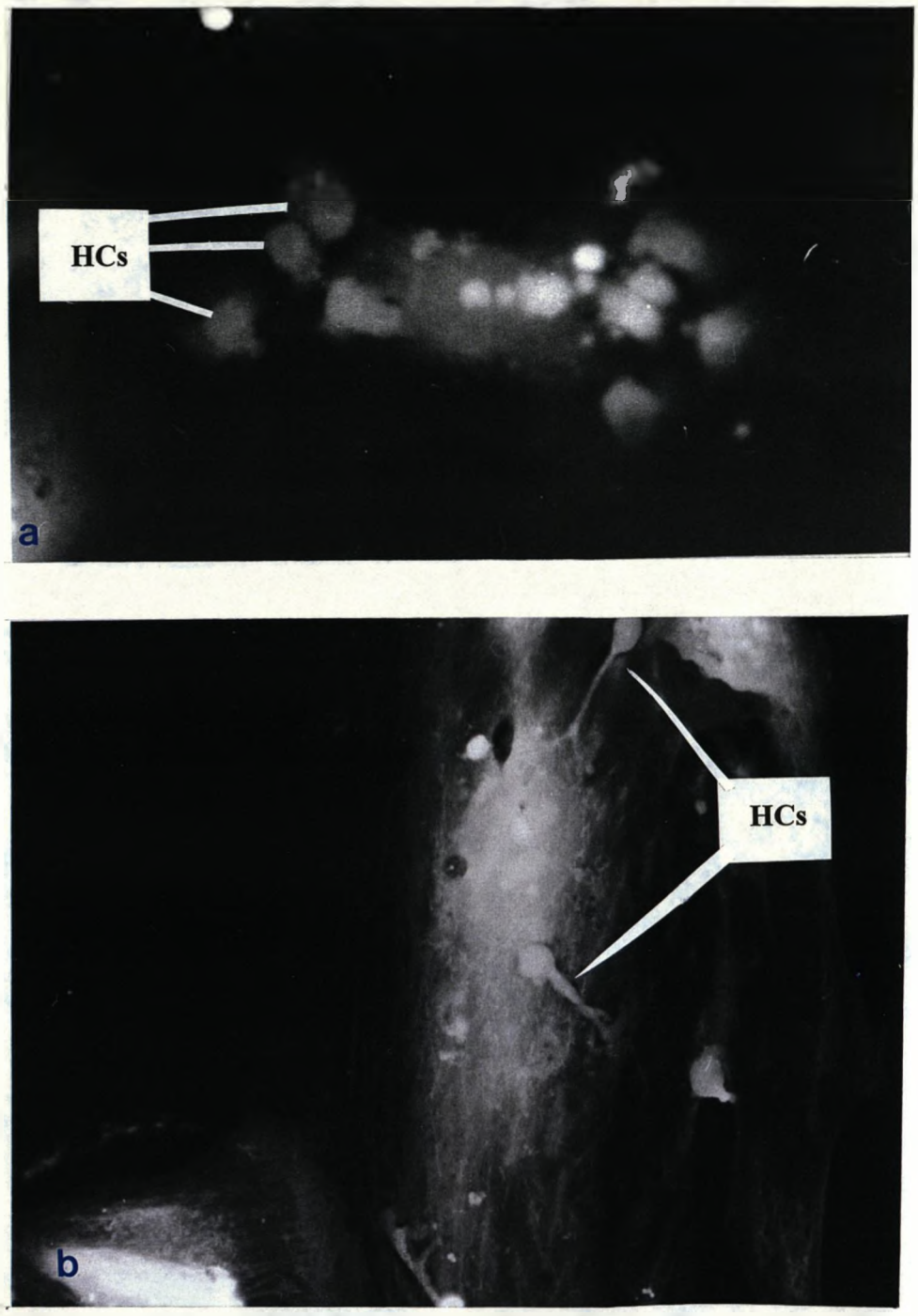



**Fig.5.6 Co-culture of HCs with HUVEC (a) or bone marrow stromal cells (b). Cells are stained with rhodamine phalloidin and visualised using fluorescence microscopy.** HCs appear sessile under HUVEC and motile under marrow stromal cells. The number of HCs under the stromal cells ranged from 0 to 12; the modal number of transmigrated cells was five. Arrows indicate HCs.

### 5.5.1 α4β1/VCAM-1 mediates the interaction of HCs with relevant stromal cells.

Both bone marrow stromal cells and splenic red pulp cells were examined. Bone marrow stromal cells were derived from long-term cultures of normal bone marrow, while the splenic red pulp cells were obtained from the outgrowth of an HCL spleen. Both cell types were found to express VCAM-1 without additional stimulation, and HCs strongly adhered to them. Following more prolonged culture, HCs were seen to have migrated under both cell types (Fig.5.6 shows migration under marrow stromal cells). Both the adhesion and transmigration of HCs were prevented by the blocking anti-VCAM-1 mAb.

### 5.5.2 α4β1/VCAM-1 supports the adhesion of HCs to splenic tissues.

The expression of VCAM-1 by splenic endothelium and splenic red-pulp macrophages has previously been reported (Rice *et al*, 1990). A Stamper-Woodruff type assay on normal spleen showed that HCs adhered to the red-pulp areas, but not to other regions; such adhesion was blocked by the anti-VCAM-1 mAb (Fig.5.7). CLL cells that did not express α4β1 did not adhere to the splenic sections, and HCs adhered poorly to control tissue (tonsil).

Since HCL spleen is greatly altered by the disease process, VCAM-1 expression was examined immunohistochemically (Fig.5.8). These studies confirmed the continued expression of VCAM-1 by endothelial cells and also revealed strongly positive cells (probably macrophages) scattered throughout the splenic stroma. This assay also revealed that HCs in the spleen themselves appeared to be weakly positive for VCAM-1.

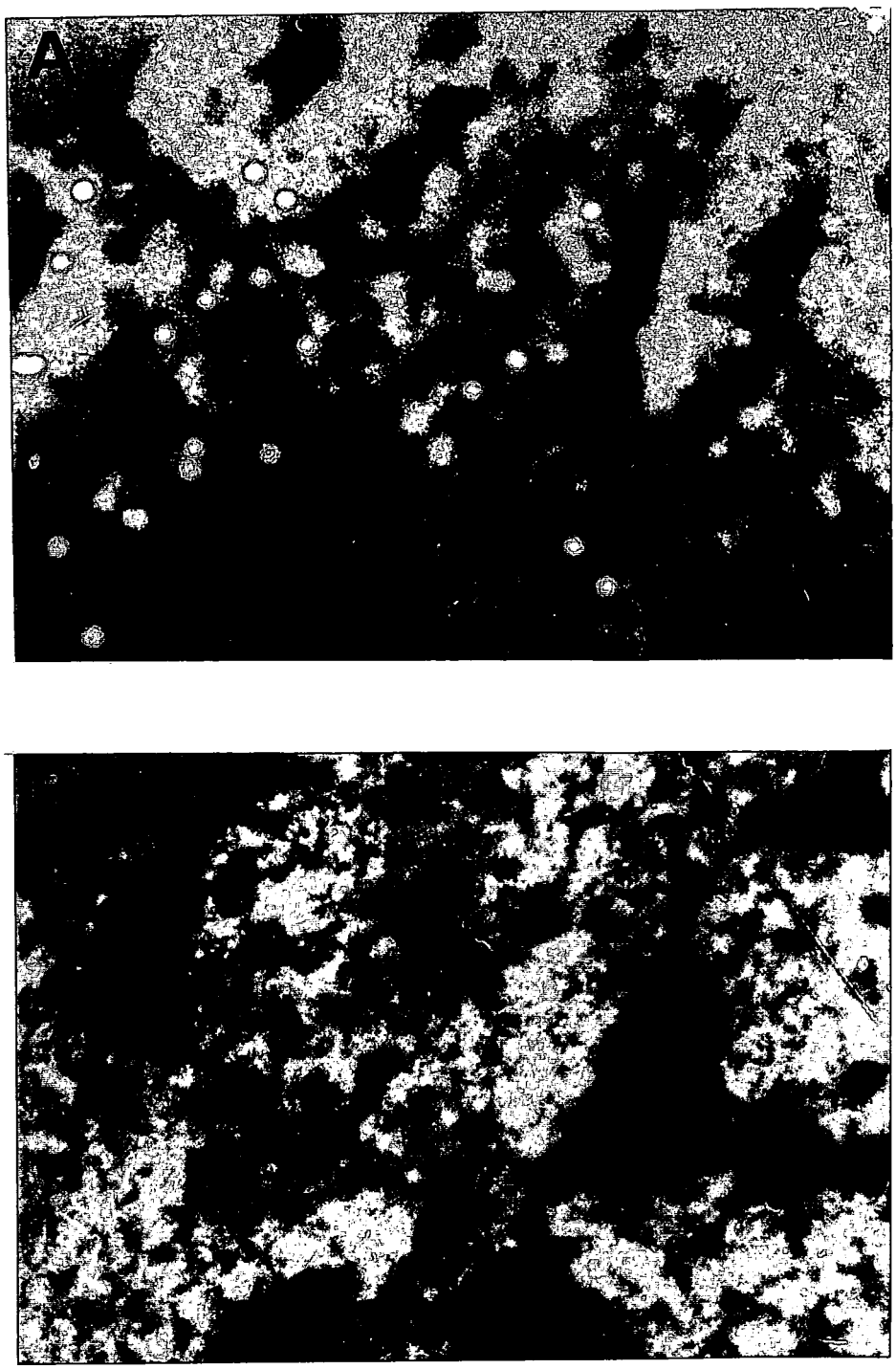



**Fig.5.7 Adherence of HCs to sections of normal spleen. Panel A shows adherence in the presence of isotype-matched control mAb. Approximately 30 HCs adhered per $mm^2$ of section. Panel B shows adherence in the presence of blocking anti-VCAM-1 mAb. No adherent HCs were found.**

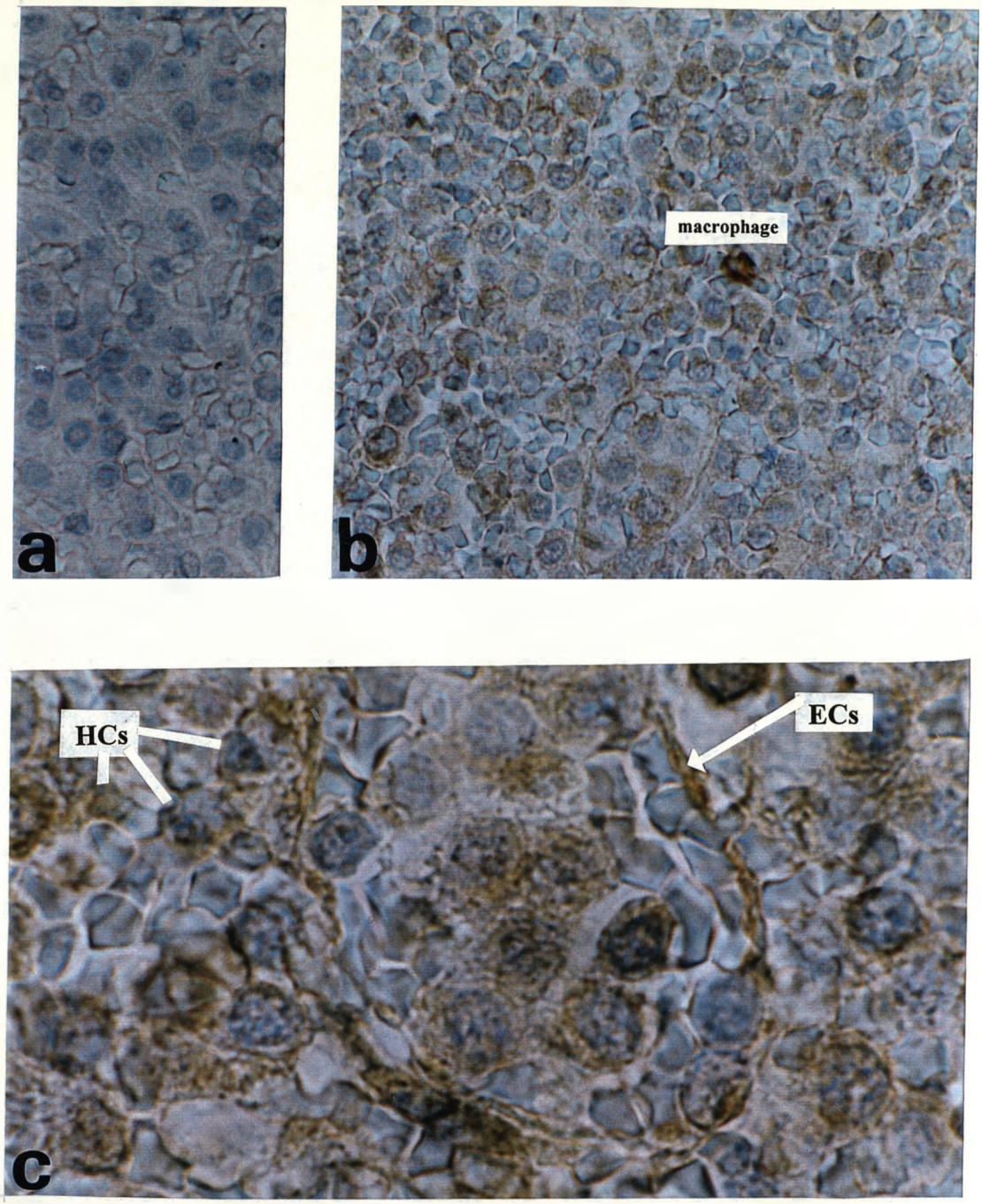



**Fig.5.8 Immunohistochemical determination of VCAM-1 expression in sections of HCL spleen (a) control mAb; (b) staining with anti-VCAM-1 low magnification; (c) VCAM-1 high magnification. Strongly positive cells are probably macrophages. Positive endothelial cells and HCs are also distinguishable.**

### 5.5.3 Expression of VCAM-1 by HCs

In order to confirm the observation that HCs express VCAM-1, peripheral blood HCs were analysed by triple-layer FACS. These cells were shown to be weakly positive. VCAM-1 was not passively bound since it was not lost following prolonged culture and was present after exposure of the cells to EDTA. In contrast to HUVEC, bone marrow stromal cells and splenic red pulp fibroblasts, VCAM-1 was not further induced by TNFα or PMA.

The functional significance of the expression of VCAM-1 on HCs is not known; it might, by binding to α4β1 on adjacent HCs, act to promote homotypic adhesion. However, in a simple adhesion assay, neither anti-VCAM-1 nor anti-β1 were able to inhibit HC aggregation (data not shown).

## Discussion

This chapter investigates the cell-cell interactions of HCs. The results show that α4β1 has an important role in interactions with other cell types, and that such binding is an essential prerequisite for entry into tissues.

First the expression of adhesion molecules was determined by sensitive triple-layer FACS. Two cell-binding integrin receptors, α4β1 and αXβ2, were expressed at high levels, while other adhesion molecules (αVβ3, αMβ2, CD31, and L-selectin) were variably or less strongly represented.

However, quantitative expression alone cannot indicate function, and transendothelial migration must be preceded by strong adhesion that facilitates spreading. Thus the assay of strong adhesion was used to examine the functional activity of the various receptors. TNFα-stimulated HUVEC were employed as a model for the expression of relevant adhesion ligands. Only the interaction between α4β1 and its ligand VCAM-1 mediated the strong binding of HCs, and when the action of this integrin was blocked using an anti-β1 mAb, both induced and constitutive binding of HCs to endothelium was reduced.

In addition to its expression on endothelia, VCAM-1 is constitutively expressed by certain stromal cells. A cell ELISA confirmed the expression of VCAM-1 on bone marrow stroma and on stromal cells derived from HCL spleen. HCs adhered strongly to these VCAM-1-expressing stromal cells. Also, following extended culture, HCs readily migrated beneath all three VCAM-1-expressing cell types. Both adhesion and migration were almost completely abrogated by anti-VCAM-1 or anti-β1.

All the above observations suggest that α4β1 is the major adhesion molecule mediating strong cell-cell interactions in HCL, and that such binding is an essential prerequisite for transmigration into tissues. In this regard, it is highly relevant that the principal areas where VCAM-1 is constitutively expressed *in vivo* are also those typically involved by the abnormal lymphocytes of HCL. These areas are splenic and hepatic endothelia, together with marrow and splenic stroma; VCAM-1 is not expressed constitutively on other endothelial sites or on HEV. Therefore frozen section assays were employed to test the *in vivo* relevance of these observations. HCs readily adhered to normal splenic red pulp, and this adhesion was blocked by anti-VCAM-1 or anti-β1 mAbs. HCs did not adhere to control tissues such as tonsil where VCAM-1 is not expressed.

Finally, VCAM-1 was shown to be expressed by HCs themselves. Expression of low levels of VCAM-1 were demonstrated both directly *ex vivo* and following freezing or extended culture; the molecule was also present on HCs in tissue sections. The level of expression was, however, significantly lower than that of α4β1 and was not upregulated following *in vitro* activation of HCs using TNFα or phorbol ester.

The simultaneous expression of α4β1 and its ligand VCAM-1 on HCs suggested that these adhesion molecules might provide a potential mechanism for HCs to bind to each other. α4β1/VCAM-1 interaction between HCs would encourage further accumulation of the HCs in tissues already involved by the disease. However, anti-VCAM-1 or anti-β1 mAbs did not prevent the aggregation of cultured HCs. Furthermore, HCs did not bind strongly to tissue sections from HCL spleen.

These experiments provide strong evidence that the α4β1 expressed by HCs plays a major role in their tissue localisation. In this regard HCs resemble

lymphoblasts which bind to VCAM-1 expressed on bone marrow stromal cells, and sickle cells which employ α4β1 to localise within splenic red pulp. However, α4β1 is also expressed on a wide range of haemopoietic cells which do not show the same pattern of tissue localisation as HCs. The question therefore arises as to why all α4β1-expressing cells do not show the same pattern of tissue localisation. This may be explained by the multi-step model of tissue entry. HCs express α4β1 in a functionally active form, unlike peripheral blood B cells which express a non-active form of the integrin and do not bind to VCAM-1 (Postigio *et al*, 1991b). HCs also have a highly active cytoskeletal structure and can respond to locally produced cytokines, enabling them to react to such adhesion; in contrast, cells such as CLL lymphocytes may bind to VCAM-1 but do not maintain this adhesion and under similar conditions do not transmigrate. Finally, the responses of HCs to adhesive proteins which have been described, favour their subsequent migration through tissues; in these tissues HCs may associate with other VCAM-1-expressing cells such as macrophages.

In this chapter, therefore, the findings support the notion that the tissue localisation of malignant cells is a multifactorial process. α4β1 is essential to the initial tissue-homing of HCs, but its final distribution depends also on intrinsic activation of the abnormal lymphocyte, its response to locally secreted cytokine, and its specific interactions with adhesive proteins and with other VCAM-1 expressing cells in stroma of tissues.

# Chapter 6

## General Conclusions

### 6.1 Results of the thesis and their interpretation

The experimental work of this thesis explored adhesion processes in CLL and HCL to show how adhesion may influence the tissue distribution of the abnormal lymphoid cells. Although CLL and HCL are both clonal proliferations of mature B-cells, they differ greatly in many respects, especially in their patterns of organ/tissue involvement. It seemed reasonable to postulate that the adhesive properties of the abnormal cells underpin these differences in distribution- a postulate made all the more plausible by recent work from this Department relating integrin/ECM interactions of HCs to organ involvement in HCL (Burthem *et al*, 1994a and 1994b).

The first three experimental chapters were concerned with CLL and investigated adhesion molecule expression, interaction with endothelial ligands, and adhesion and response to extracellular matrix elements respectively.

Expression of adhesion molecules (Chapter 2) varied between cases and did not correlate with *in vivo* clinical disease features or with the degree of activation of the cells. It is not clear why expression varied from case-to-case in this way, but differences in the extent of differentiation of the abnormal clone may be relevant (Dighiero, 1993). These findings indicate that different patterns of clinical disease in CLL are not simply related to adhesion molecule expression. The function of these molecules and the external factors such as cytokines must be important. Chapters 3 and 4 considered some of these issues.

Chapter 3 considered interaction of CLL cells with endothelium. Chapter 2 had already demonstrated that CLL cells possess receptors for endothelial ligands (all cases expressed αLβ2 and L-selectin and any combination of αMβ2, αXβ2, α4β1, α4β7, or CD31). The function of these receptors was therefore explored. Adhesion to HUVEC was generally low, but was greater in cases with a more activated phenotype. Binding involved adhesion to ICAM-1, E-selectin, and possibly other endothelial ligands. This constitutive adherence was enhanced by exogenous B-cell activating agents. Only CLL cases expressing α4 integrins bound well to activated endothelium without exogenous stimulation. These findings indicate that CLL cells have a variable, but sometimes substantial, capacity to interact with endothelium and that this capacity is influenced both by the intrinsic activation state of the CLL clone and by exogenous stimulation ( e.g. by cytokines such as TNFα).

In Chapter 4 CLL cells were shown to bind variably to purified extracellular matrix proteins, especially fibronectin and vitronectin. The CLL cells expressed β1 integrins, and α3β1, α4β1, and α5β1 can all bind to fibronectin. Constitutive binding to fibronectin was correlated with intrinsic activation state, and adhesion increased following *in-vitro* stimulation of CLL cells. Binding to vitronectin was more difficult to explain; the CLL cells did not express the classic vitronectin receptor, αVβ3, although binding to vitronectin could be high. The unidentified vitronectin receptor on the CLL cells did not depend on cell activation in order to bind. CLL cells did not bind to the basement membrane proteins, collagen and laminin. These observations are in keeping with the fact that CLL cells, unlike HCs, do not show any propensity to associate with basement membrane during tissue invasion (Rai, 1993). The significance of the potential of CLL cells to interact with fibronectin and vitronectin is

not clear, but the present findings indicate that ECM has the potential to modify CLL cell behaviour.

Following binding to matrix proteins, CLL cells showed no morphological changes which would indicate a cytoskeletal response. In addition, CLL cells did not migrate under endothelial cells during co-culture experiments. Stimulation of the CLL cells with IL-10 and, to a lesser extent with TNFα, caused pronounced polarity in the adherent CLL cells, and accumulation of CLL cells beneath the endothelium. These findings indicate that CLL cells have the potential for significant migratory responses. However, an external stimulus (e.g. cytokine) is required for this potential to be realised. As will be further considered in Chapter 5, HCs differ markedly in this respect in that they have the capacity to migrate without any obvious additional stimulus; such behaviour is, presumably, related to the nature of their intrinsic activation.

The observation that HCs form close associations with endothelial cells and macrophages *in vivo* led to an investigation, in Chapter 5, into the interactions of hairy-cells with cellular ligands. An important role for α4β1/VCAM-1 ligation was identified. HCs bound efficiently to unstimulated endothelium, and very strongly to stimulated endothelium via α4β1/VCAM-1 interaction. Furthermore, HCs showed a markedly polar (motile) morphology on VCAM-1, and required interaction via VCAM-1 on endothelium in order to migrate under these cells. HCs migrated under other VCAM-1-expressing cell types, such as stromal cells in bone marrow and splenic red pulp- cell types known to form important associations with HCs. VCAM-1 was also found to be expressed at low levels on the HCs themselves. The functional significance of this observation was not established, but is worth further investigation.

All the data of Chapter 5 indicate that interaction of $\alpha 4\beta 1$ on HCs with VCAM-1 can facilitate adhesion to VCAM-1-expressing endothelium, subsequent tissue entry, and then migration within VCAM-1-rich environments. These results, at least in part, provide a basis for the long-known specific interaction of HCs with endothelium and other accessory cell types. Furthermore the data add significantly to previous work from this Department concerning HC adhesion.

## 6.2 Future Directions

The present work suggests at least two important areas for further work. First of all, neither this thesis, nor previous work from this Department have considered the question of tissue remodelling. A general process is clearly important both for the lymphocyte to be able to migrate through the ECM and for the pathogenesis of the various lymphoproliferative disorders (e.g. psuedosinus formation in HCL). It has recently become clear that matrix metalloproteinases are important in these processes and that lymphocytes carry a number of such enzymes. It is likely that investigation of this area of research will be fruitful.

Secondly, localisation of lymphocytes to particular microenvironments will conceivably influence their proliferation and or survival; these processes are central to the pathogenesis of lymphoproliferative disorders. Such survival/proliferation is likely to be controlled by signals induced through adhesion molecules. Investigation of the different signals generated by different integrin receptors is therefore also likely to be a productive area for future study.

# APPENDIX 1

## Materials and Methods

### 1.1 APAAP

**(1) Cytospin preparations**

Purified CLL or Hairy cells were suspended at $1.5x10^5$/ml in RPMI containing 10% FCS. 100µl of suspension was added to buckets of a Shandon *Cytospin 2*. CLL cells were spun at 200 rpm for 3 minutes, HCs at 500 rpm for 5 minutes, both at low acceleration. Slides were air-dried overnight, and CLL preparations used the following day. HCL samples were more robust, and cytospun preparations could be wrapped and stored at -20°C for several months.

**(2) Fixation**

This was usually in acetone-methanol (50:50) for 2 minutes. Once fixed the slides were not allowed to dry, and were wetted with TBS (0.15M NaCl, 0.05M TRIS, pH 7.4).

**(3) Primary mAb**

Optimal concentrations of each individual mAb was determined using immunofluorescence. mAb were diluted in TBS containing 4% human AB serum, and were centrifuged before use to remove Ig complexes. 50µl of mAb was added to the cytospin preparation for 30 minutes at room temperature. Slides were then washed in TBS, pH 7.4.

**(4) Second layer antibody**

50µl rabbit anti-mouse antibody diluted 1:20 in TBS containing 4% human AB serum, 30 minutes. Washed in TBS.

**(5) Tertiary layer APAAP complex**

50µl mouse anti-alkaline phosphatase conjugated to alkaline phosphatase (1:20 in TBS), 30 minutes. Washed in TBS.

**(6) Steps (4) and (5) were repeated** to improve the intensity of staining. Incubation times were reduced to 10 minutes.

**(7) Substrate**

a) 2mg naphthol As-Mx phosphate dissolved in 200µl N,N dimethyl formamide

b) Added 10 ml TRIS/HCl, (0.1M, pH 8.2).

c) Added 10 mg Fast red TR.

d) Added 50 µl 1M levamisole.

e) Substrate was filtered (0.2µm pores) directly onto slide

f) Colour was allowed to develop for up to 15 minutes.

g) Slide was washed in TBS followed by $dH_2O$.

**(8) Counterstain**

Harris haematoxylin was added for 2 minutes, colour developed in running tap water for 10 minutes, then samples were mounted in Apathy's mounting medium.

## 1.2 Immunoprecipitation

**(1) Cell labelling: lactoperoxidase catalysed radio iodination**

Purified CLL cells ($10^8$/ml) were washed and suspended in phosphate buffered saline (PBS: 0.14M NaCl, 2.7mM KCl, 1.5mM $KH_2PO_4$, 8.1mM $Na_2HPO_4$ in sterile $H_2O$, pH 7.4). 1mCi $Na^{125}I$, and final concentrations of 0.02mg/ml lactoperoxidase, and 0.2 IU/ml glucose oxidase were added. The reaction was initiated by addition of 5mM glucose, and allowed to proceed for ten minutes. Reaction was stopped using ice-cold PBS. Cells were then washed four times with cold PBS to remove unbound iodine.

**(2) Preparation of cell lysate**

Cells were lysed in PBS/1% NP40/1% BSA buffer (2ml) plus enzyme inhibitors (PMSF, final conc. 2mM; E-64 (L-trans-Epoxysuccinyl leucylamido (4 guanodino)-butane, final conc. 10uM; Aprotinin - final conc. 10ug/ml) on ice for 30mins.

**(3) Removal of cellular debris**

The lysis mixture was then centrifuged at 13000g to clear the supernatant (for 30mins by microfuge). The supernatant was then dialysed overnight into PBS + 0.05% azide at 4°C to remove free $^{125}I$, small peptides and other small radio labelled products.

**(4) Pre-clearance of sample**

The pre-clearance was extensive. First with protein G sepharose alone (twice) to remove non-specific and immunoglobulin material. Then with protein G

sepharose pre-incubated with purified mouse IgG1 and IgG2b (twice) to remove Ig binding proteins.

**(5) Immunprecipitation**

Test antibodies complexed particular integrins (class-specific control antibodies were also used). Antibody/antigen complexes were then collected using protein G sepharose beads. These beads were then extensively washed using wash buffer (0.5mM LiCl, 100mM Tris pH 8, 0.5% nonidet P 40) to remove non-specifically bound proteins and the immunoprecipitated material was released by boiling the beads in DSSB for analysis by SDS PAGE (see section A.1.8).

## 1.3 Triple-layer Flow Cytometry

This method of preparing samples for flow cytometry has been reported to enhance sensitivity, and thus to detect surface molecules present in low copy number (Zola *et al*, 1990).

**(1) Primary antibody**

An appropriate amount of mAb, previously determined, was added to the bottom of a well of a 96-well tissue-culture plate. 100µl of purified CLL or Hairy cells ($2x10^6$/ml) was added and incubated at room temperature for 10 minutes.

**(3) Washing**

Cells were washed twice by filling the well with phosphate buffered saline (PBS: 0.14M NaCl, 2.7mM KCl, 1.5mM $KH_2PO_4$, 8.1mM $Na_2HPO_4$ in sterile $H_2O$, pH 7.4) containing 1% BSA and 0.1% azide and centrifugation up to 2000 rpm followed by dumping off the supernatant.

**(4) Second layer**

Second layer was 200µg/ml biotin-conjugated horse-anti-mouse mAb (Vector Laboratories, Peterborough, UK) in PBS/BSA/azide, 10 minutes (room temperature). Cells were washed as before

**(5) Third layer**

Third layer was streptavidin conjugated to phycoerythrin (Becton Dickinson, Ltd. used at recommended working dilution). Cells were washed before analysis.

**(6) Analysis**

Cells were diluted to 1ml with FACS fluid and analysed by flow cytometry using Becton Dickinson FACS analyser with Consort 30 computer programme. 5000 events were routinely analysed. The fluorescence marker was set to indicate 2%

positivity for appropriate Ig control samples. As there was a pure population of CLL or HCs, and expression was uniform on the abnormal cells, expression was determined by the percentage of cells to the right of the isotypic control marker following a unimodal shift of the fluorescence peak.

## 1.4 HUVEC culture

Umbilical cords were generously provided by Christiana Hartley Maternity Unit of Southport & Formby D.G.H.. Umbilical veins were washed out using PBS, then endothelial cells detached using 0.7% trypsin/14% EDTA for 8 minutes at 37°C. Detached cells were collected and washed in complete medium (Iscove's modification of Dulbecco's medium, 20% newborn calf serum, 200IU/ml Penicillin, 200μg/ml Streptomycin). HUVEC were resuspended in complete medium, and placed in T25 Falcon tissue culture flasks in an incubator at 37°C, 5% $CO_2$. Medium was replaced after 24hrs, then HUVEC were grown to confluence.

HUVEC were used at first passage; cells were detached using Trypsin/EDTA (Gibco, LTD.), and placed in 96-well plates (Falcon flexible microtitre plates, coated with Promega ECL attachment Matrix), at a dilution of approximately 1:2.5. Immunohistochemistry was used to confirm that these were endothelial cells using antibodies to smooth muscle actin and to factor VIII-related antigen.

## 1.5 Endothelial cell line

A vascular endothelial cell line was generously donated by D. Pumford (Department of Haematology, Royal Liverpool University Hospital). The original source and method of transformation of this cell line was not known. These cells were shown to be endothelial cells morphologically, as well as by expression of smooth muscle actin, factor VIII-related antigen and CD31. These were stored in liquid nitrogen, and cultured in Iscove's modification of Dulbecco's medium containing 10% new-born calf serum, 200 IU/ml penicillin and 200μg/ml Streptomycin. These cells were used at up to eighth passage.

## 1.6 ELISA

A cell ELISA technique was used to identify surface antigens on cells (such as HUVEC, the endothelial cell line, bone marrow stromal cells, and splenic red pulp fibroblasts) cultured in 96-well plates. Secondary and tertiary reagents were supplied as a kit from Sigma, Ltd., and used according to the manufacturer's recommendations. Between each step of the following protocol, wells were washed three times with PBS/1%BSA.

**(1) Cell preparation**

Cells were grown to confluence in the wells. For identification of upregulation of adhesion molecules due to cytokine stimulation, 10U/ml of TNFα was added to wells in complete medium for the appropriate times before the assay.

**(2) Fixation**

The fixative was 4% paraformaldehyde in iso-osmotic buffer (0.13M phosphate buffer, pH 7.4-7.6 containing 30mM glucose. 100μl was added to wells for 5 minutes.

**(3) Endogenous peroxidase**

In some samples, e.g. splenic fibroblasts and bone marrow stromal cells, but not HUVEC, it was necessary to block endogenous peroxidase activity. This was achieved by adding fresh aliquots of 3% $H_2O_2$ to the wells every few minutes. Peroxidase activity caused bubbles of oxygen to form in the wells; when bubbling stopped (2-40 minutes, depending on the tissue sample), the endogenous peroxidase activity had been lost.

**(4) Primary mAb**

Antibodies were diluted according to suppliers' directions in PBS/4% human AB serum/0.1% azide, and microfuged before use. Incubation was at 4°C overnight.

**(5) Second layer**

Biotin-conjugated goat-anti-mouse Ig, diluted 1:1000 in PBS/BSA/azide. Incubated 1hr, room temperature.

**(6) Third layer**

ExtrAvidin-peroxidase (1:400) diluted as above, 30 minutes, room temperature.

**(7) Substrate**

OPD tablets (DAKO Ltd.) dissolved in 0.1M phospho-citrate buffer, pH 5.0 containing 12% $H_2O_2$. 100µl was added to wells and colour was allowed to develop for around 3 minutes, then the reaction was stopped using 100µl 12.5% $H_2SO_4$.

**(8) Analysis**

Absorbances were read at 492nm using a Titertek *Multiscan Plus* plate reader against blank wells containing 100µl substrate and 100µl 12% $H_2SO_4$.

## 1.7 Adhesion assay

This method was developed to compare the ability of CLL and HCs to adhere to various substrata. The technique is a distraction assay, which gives an indication of the strength of adhesion, and does not necessarily identify weak interactions. In order to optimise the washing step, an assay was performed using a range of centripetal forces. A chart was generated showing number of cells remaining adherent against rpm, and a point selected in the middle of the plateau for all subsequent experiments.

**(1) Preparation of wells**

For cell-cell adhesion assays, HUVEC or bone marrow stromal cells were grown to confluence in flat-bottomed 96-well assay plates as described in Appendix 1.4. Alternatively, wells were coated with purified extracellular matrix proteins (20μg/ml in PBS overnight) (Burthem *et al*, 1994a).

**(2) Cell labelling**

Purified CLL cells or HCs ($10^7$ cells in 5ml) were suspended in methionine-free RPMI containing 10% foetal calf serum, 200 IU/ml penicillin and 200μg/ml Streptomycin, and supplemented with 20μCi $^{35}$S-labelled methionine (purchased from Amersham Ltd.). These were incubated overnight at 37°C in a humidified atmosphere of 5% $CO_2$. Cells were used for assay on the same day; and were washed in RPMI containing serum and antibiotics five times, and allowed to recover in this (methionine-containing) medium for at least 1hr.

**(3) Adhesion assay**

Directly before the assay cells were suspended in RPMI without serum at $2x10^6$ cells/ml. 100μL of this suspension was added to the prepared wells. This concentration gave a nearly confluent layer of cells in the well with no overlapping,

and thus theoretically could allow 100% of cells to adhere. Plates were incubated for 10 minutes at 37°C and 5%$CO_2$. Plates were then sealed with Wallac acetate plate-sealers, inverted, and centrifuged at 250g for five minutes. Plate sealers were quickly removed, then most of the medium was gently aspirated and replaced with warm iso-osmotic fixative (4% paraformaldehyde in 0.13M phosphate buffer, pH 7.4-7.6 containing 30mM glucose) for 10 minutes. Next wells were washed and 100μL of scintillant added (Wallac 'Hi-load') and plates were counted in a Wallac 1450 MicroBeta liquid scintillation counter. Adherence was quantitated from the percentage of counts left in test wells compared to the total counts due to $10^5$ cells (100%).

## 1.8 SDS-PAGE

This method was used when analysing the purity of fibronectin isolated from human plasma (A.1.9), and for separating integrin chains immunoprecipitated from CLL cell lysates (A.1.2).

***Reagents:***

**Double Strength Sample Buffer (DSSB).** 20% v/v glycerol; 10% v/w sodium dodecyl sulphate; 25% stacking gel buffer; 0.1mM EDTA; 4.5g bromophenol blue; $ddH_2O$ to 100%.

**Running gel buffer.** 1.5mM Tris-HCL, pH8.8; 4% SDS.

**Running gel.** Acrylamide at 8% (from 30% stock in $ddH_2O$); 25% running gel buffer; 0.1% TEMED; 0.3% ammonium persulphate (fresh); distilled water to make 100%.

**Stacking gel buffer.** 0.5M Tris-HCL, pH6.8; 4% SDS.

**Stacking gel.** 4% acrylamide; 25% stacking gel buffer; 0.1% TEMED; 0.3% APS; $ddH_2O$ to make 100%.

**Electrode buffer.** 1% SDS; 0.2M glycine; 25mM Tris, pH 8.3.

**Coomassie blue stain.** 0.12% w/v brilliant blue; 25% ethanol; 10% acetic acid.

**Destain.** 10% methanol; 10% acetic acid; $ddH_2O$ to make 100%.

***Procedure:***

**(1)** Samples were prepared in Eppendorf tubes by heating to 95°C for 5mins with an equal volume of DSSB. These were then loaded directly onto gels.

**(2)** 16cm gels were cast using the Bio-Rad gel casting system and run using the Bio-Rad Protean II xi Slab Cell system. A 15 well 0.75mm comb allowed a well capacity of up to 120µl for each sample. Sample volumes of 30 to 60 µl were used. A linear 8% gel was employed, which gave good separation in the molecular weight range

30kD to 200kD. Gels were routinely run at a constant voltage of 200V giving a standard run time of 6hrs.

**(3)** Gels were stained for 15 minutes and destained overnight to reveal bands, and then dried under vacuum using a BioRad gel drier. Sigma "high molecular weight markers" were employed, giving a molecular weight series of 205kD, 116kD, 97kD, 66kD, and 29kD. These provided a linear plot using log/normal axes. Molecular weights of sample bands were calculated by reference to these values.

**(4)** Where radiolabelled samples were used these were visualised by autoradiography. Autoradiography film was pre-flashed to linearise response; the film was then placed with the gel in a sealed cassette at -80$^{o}$C. Appropriate exposure times were individually determined.

## 1.9 Purification of fibronectin

FN was purified by gelatin-sepharose affinity chromatography according to the method of Engvall and Ruoslahti, 1977.

**1) Preparation of plasma.**

Fresh frozen plasma unsuitable for human therapeutic use was provided by BTS Liverpool, UK. Serine and metalloproteases were inhibited by the addition of EDTA to a final concentration of 5mM, and PMSF to a final concentration of 1mM. Plasma was stored at –20°C, and before use was thawed and filtered to remove precipitated material which could clog the affinity columns. The filtered plasma was applied immediately to a gelatin sepharose column.

**(2) Preparation of gelatin-sepharose.**

***Principle:***

Cyanogen bromide activated sepharose 4B will bind proteins via free amino groups, immobilising them by a stable covalent linkage. Reactions proceed best in the pH range 8-10 where amino groups are unprotonated; therefore, coupling buffer should not contain free amino groups. The addition of 0.5M sodium chloride minimises non-specific protein/sepharose interaction which may interfere with chromatography. After coupling, the remaining active groups are blocked with excess ethanolamine (a primary amine).

***Method:***

2g of CNBr activated 4B sepharose (giving approx 7ml of swollen gel) were swollen in $dH_2O$ then washed over synthaglass with 400ml of 1mM HCl.

20mg of gelatin were dissolved in coupling buffer (0.1M $NaHCO_3$, pH8.3 and 0.5M NaCl), and this was mixed with the swollen washed gel on a rotatory mixer overnight at 4°C.

Excess protein was washed away with coupling buffer, then free amino groups were blocked with ethanolamine (1M pH 9 for 2 hours at room temperature). Efficiency of coupling was estimated by determining the difference in optical absorbance at 280nm.

The gel was then packed in a column and washed with 4 cycles of alternating pH using coupling buffer (pH 8.3) and acetate buffer 0.1M (pH 4).

**(3) Chromatography.**

Plasma was applied to the column at a rate of 2ml/hr by peristaltic pump. The column was then washed with PBS until optical absorbance at 278nm had returned to its stable baseline. Specific elution of FN was achieved by the application of PBS/4M urea to the column. FN was eluted in the leading front. Purity was determined by SDS PAGE electrophoresis (A1.8).

## 1.10 Image analysis

Glass coverslips were coated with fibronectin or vitronectin as described in the adhesion assay (A.1.7). The coverslips were then transferred to 24-well plates where CLL cells or HCs were allowed to adhere for 5-10 minutes. Non-adherent cells were removed by washing and the remaining cells were cultured for up to 90 minutes in RPMI 1640 medium containing 10% foetal calf serum. Coverslips were then washed and fixed directly by adding 4% glutaraldehyde to the culture medium for 1hr. The coverslips were then washed, air dried, and mounted on glass slides using clear cyanacrylate adhesive. Cells were stained with Harris haematoxylin. This stain was specifically selected for these experiments as it provided a good uniform staining of the cell including fine cytoplasmic processes, was highly visible to camera and image grabbing software, and gave very low background staining.

(4) An image analysis system (PC image, version 2.1, Foster Findlay Associates, UK.) was used to quantify cell morphology automatically, without subjective human interaction. Preparations were examined with an Olympus BH-2 microscope (magnification X400) and an attached JVC TK-1070E video camera. The final image was analysed using a Dell 486D/33 computer.

(5) The preparations were scanned and images of 200 cells were taken for each sample. In PC Image, 2.1, the field of view from the slide was converted into a binary image. A selective deletion was carried out removing non-cellular objects as well as touching or overlapping cells from the binary image. If cells were not stained absolutely evenly throughout the fill procedure was used to fill in the less strongly stained areas. The morphology of each individual cell was then measured. The

parameters which best described cell form in this study were selected from the multiple parameters available. These were:

Area (= $\frac{1}{2}(X_k Y_{k+1} - X_{k+1} Y_k)$) a simple measure of spread cell area

Circularity (= $4\pi$Area/Perimeter$^{2)}$ a measure of the deviation of cell shape from that of a perfect circle (1=circular, 0=straight line)

To give an indication of the polarisation of cells, results were often expressed as area divided by circularity, and this parameter was described as elongation.

# APPENDIX 2

## References


Airas L, Salmi M, Jalkanen S (1993) Lymphocyte-vascular adhesion protein-2 is a novel 70-kDa molecule involved in lymphocyte adhesion to vascular endothelium. *J. Immunol.* **151:** 4228-4238.

Akiyama SK, Yamada SS, Chen W, Yamada KM (1989) Analysis of fibronectin receptor function with monoclonal antibodies: roles in cell adhesion, migration, matrix assembly, and cytoskeletal organisation. *J. Cell Biol.* **109:** 863-875.

Albeda SM, Buck CA (1990) Integrins and other cell adhesion molecules. *FASEB J.* **4:** 2868-2880.

Albeda SM, Muller WA, Buck CA, Newman JJ (1991) Molecular and cellular properties of PECAM-1 (endoCAM/CD31): a novel vascular cell-cell adhesion molecule. *J. Cell Biol.* **114:** 1059-1068.

Alexander E, Henkart P (1976). The adherence of human Fc receptor-bearing lymphocytes to antigen-antibody complexes II. Morphologic alterations induced by the substrate. *J. Exp. Med.* **143:** 329-347.

Anderson AO, Anderson ND, White JD (1982) Lymphocyte locomotion, lymphatic tissues and lymphocyte circulation in the rat. *In "Animal Models of Immunlogical Processes"* (J.B.Hay, Ed.) pp25-95. Academic Press, New York.

Andreeff M, Darzynkiewicz Z, Sharpless TK, Clarkson BD, Melamed MR (1980) Discrimination of human leukemia subtypes by flow cytometric analysis of cellular DNA and RNA. *Blood* **55:** 282.

Andrew DP, Berlin C, Honda S, Yoshino T, Hamann A , Holzmann B, Kilshaw PJ, Butcher EC (1994) Distinct but overlapping epitopes are involved in α4β7-mediated adhesion to vascular cell adhesion molecule-1, mucosal addressin-1, fibronectin, and lymphocyte aggregation. *J Immunol* **153:** 3847-3861.

Aubry J-P, Pochon S, Graber P, Jansen KU, Bonnefoy J-Y (1992) CD21 is a ligand for CD23 and regulates IgE production. *Nature* **358:** 505-507.

Baggiolini M, Dewald B, Moser B (1994) Interleukin-8 and related chemotactic cytokines-CXC and CC chemokines. *Adv. Immunol.* **55:** 97-179.

Baldini L, Cro L, Calori R, Nobili L, Silvestris L, Maiolo AT (1992) Differential expression of very late activation antigen-3 (VLA-3)/VLA-4 in B-cell non-Hodgkin lymphoma and B-cell chronic lymphocytic leukemia. *Blood* **79:** 2688-2693.

Baldini LGM, Cro LM (1994) Structure and function of VLA integrins: differential expression in B-cell leukemia/lymphoma. *Leuk Lymphoma* **12:** 197-203.

Ballard LL, Brown EJ, Holers VM (1991) Expression of the fibronectin receptor VLA-5 is regulated during human B cell differentiation and activation. *Clin. Exp. Immunol.* **84:** 336-346.

Bauer JS, Schreiner CL, Giancotti FG, Ruoslahti E, Juliano RL (1992). Motility of fibronectin receptor deficient cells on fibronectin and vitronectin: collaborative interactions among integrins. *J. Cell Biol.* **116:** 477-487.

Baumhueter S, Singer MS, Henzel W, Hemmerich S, Renz M, Rosen SD, Lasky LA (1993) Binding of L-selectin to the vascular sialomucin CD34. *Science* **262:** 436-438.

Berg EL, McEvoy LM, Berlin C, Bargatze RF, Butcher EC (1993) L-selectin mediated lymphocyte rolling on MAdCAM-1. *Nature (London)* **366:** 695-698.

Berlin CE, Berg EL, Briskin MJ, Andrew DP, Kilshaw PJ, Holzmann B, Weissman IL, Hamann A, Butcher EC (1993) $\alpha 4\beta 7$ integrin mediates lymphocyte binding to the mucosal vascular addressin MAdCAM-1. *Cell* **74:** 185-195.

Berlin C, Bargatze RF, Campbell JJ, von Andrian UH, Szabo MC, Hasslen SR, Nelson RD, Berg EL, Erlandsen SL, Butcher EC (1995) $\alpha 4$ integrins mediate lymphocyte attachment and rolling under physiologic flow. *Cell* **80:** 413-422.

Berneman ZN, Chen Z, Peetermans ME (1990) Morphological evidence for motile behavior by plasma cells. *Leukemia* **4:** 53-59.

Bevilacqua MP, Pober JS, Mendrick DL, Cotran RS, Gimbrone MA (1987) Identification of an inducible endothelial-leukocyte adhesion molecule. *Proc. Natl. Acad. Sci. USA* **84:** 9238-9242.

Bevilacqua M, Butcher EC, Furie B, Furie B, Gallatin M, Gimbrone M, Harlan J, Kishimoto K, Lasky L, McEver R, Paulson J, Rosen S, Seed B, Siegelman M, Springer T, Stoolman L, Tedder T, Varki A, Wagner D, Weissman I, Zimmerman G (1991) Selectins: a family of adhesion receptors. *Cell* **67:** 233.

Binet JL, Auquier A, Dighiero G, (1981) A new prognostic classification of chronic lymphocytic leukemia derived from a multivariate survival analysis. *Cancer* **48:** 198-206.

Binet JL, Catovsky D, Dighiero G, Gale RP, Montserrat E, Rai KR, Rozman C, Sawitsky A (International Workshop on Chronic Lymphocytic Leukemia, 1989) Chronic lymphocytic leukaemia: recommendations for diagnosis, staging, and response criteria. *Ann. Intern. Med.* **110:** 236-238.

Blase L, Daniel PT, Koretz K, Schwartz-Albiez R, Moller P (1995) The capacity of human malignant B-lymphocytes to disseminate in SCID mice is correlated with

functional expression of the fibronectin receptor α5β1 (CD49e/CD29). *Int. J. Cancer.* **60:** 860-866.

Braylan RC, Jaffe ES, Triche TJ, Nanba K, Fowlkes BJ, Metzger H, Frank MMF, Dolan MS, Yee CL, Green I, Berard CW (1978). Structural and functional properties of the "hairy" cells of leukaemic reticuloendotheliosis. *Cancer* **41:** 210-227.

Buckley RH (1993) Primary immunodeficiency diseases. *In* "Fundamental Immunology" (W.E.Paul, Ed.) 3rd ed. Pp.1353-1374. Raven Press, New York.

Burke JS (1978). The value of the bone marrow biopsy in the diagnosis of hairy cell leukaemia. *Am. J. Clin. Pathol.* **70:** 876-884.

Burns GF, Cawley JC, Worman CP, Hayhoe FG (1978). Multiple heavy chain isotypes on the surface of the cells of hairy-cell leukaemia. *Blood* **52:** 1132-1147.

Burthem J, Baker PK, Hunt JA, Cawley JC (1994a) Hairy cell interactions with extracellular matrix: Expression of integrin receptors and their role in the cell's response to specific adhesive proteins. *Blood* **84:** 873-882.

Burthem J, Baker PK, Hunt J, Cawley JC (1994b) The function of *c-fms* in hairy cell leukaemia: M-CSF stimulates hairy cell movement. *Blood* **83:** 1381-1389.

Burton GF, Kupp LI, McNalley EC, Tew JG (1995) Follicular dendritic cells and B cell chemotaxis. *Eur. J. Immunol.* **25:** 1105-1108.

Butcher EC (1991) Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. *Cell* **67:** 1033-1036.

Caligaris-Cappio FG, Bergui L, Corbascio G, Tousco F, Marchisio PC (1986). Cytoskeleton organisation is aberrantly rearranged in the cells of B chronic lymphocytic leukemia and hairy cell leukemia. *Blood* **67:** 233-239.

Caligaris-Cappio FG, Riva M, Tesio L, Shena M, Gaidano GL, Bergui L (1989) Human normal CD5-positive B cells can be induced to differentiate to CD5-negative B cells with germinal centre cell features. *Blood* **73:** 1259.

Camerini D, James SP, Stamenkovic I, Seed B (1989) Leu-8/TQ1 is the human equivalent of the Mel-14 lymph node homing receptor. *Nature* **342:** 78-82.

Cawley JC, and Burthem J (1992) Hairy cell leukaemia. In: Leukaemia Ed. J.A.Whittaker chap 21, 495.

Cepek KL, Shaw SK, Parker CM, Russel GJ, Morrow JS, Rimm DL, Brenner MB (1994) Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and an integrin αEβ7. *Nature* **372:** 190-193.

Cerf-Bensussan N, Begue B, Gagnon J, Meo T (1992) The human intraepithelial lymphocyte marker HML-1 is an integrin consisting of a β7 subunit associated with a distinctive α chain. *Eur. J. Immunol.* **22:** 273-278.

Chadburn A, Inghirami G, Knowles DM (1990) Hairy cell leukaemia associated antigen Leu M5 (CD 11c) is preferentially expressed by benign activated neoplastic T-cells. *Am. J. Path.* **136:** 29-37.

Chan PY, Aruffo A (1993) VLA-4 integrin mediates lymphocyte migration on the inducible endothelial cell ligand VCAM-1 and the extracellular matrix ligand fibronectin. *J. Biol. Chem.* **268:** 24655-24664.

Chuluyan HE, Osborn L, Lobb R, Issekutz AC (1995) Domains 1 and 4 of vascular cell adhesion molecule-1 (CD106) both support very late activation antigen-4 (CD49d/CD29)-dependent monocyte transendothelial migration. *J. Immunol.* **155:** 3135-3144.

Clark EA, Lane PJL (1991) Regulation of human B-cell activation and adhesion. *Annu. Rev. Immunl.* **9:** 97-127.

Csanaky G, Kalasz V, Keleyi G, Losonczy H, Baliko Z, Toth A (1993) Expression of an adhesion molecule and homing in B-cell chronic lymphocytic leukaemia: I. Application of the HEV-binding assay to a clinical series. *Med Oncol Tumor Pharmacother* **10:** 125-129.

Csanaky G, Vass JA, Milosevits J, Ocsovszki I, Szomor A, Schmelczer M (1994) Expression and function of L-selectin molecules (Lecam-1) in B-cell chronic lymphocytic leukemia. *Haematologica* **79:** 132-136.

Csanaky G (1994) High endothelial venules and cell adhesion molecules in B-cell chronic lymphocytic leukaemia and related low grade B-cell lymphoma/leukaemia: II. Expression of cell adhesion molecules in lymph nodes biopsies. *Histol. Histopath.* **9:** 577-582.

Cybulsky MI, Fires JWU, Williams AJ, Sultan P, Eddy R, Byers M, Shows T, Gimbrone MA, Collins T (1991) Gene structure, chromosomal location and basis for alternative mRNA splicing of the human VCAM-1 gene. *Proc. Natl. Acad. Sci.* (Wash.) **88:** 7859-7863.

Cyster JG, Williams AF (1992) The importance of cross-linking in the homotypic aggregation of lymphocyte induced by antileukosialin (CD43) antibodies. *Eur. J. Immunol.* **22:** 2565-2572.

Dameshek W (1967) Chronic lymphocytic leukemia-an accumulative disease of immunologically incompetent lymphocytes. *Blood* **29:** 566-584.

Davies JM, St.John J, Cheung HT (1990). Haptotactic activity of fibronectin on lymphocyte migration in vitro. *Cell. Immunol.* **129:** 67-79.

Defilippi P, Truffa G, Stefanuto G, Altruda F, Silengo L, Tarone G (1991) TNFα and IFNγ modulate expression of the vitronectin receptor (integrin β3) in human endothelial cells. *J. Biol. Chem.* **266:** 7638-7645.

Defilippi P, Silengo L, Tarone G (1992) α6β1 integrin (laminin receptor) is down regulated by TNFα and IL-1β in human endothelial cells. *J. Biol. Chem.* **267:** 18303-18307.

Defrance T, Aubry JP, Rousset F, Vanbervliet B, Bonnefoy JY, Arai N, Takebe Y, Yokota T, Lee F, Arai K, DeVries J, Banchereau J (1987) Human recombinant interleukin 4 induces Fcε receptors (CD23) on normal human B lymphocytes. *J. Exp. Med* **165:** 1459-1467.

Delsepesse G, Sarfati M, Wu CY, Fournier S, Letellier M (1992) The low affinity receptor for IgE. *Immunol. Rev.* **125:** 77-97.

DeLisser HM, Yan HC, Newman PJ, Muller WA, Buck CA Albeda SM (1993) PECAM-1 (CD31)-mediated cellular aggregation involves cell surface glycosaminoglycans. *J. Biol. Chem.* **268:** 16037-16046.

De Rossi G, Zarcone D, Mauro F, Cerruti G, Tenca C, Puccetti A, Mandelli F, Grossi CE (1993) Adhesion molecule expression on B-cell chronic lymphocytic leukemia cells: malignant cell phenotypes define distinct disease subsets. *Blood* **81:** 2679-

De Rossi G, Tenca C, Cerruti G, Favre A, Zarcone D, Tabilio A, Mauro FR, Annino L, Grossi CE (1994) Adhesion molecule expression on B-cells from acute and chronic lymphoid leukemias. *Leuk. Lymphoma* **16:** 31-36.

Diamond MS, Staunton DE, Stacker FA, Garcia AJ, Hibbs ML, Springer TA (1990) ICAM-1 (CD54): a counter receptor for Mac-1 (CD11b/CD18). *J. Cell Biol.* **111:** 3129-3139.

Dighiero G, Travade Ph, Chevret S, Fenaux P, Chastang C, Binet JL (French Co-operative Group on CLL (1991) B-cell chronic lymphocytic leukemia: present status and future directions. *Blood* **78:** 1901-1914.

Dighiero G (1993) Biology of the neoplastic lymphocyte. *Bailliere's Clin. Haem.* **6:** 807-820.

Dougherty GJ, Cooper DL, Memory JF, Chiu RK (1994) Ligand binding specificity of alternatively spliced CD44 isoforms. *J. Biol. Chem.* **269:** 9074-9078.

Duijvestijn A, Hamann A (1989) Mechanisms and regulation of lymphocyte migration. *Immunol. Today* **10:** 23-28.

Durum SK, Oppenheim JJ (1993) Proinflammatory cytokines and immunity. *In* "Fundamental Immunology" (W.E.Paul, Ed.), 3rd ed. Pp.801-835.. Raven Press, New York.

Dustin ML, Rothlein R, Bhan AK, Dinarello CA, Springer TA (1986) Induction by IL-1 and interferon-gamma: tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1). *J. Immunol.* **137:** 245-254.

Ebert EC (1995) Human intestinal intraepithelial lymphocytes have potent chemotactic activity. *Gastroenterol.* **109:** 1154-1159.

Elgabalawy H, Gallatin M, Vazeux R, Peterman G, Wilkins J (1994) Expression of ICAM-R (ICAM-3), a novel counter-receptor for LFA-1 in rheumatoid and nonrheumatoid synovium-comparison with other adhesion molecules. *Arthritis and Rheumatism.* **37:** 846-854.

Elenstrom-Magnusson C, Chen WC, Clinchy B, Obrink B, Severinson E (1995) IL-4-induced B cell migration involves transient interactions between beta 1 integrins and extracellular matrix components. *Internat. Immunol.* **7:** 567-573.

Elices MJ, Osborn L, Takada Y, Crouse C, Luhowskyj S, Hemler ME, Lobb RR (1990) VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. *Cell* **60:** 577-584.

Engvall E, Ruoslahti E. (1977) Binding of the soluble form of fibroblast surface protein, fibronectin, to collagen. *Int J Cancer* **20:** 1-5.

Engel P, Nojima Y, Rothstein D, Zhou LJ, Wilson GL, Kehrl JH, Tedder TF (1993) The same epitope on CD22 of B lymphocytes mediates the adhesion of erythrocytes, T and B lymphocytes, neutrophils, and monocytes. *J. Immunol.* **150:** 4719-4732.

Erle DJ, Briskin MJ, Butcher EC, Garcia-Pardo A, Lazarovits AI, Tidswell M (1994) Expression and function of the MAdCAM-1 receptor, integrin α4β7, on human leukocytes. *J Immunol* **153:** 517-528.

Flores-Romo L, Wallace L, Gordon J. (1989) Evidence that CD23 and CD39 can function as accessory molecules in antigen presentation by B lymphocytes. *Leucocyte typing* IV. *White cell differentiation antigens.* Oxford: Oxford University. pp77-79.

Foon KA, Rai KR, and Gale RP (1990) Chronic lymphocytic leukemia: new insights into biology and therapy. *Annals of Internal Medicine* **113:** 525-539.

de Fougerolles AR, Stacker SA, Schwarting R, Springer TA (1991) Characterization of ICAM-2 and evidence for a third counter-receptor for LFA-1. *J. Exp. Med.* **174:** 253-267.

de Fougerolles AR, Klickstein LB, Springer T (1993) Cloning and expression of intercellular adhesion molecule 3 reveals strong homology to other immunoglobulin family receptors for lymphocyte function-associated antigen 1. *J. Exp. Med.* **177:** 1187-1192.

de Fougerolles AR, Quin X, Springer TA (1994) Characterization of the function of intercellular adhesion molecules (ICAM)-3 and comparison with ICAM-1 and ICAM-2 in immune responses. *J. Exp. Med.* **179:** 619-629.

Fournier S, Delespesse G, Rubio M, Biron G, Sarfati M (1992) CD23 antigen regulation and signalling in chronic lymphocytic leukemia. *J. Clin. Invest.* **89:** 1312-1321.

Fournier S, Rubio M, Delespesse G, Sarfati M (1994) Role for low-affinity receptor for IgE (CD23) in normal and leukemic B-cell proliferation. *Blood* **84:** 1881-1886.

Freedman AS (1990) Immunobiology of chronic lymphocytic leukaemia. *Hematology* **4:** 405-429.

Freedman AS, Munro JM, Rice GE, Bevilacqua MP, Morimoto C, McIntyre BW, Rhynart K, Pober JS, Nadler LM (1990) Adhesion of human B cells to germinal centres in vitro involves VLA-4 and INCAM-110. *Science* **249:** 1030-1032.

Freedman AS, and Nadler LM (1993) Immunologic markers in B-cell chronic lymphocytic leukaemia. In Cheson BD (ed.) *Chronic Lymphocytic Leukemia: Scientific Advances and Clinical Developments,* pp 1-32. New York: Marcel Dekker.

Freeman GJ, Freedman AS, Segil JM, Lee G, Whitman JF, Nadler LM (1989) B7, a new member of the Ig superfamily with unique expression on activated and neoplastic B cells. *J. Immunol.* **143:** 2714-2722.

Fries JW, Williams AJ, Atkins RC, Newman W, Lipscomb MF, Collins T (1993) Expression of VCAM-1 and E-selectin in an in vivo model of endothelial activation. *Am. J. Pathol.* **143:** 725-737.

Gale PG, Foon KA (1985) Chronic lymphocytic leukemia: recent advances in biology and treatment. *Ann Intern. Med.* **103:** 101.

Geng JG, Bevilacqua MP, Moore KL, McIntyre TM, Prescott SM, Kim JM, Bliss GA, Zimmerman GA, McEver RP (1990) Rapid neutrophil adhesion to activated endothelium mediated by GMP-140. *Nature* **343:** 757-760.

Gold MR, DeFranco AL (1994) Biochemistry of B lymphocyte activation. *Adv. Immunol.* **55:** 221-295.

Gordon J (1984). Molecular aspects of immunoglobulin expression by human B cell lymphomas. *Adv. Cancer Res.* **41:** 71-154.

Gray D (1992) The dynamics of immunological memory. *Sem. Immunol.* **4:** 29-34.

Griffiths SD, Cawley JC (1991). Monocyte/macrophages stimulate hairy cell proliferation. *Leuk. Lymph.* **4:** 325-330.

Hall JG, Morris B (1965) The origin of cells in the efferent lymph from a single lymph node. *J. Exp. Med.* **121:** 901-910.

Han T, Ozer H, Bloom M, Sagawa K, Minowada J (1982) The presence of monoclonal cytoplasmic immunoglobulins in leukemic B cells from patients with chronic lymphocytic leukemia. *Blood* **59:** 435-438.

Hanson CA, Gribbin TE, Schnitzer B, Schlegelmilch JA, Mitchell BS, Stoolman LM (1990) CD11c (LEU-M5) expression characterizes a B-cell chronic lymphoproliferative disorder with features of both chronic lymphocytic leukemia and hairy cell leukemia. *Blood.* **76:** 2360-2367.

Hemler ME (1988) Adhesive protein receptors on hematopoietic cells. *Immunol. Today* **9:** 109-113.

Hemler ME (1990) VLA proteins in the integrin family: structures, functions, and their role on leukocytes. *Annu. Rev. Immunol.* **8:** 365-400.

Hemler ME, Elices MJ, Parker C, Takada Y (1990) Structure of the integrin VLA-4 and its cell-cell and cell-matrix adhesion functions. *Immunol. Rev.* **114:** 45-65.

Hession C, Tizard R, Vassallo C, Schiffer SB, Goff D, Moy P, Chi-Rosso G, Luhowskyj S, Lobb R, Osborn L (1991) Cloning of an alternative form of vascular cell adhesion molecule-1 (VCAM-1) *J. Biol. Chem.* **266:** 6682-6685.

Hession C, Moy P, Tizard R, Chisholm P, Williams C, Wysk M, Burkly L, Miyake K, Kincade P, Lobb R (1992) Cloning of murine and rat vascular cell adhesion molecule-1. *Biochem. Biophys. Res. Commun.* **183:** 163-169.

Holzmann B, McIntyre BW, Weissman IL (1989) Identification of a murine Peyer's patch-specific lymphocyte homing receptor as an integrin molecule with an alpha chain homologous to human VLA-4 alpha. *Cell* **56:** 37-46.

Hynes RO (1987) Integrins: a family of cell surface receptors. *Cell* **48:** 549-554.

Hynes RO (1992) Integrins: versatility, modulation, and signalling in cell adhesion. *Cell* **69:** 11-25.

Ikuta K, Uchida N, Friedman J, Weissman IL (1992) Lymphocyte development from stem cells. *Annu. Rev. Immunol.* **10:** 759-783.

Imai Y, Singer MS, Fennie C, Lasky LA, Rosen SD (1991) Identification of a carbohydrate-based endothelial ligand for a lymphocyte homing receptor. *J. Cell. Biol.* **113:** 1213-1221.

Jalkanen S, Bargatze RF, del los Toyos J, Butcher EC (1987) Lymphocyte recognition of high endothelium: antibodies to distinct epitopes of an 85-95kD glycoprotein antigen differentially inhibit lymphocyte binding to lymph node, mucosal or synovial endothelial cells. *J. Cell Biol.* **105:** 983-990.

Jalkanen S, Jalkanen M (1992) Lymphocyte CD44 binds the COOH-terminal heparin-binding domain of fibronectin. *J. Cell Biol.* **116:** 817-825.

Jondal M, Wigzell H, Aiuti F (1973) Human lymphocyte subpopulations: classification according to surface markers and/or functional characteristics. *Transplant Rev.* **16:** 163-195.

Jones DA, Abbassi O, McIntire LV, McEver RP, Smith CW (1993) P-selectin mediates neutrophil rolling on histamine-stimulated endothelial cells. *Biophys. J.* **65:** 1560-1569.

Jumper MD, Nishioka Y, Davis LS, Lipsky PE, Meek K (1995) Regulation of human B cell function by recombinant CD40 ligand and other TNF-related ligands. *J. Immunol.* **155:** 2369-2378.

Jutila MA (1994) Function and regulation of leukocyte homing receptors. *J. Leukoc. Biol.* **55:** 133-140.

Kapiotis S, Quehenberger P, Sengoelge G, Partan C, Eher R, Strobl H, Bevec D, Zapolska D, Schwarzinger I, Soeiser W (1994) Modulation of pyrogen-induced upregulation of endothelial cell adhesion molecules (CAMs) by interleukin-4: transcriptional mechanisms and CAM-shedding. *Ciculatory Shock* **43:** 18-25.

Katayama I., Schneider GB (1977). Further ultrastructural characterization of hairy cells of leukemic reticuloendotheliosis. *Am. J. Pathol.* **86:** 163-182.

al Katib A, Wang CY, McKenzie S, Clarkson BD, Koziner B (1992) Phorbol ester-induced hairy cell features on chronic lymphocytic leukemia cells in vitro. *Am. J. Haematol.* **40:** 264-269.

Keizer GD, Borst J, Visser W, Schwarting R, de Vries JE, Figdor CG (1987) Membrane glycoprotein p150,95 of human cytotoxic T cell clone is involved in conjugate formation with target cells. *J. Immunol.* **138:** 3130-3136.

Keller H, Niggli V, Zimmerman A (1992). Diversity in motile responses of human neutrophil granulocytes: functional meaning and cytoskeletal basis. *Adv. Exp. Med. Biol.* **297:** 23-37.

Kimby E, Rincon J, Pattarroyo M, Mellstedt H (1994) Expression of adhesion molecules CD11/CD18 (LeuCAMs, β2-integrins), CD54 (ICAM-1) and CD58 (LFA-3) in B-chronic lymphocytic leukemia. *Leuk. Lymphoma* **13:** 297-306.

Kinashi T, St.Pierre Y, Springer TA (1995) Expression of glycophosphatidylinositol-anchored and -non-anchored isoforms of vascular cell adhesion molecule 1 in murine stromal and endothelial cells. *J. Leukoc. Biol.* **57:** 168-173.

Kishimoto TK, Jutila MA, Butcher EC (1990) Identification of a human peripheral lymph node homing receptor: a rapidly down-regulated adhesion molecule. *Proc. Natl. Acad. Sci. USA* **87:** 2244-2248.

Knapp W, Dorken B, Gilks WR, Reiber EP, Schmidt RE, Stein H, von dem Borne AEGK, eds (1989) Leukocyte typing IV. White cell differentiation antigens. Oxford, UK, Oxford University Press.

van Kooyk Y, Weder P, Hogervorst F, Verhoeven AJ, van Seventer G, te Velde AA, Borst J, Keister GD, Figdor CG (1991) Activation of LFA-1 through a $Ca^{2+}$-dependent epitope stimulates lymphocyte adhesion. *J. Cell Biol.* **112:** 345-354.

Kosco MH, Pflugfelder E, Gray D (1992) Follicular dendritic cell-dependent adhesion and proliferation of B cells in vitro. *J. Immunol.* **148:** 2331-2339.

Kovach NL, Carlos TM, Yee E, Harlan JM (1992) A monoclonal antibody to beta 1 integrin (CD29) stimulated VLA-dependent adherence of leukocytes to human umbilical vein endothelial cells and matrix components. *J. Cell Biol.* **116:** 499-509.

Kurzinger K, Reynolds T, Germain RN, Davignon D, Martz E, Springer TA (1981) A novel lymphocyte function-associated antigen (LFA-1): cellular distribution, quantitative expression, and structure. *J. Immunol.* **127:** 596-602.

Lampert I, Catovsky D, Marsh GW, Child JA, Galton DA (1980) The histopathology of prolymphocytic leukemia with particular reference to the spleen. A comparison with chronic lymphocytic leukemia. *Histopathology* **4:** 3-19.

Lampert IA (1983) Splenectomy as a diagnostic technique. *Clin. Haematol.* **12:** 535-563.

Larkin M, Ahern TJ, Stoll MS, Shaffer M, Sako D, O'Brien J, Yuen CT, Lawson AM, Childs RA, Barone KM, Langer-Safer PR, Hasegawa A, Kiso M, Larsen GR, Feizi T (1992) Spectrum of sialylated and nonsialylated fuco-oligosaccharides bound by the endothelial-leukocyte adhesion molecule E-selectin. *J. Biol. Chem.* **267:** 13661-13668.

Larsen E, Palabrica T, Sajer S, Gilbert GE, Wagner DD, Furie BC, Furie B (1990) PADGEM-dependent adhesion of platelets to monocytes and neutrophils is mediated by lineage-specific carbohydrate, LNF III (CD15). *Cell* **63:** 467-474.

Larson RS, Corbi AL, Berman L, Springer T (1989) Primary structure of the leukocyte function-associated molecule-α subunit: an integrin with an embedded domain defining a protein superfamily. *J. Cell Biol.* **108:** 703-712.

Larson RS, Springer TA (1990) Structure and function of leukocyte integrins. *Immunol. Rev.* **114:** 181-217.

Larsson LG, Gray HE, Totterman T, Pettersson U, Nilsson K (1987) Drastically increased expression of MYC and FOS protooncogenes during in vitro differentiation of chronic lymphocytic leukemia cells. *Proc. Natl. Acad. Sci. USA* **84:** 223.

Lasky LA (1992) Selectins: interpreters of cell-specific carbohydrate information during inflammation. *Science* **258:** 964-969.

Lasky LA, Singer MS, Dowbenko D, Imai Y, Henzel WJ, Grimley C, Fennie C, Gillett N, Watson SR, Rosen SD (1992) An endothelial ligand for L-selectin is a novel mucin-like molecule. *Cell* **69:** 927-938.

Lenter M, Vestweber V (1994) The integrin chains β1 and α6 associate with the chaperone calnexin prior to integrin assembly. *J. Biol. Chem.* **269:** 12263-12268.

Lenter M, Levinovitz A, Isenmann S, Vestveber D, (1994) Monospecific and common glycoprotein ligands for E- and P-selectin on myeloid cells. *J. Cell Biol.* **125:** 471-481.

Levinovitz A, Muhlhoff J, Isenmann S, Vestweber D. (1993) Identification of a glycoprotein ligand for E-selectin on mouse myeloid cells. *J. Cell Biol.* **121:** 449-459.

Li YY, Cheung HT (1992) Basement membrane and its components on lymphocyte adhesion, migration, and proliferation. *J. Immunol.* **149:** 3174-3181.

Lipshutz MD, Mir R, Rai KR, Sawitsky A (1980) Bone marrow biopsy and clinical staging in chronic lymphocytic leukemia. *Cancer* **46:** 1422-1427.

Lorant DE, Topham MK, Whatley RE, McEver RP, McIntyre TM, Prescott SM, Zimmerman GA (1993) Inflammatory roles of P-selectin. *J. Clin. Invest.* **92:** 559-570.

Mackay CR, Marston WL, Dudler L (1990) Naive and memory T cells show distinct pathways of lymphocyte recirculation. *J. Exp. Med.* **171:** 801-817.

Mackay CR, Imhof BA (1993) Cell adhesion in the immune system. *Immunol. Today* **14:** 99-102.

Marlin SD, Springer TA (1987) Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1). *Cell* **51:** 813-819.

Masinovsky B, Urdal D, Gallatin WM (1990) IL-4 acts synergistically with IL-1β to promote lymphocyte adhesion to microvascular endothelium by induction of vascular cell adhesion molecule-1. *J. Immunol.* **145:** 2886-2895.

Masumoto A, Hemler ME (1993) Mutation of putative divalent cation sites in the α4 subunit of the integrin VLA-4: distinct effects on adhesion to CS1/fibronectin, VCAM-1, and invasin. *J. Cell Biol.* **123:** 245-253.

Mauro FR, De Rossi G, Burgio VL, Caruso R, Giannarelli D, Monarca B, Romani C, Baroni CD, Mandelli F (1994) Prognostic value of bone marrow histology in chronic lymphocytic leukemia. A study of 335 untreated cases from a single institution. *Haematologica* **79:** 334-341.

McEver RP (1992) Leukocyte-endothelial cell interactions. *Curr. Opinion Cell Biol.* **4:** 840-849.

Merle-Beral H. (1990) A further characterization of B-CLL lymphocytes: phenotypic and functional studies. *Nouv-Rev-Fr-Hematol,* **32:** 319-321.

Micklem KJ, Sim RB (1985) Isolation of complement-fragment-iC3b-binding proteins by affinity chromatography: the identification of p150,95 as an iC3b-binding protein. *Biochem. J.* **231:** 233-236.

Miller LJ, Schwarting R, Springer TA (1986) Regulated expression of the Mac-1, LFA-1, p150,95 glycoprotein family during leukocyte differentiation. *J. Immunol.* **137:** 2891-2900.

Miyake K, Weissman IL, Greenberger JS, Kincade PW (1991) Evidence for a role of the integrin VLA-4 in lympho-hemopoiesis. *J. Exp. Med.* **173:** 599-607.

Miyake K, Median K, Ishihara K, Kimoto M, Auerback R, Kincade PW (1992) A VCAM-like adhesion molecule on murine bone marrow stromal cells mediates binding of lymphocyte precursors in culture. *J. Cell Biol.* **114:** 557-565.

Moller P, Eichelmann A, Koretz K, Mechtersheimer G (1992) Adhesion molecules VLA-1 to VLA-6 define discrete stages of peripheral B lymphocyte development and characterize different types of B cell neoplasia. *Leukemia* **6:** 256-264.

Molossi S, Elices M, Arrhenius T, Rabinovitch M (1995) Lymphocyte transendothelial migration toward smooth muscle cells in interleukin-1 beta-stimulated co-cultures is related to fibronectin interactions with $\alpha 4\beta 1$ and $\alpha 5\beta 1$. *J. Cellular Physiol.* **164:** 620-633.

Montserrat E, Rozman C (1987) Clinicopathologic staging of chronic lymphocytic leukemia. In Gale KR (eds) *Chronic lymphocytic leukemia: recent progress and future directions,* pp 215-224. New York: Alan R. Liss.

Moore KL, Stults NL, Diaz S, Smith DF, Cummings RD, Varki A, McEver RP (1992) Identification of a specific glycoprotein ligand for P-selectin (CD62) on myeloid cells. *J. Cell. Biol.* **118:** 445-456.

Moy P, Lobb R, Tizard R, Olson D, Hession C (1993) Cloning of an inflammation-specific phosphatidyl inositol-linked form of murine vascular cell adhesion molecule-1. *J. Biol. Chem.* **268:** 8835-8841.

Muller WA, Berman ME, Newman PJ, DeLisser HM, Albeda SM (1992) A heterophilic adhesion mechanism for platelet/endothelial cell adhesion molecule 1 (CD31). *J. Exp. Med.* **175:** 1401-1404.

Muller WA, Weigl SA, Deng X, Phillips DM (1993) PECAM-1 is required for transendothelial migration of leukocytes. *J. Exp. Med.* **178:** 449-460.

Mulligan SP, Trevada P, Malutes E (1990). B-Ly-7 a monoclonal antibody reactive with hairy cell leukemia also defines an activation antigen on normal CD8+ T-cells. *Blood* **76:** 959-964.

Nagafuchi A, Shirayoshi Y, Okazaki K, Yasuda K, Takeichi M (1987) Transformation of cell adhesion properties by exogenously introduced E-cadherin cDNA. *Nature* **329:** 341-343.

Nakamura N, Suzuki S, Segami H, Nozawa Y, Tominaga K, Wachi E, Hojo H, Abe M, Sakuma H, Wakasa H (1994) Clinicopathological and immunophenotypic studies in 12 cases with B cell chronic lymphocytic leukemia. *Pathol. Internat.* **44:** 779-784.

Nanba K, Soban EJ, Bowling MC, Berard CW (1977a) Splenic pseudosinuses and hepatic angiomatous lesions: distinctive features on hairy cell leukaemia. *Am. J. Clin. Pathol.* **67:** 415-426.

Nanba K, Jaffe ES, Soban EJ, Braylan RC, Berard CW (1977b) Hairy cell leukemia. Enzyme histochemical characterisation, with special reference to splenic stromal changes. *Cancer* **39:** 2323-2336.

Newman PJ, Berndt MC, Gorski J, White GC, Lyman S, Paddock C, Muller WA (1990) PECAM-1 (CD31) cloning and regulation to adhesion molecules of the immunoglobulin gene superfamily. *Science* **247:** 1219-1222.

Newman PJ, Hillery CA, Albrecht R, Parise LV, Berndt MC, Mazurov AV, Dunlop LC Zhang J, Rittenhouse SE (1992) Activation-dependent changes in human platelet PECAM-1: phosphorylation, cytoskeletal association, and surface membrane redistribution. *J. Cell Biol.* **119:** 239-246.

Norgard KE, Moore KL, Diaz S, Stults NL, Ushiyama S, McEver RP (1993) Characterization of a specific ligand for P-selectin on myeloid cells. A minor glycoprotein with sialylated O-linked oligosaccharides. *J. Biol. Chem.* **268:** 12764-12774.

Norgard-Sumnicht KE, Varki NM, Varki A. (1993) Calcium-dependent heparin-like ligands for L-selectin in nonlymphoid endothelial cells. *Science* **261:** 480-483.

Nortamo P, Li R, Renkonen R, Timonen T, Prieto J, Patarroyo M, Gahmberg CG (1991) The expression of human intercellular adhesion molecule-2 is refractory to inflammatory cytokines. *Eur. J. Immunol.* **21:** 2629-2632.

Osborn L, Hession C, Tizard R, Vassallo C, Luhowskyj S, Chi Rosso G, Lobb R (1989) Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. *Cell* **59:** 1203-1211.

Pabst R, Binns RM, Rothkotter HJ, Westermann J (1993) Quantitative analysis of lymphocyte fluxes *in vivo*. *Cur. Topics Microbiol. Immunol.* **184:** 151-159.

Paloczi K, Demeter J, Hokland P, Petranyi Gy. (1990) Expression of differentiation and activation antigens on peripheral blood mononuclear cells in chronic lymphocytic leukaemia (CLL) and in hairy cell leukaemia (HCL). *Eur. J. Haematol.* **45:** 285-286.

Peach RJ, Hollenbaugh D, Stamenkovic I, Aruffo A (1993) Identification of hyaluronic acid binding sites in the extracellular domain of CD44. *J. Cell Biol.* **122:** 257-264.

Piali L, Albeda SM, Baldwin HS, Hammel P, Gisler RH, Imhof BA (1993) Murine platelet endothelial cell adhesion molecules (PECAM-1)/CD31 modulates β2 integrins on lymphokine-activated killer cells. *Eur. J. Immunol.* **23:** 2464-2471.

Piali L, Hammel P, Uherek C, Bachmann F, Gisler RH, Dunon D, Imhof BA (1995) CD31/PECAM-1 is a ligand for αVβ3 integrin involved in adhesion of leukocytes to endothelium. *J. Cell Biol.* **130:** 451-460.

Pigott R, Dillon LP, Hemingway IH, Gearing AJH (1992) Soluble forms of E-selectin, ICAM-1 and VCAM-1 are present in the supernatants of cytokine activated cultured endothelial cells. *Biochem. Biophys. Res. Commun.* **187:** 584-589.

Pilon VA, Davey FR, Gerald GB (1981) Splenic alterations in hairy cell leukemia. *Arch. Pathol. Lab. Med.* **105:** 577-581.

Pilon VA, Davey FR, Gordon GB, Jones DB (1982) Splenic alterations in hairy-cell leukemia: II. An electron microscopic study. *Cancer* **49:** 1617-1623.

Pohlman TH, Munford RS, Harlan JM (1987) Deacylated lipopolysaccharide inhibits neutrophil adherence to endothelium induced by lipopolysaccharide in vitro. *J. Exp. Med.* **165:** 1393-1402.

Polte T, Newman W, Gopal TV (1990) Full length vascular cell adhesion molecule-1. *Nucl. Acid Res.* **18:** 5901.

Posnett DN, Duggan A, McGrath H (1990) Hairy-cell leukaemia-associated antigen (HC-2) is an activation antigen of several haemopoetic cell lineages, inducible on monocytes by IFN α. *J. Immunol.* **144:** 929-933.

Postigio AA, Corbi AL, Sanchez-Madrid, de Landazuri O (1991a) Regulated expression and function of CD11c/CD18 integrin on human B lymphocytes. Relation between attachment to fibrinogen and triggering of proliferation through CD11c/CD18. *J. Exp. Med.* **174:** 1313-1322.

Postigio AA, Pulido R, Campanero MR, Acevedo A, Garcia-Pardo A, Corbi AL , Sanchez-Madrid F, De-Landazuri MO (1991b) Differential expression of VLA-4 integrin by resident and peripheral blood receptors upon B cell activation. *Eur. J. Immunol.* **21:** 2437-2445.

Preud'homme JL, Seligman M (1974) Surface immunoglobulins on human lymphoid cells. *Prog. Clin. Immunol.* **2:** 121.

Rai K (1993) Progress in chronic lymphocytic leukaemia: a historical perspective. *Bailliere's Clin Haem* **6:** 757.

Rai KR and Sawitsky A, Cronkite EP (1975) Clinical staging of chronic lymphocytic leukemia. *Blood* **46:** 219-234.

Randolph GJ, Furie MB (1995) A soluble gradient of endogenous monocyte chemoattractant protein-1 promotes the transendothelial migration of monocytes in vitro. *J. Immunol.* **155:** 3610-3618.

Ratner S, Patrick P, Bora G. (1992). Lymphocyte development of adherence and motility in extracellular matrix during IL-2 stimulation. *J. Immunol.* **149:** 681-688.

Re G, Pileri S, Cau R, Bucchi ML, Casali AM, Calvari G. (1988) Histometry of splenic microvascular architecture in hairy cell leukaemia. *Histopathol.* **13:** 425-434.

Reuss-Borst MA, Ning Y, Klein G, Muller CA (1995) The vascular cell adhesion molecule (VCAM-1) is expressed on a subset of lymphoid and myeloid leukaemias. *Br. J. Haematol.* **89:** 299-305.

Rice GE, Munro JM, Bevilacqua MP (1990) Inducible cell adhesion molecule (INCAM-110) is an endothelial receptor for lymphocytes. A CD11/CD18-independent adhesion mechanism. *J. Exp. Med.* **171:** 1369-1374.

Ringwald M, Schuh R, Vestweber D, Eistetter H, Lottspeich F, Engel J, Dolz R, Jahnig F, Epplen J, Mayer S, Muller C, Kemler R (1987) The structure of cell adhesion molecule uvomorulin. Insights into the molecular mechanism of $Ca^{2+}$-dependent cell adhesion. *EMBO J.* **6:** 3647-3653.

Rohlein R, Czajkowski M, O'Neill MM, Marlin SD, Mainolki E, Merluzzi MJ (1988) Induction of cellular adhesion molecule-1 on primary and continuous cell lines by proinflammatory cytokines. Regulation by pharmacologic agents and neutralizing antibodies. *J. Immunol.* **141:** 1665-1669.

Rosenstein Y, Park JK, Hahn WC, Rosen FS, Bierer BE, Burakoff SJ (1991) CD43, a molecule defective in Wiskott-Aldrich syndrome, binds ICAM-1. *Nature* **354:** 233-255.

Rot A (1993) Chemokines link the two steps of leukocyte adhesion to endothelium. *Immunologist* **1:** 145-149.

Rozman C, Hernandez-Nieto L, Montserrat E, Brugues R (1981) Prognostic significance of bone marrow patterns in chronic lymphocytic leukemia. *Br. J. Haematol* **47:** 529-537.

Rozman C, Montserrat E, Rodriguez-Fernandez JM, Ayats R, Vallespi T, Parody R, Rios A, Prados D, Morey M, Gomis F, Alcala A, Gutierrez M, Maldonado J, Gonzalez C, Giralt M, Hernandez-Nieto L, Cabrera A, and Fernandez-Ranada JM (1984) Bone marrow histologic pattern-the single best prognostic parameter in chronic lymphocytic leukemia: a multivariate survival analysis of 329 cases. *Blood,* **64:**642-648.

Ruegg C, Postigio AA, Sikorski EE, Butcher EC, Pytela R, Erle DJ (1992) Role of integrin alpha 4 beta7/alpha4 beta P in lymphocyte adherence to fibronectin and VCAM-1 and in homotypic cell clustering. *J. Cell Biol.* **117:** 179-189.

Ruoslahti E, Pierschbacher MD (1987) New perspectives in cell adhesion: RGD and integrins. *Science* **238:** 491-497.

Ryan DH, Nuccie BL, Abboud CN, Winslow JM (1991) Vascular cell adhesion molecule-1 and the integrin VLA-4 mediate adhesion of human B cell precursors to cultured bone marrow adherent cells. *J. Clin. Invest.* **88:** 995-1004.

Salles G, Zain M, Jiang W, Boussiotis VA, Shipp MA (1993) Alternatively spliced CD44 transcripts in diffuse large-cell lymphomas: characterisation and comparison with normal activated B cells and epithelial malignancies. *Blood* **82:** 3539-3547.

Salmi M, Jalkanen S (1992) A 90 kilodalton endothelial molecule mediating lymphocyte binding in humans. *Science* **257:** 1407-1409.

Salmi M, Kalimo K, Jalkanen S (1993) Induction and function of vascular adhesion protein-1 at sites of inflammation. *J. Exp. Med.* **178:** 2255-2260.

Sanchez-Madrid F, Corbi AL (1992) Leukocyte integrins: structure, function and regulation of their activity. *Semin. Cell. Biol.* **3:** 199-210.

Sanchez-Mateos P, Arroyo AG, Balboa MA, Sanchez-Madrid F (1993) Post-receptor occupancy events in leukocytes during beta 1 integrin-ligand interactions. *Eur. J. Immunol.* **23:** 2642-2648.

Sano H, Nakagawa N, Nakajima H, Yoshida S, Iwamoto I (1995) Role of vascular cell adhesion molecule-1 and platelet-activating factor in selective eosinophil migration across vascular endothelial cells. *Int. Arch. Allergy and Immunol.* **107:** 533-540.

Sastry SK, Horwitz AF (1993) Integrin cytoplasmic domains: mediators of cytoskeletal linkages extra- and intracellular initiated transmembrane signalling. *Curr. Opin. Cell. Biol.* **5:** 819-831.

Schimmenti LA, Yan HC, Madri JA, Albeda SM (1992) Platelet endothelial cell adhesion molecule, PECAM-1, modulates cell migration. *J. Cell Physiol.* **153:** 417-428.

Schleiffenbaum B, Spertini O, Tedder TF (1992) Soluble L-selectin is present in human plasma at high levels and retains functional activity. *J. Cell Biol.* **119:** 229-238.

Schwarting R, Stein H (1989) in Leukocyte typing IV. White cell differentiation antigens., Oxford University Press, Oxford, UK, pp455-460.

Schweighoffer T, Tanaka Y, Tidswell M, Erle DJ, Horgan KJ, Ginther Luce GE, Lazarovits A, Buck D, Shaw S (1993) Selective expression of $\alpha 4\beta 7$ on a subset of human CD4+ memory T cells with hallmarks of gut-trophism. *J. Immunol.* **151:** 717-729.

Schweitzer KM, Drager AM, Vandervalk P, Thijsen SFT, Zevenbergen A, Theijsmeijer AP, Vanderschoot CE, Langenhuijsen MMAC (1996) Constitutive expression of E-selectin and vascular cell adhesion molecule-1 on endothelial cells of hematopoietic tissues. *Am.J.Pathol.* **148:** 165-175.

Seftor RE, Seftor SA, Gilsen KR, Stetler-Stevenson WG, Brown PD, Ruoslahti E, Hendrix MJC (1992) Role of the αvβ3 integrin in human melanoma cell invasion. *Proc. Natl. Acad. Sci. USA* **89:** 1557-1561.

Seshadri RS, Brown EJ, Zipursky A. (1976) Leukaemic reticuloendotheliosis a failure of monocyte production. *N. Eng. J. Med.* **295:** 181-184.

Shaw SK, Cepek KL, Murphy EA, Russel GJ, Brenner MB, Parker CM (1994) Molecular cloning of the human mucosal lymphocyte integrin αE subunit. *J. Biol. Chem.* **269:** 6016-6025.

Shi-Hua W, Vedantham S, Rosner MD, Lovis RM, Golomb HM, Gamliel H. (1992) Ultrastructural characteristics of the spleen in hairy cell leukaemia. *Leuk. Lymph.* **8:** 137-142.

Shimizu Y, Newman W, Gopal TV, Horgan KJ, Graber N, Beall LD, van Seventer SG, Shaw S (1991) Four molecular pathways of T cell adhesion to endothelial cells: roles of LFA-1, VCAM-1, and ELAM-1 and changes in pathway hierarchy under different activation conditions. *J. Cell Biol.* **113:** 1203-1212.

Shimizu Y, Shaw S (1991) Lymphocyte interactions with extracellular matrix. *FASEB J.* **5:** 2292-2299.

Shimizu Y, Newman W, Tanaka, Y, Shaw S (1992) Lymphocyte interactions with endothelial cells. *Immunol. Today* **13:** 11401-11408.

Simmons D, Makgoba MW, Seed B (1988) ICAM, an adhesion ligand of LFA-1 is homologous to the neural cell adhesion molecule NCAM. *Nature* **331:** 624-627.

Simmons PJ, Masinovsky B, Longnecker BM, Berenson R, Torok-Storob B, Gallatin WM (1992) Vascular cell adhesion molecule-1 expressed by bone marrow stromal cells mediates the binding of haematopoietic progenitor cells. *Blood* **80:** 388-395.

Smith JW, Cheresh DA (1988) The Arg-Gly-Asp-binding domain of the vitronectin receptor. *J. Biol. Chem.* **263:** 18726-18731.

Smith CW (1992) Transendothelial migration, in Harlan JM, Liu DY (eds): Adhesion: its role in inflammatory disease, New York, NY, Freeman, p83.

Smith CH, Barker JN, Morris RW, MacDonald DM, Lee TH (1993) Neuropeptides induce rapid expression of endothelial cell adhesion molecules and elicit granulocytic infiltration in human skin. *J. Immunol.* **151:** 3274-3282.

Sonnenberg A (1993) Integrins and their ligands. *Curr. Topics Microbiol. Immunol.* **184:** 7-35.

Sprent J (1973) Circulating T and B lymphocytes of the mouse. I. Migratory properties. *Cell. Immunol.* **7:** 10-39.

Springer TA, Lasky LA (1991) Sticky sugars for selectins. *Nature* **349:** 196-197.

Springer TA (1994) Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* **76:** 301-314.

Stacker SA, Springer TA (1991) Leukocyte integrin p150,95 (CD11c/CD18) functions as an adhesion molecule binding to a counter-receptor on stimulated endothelium. *J. Immunol.* **146:** 648-655.

Stamenkovic I, Aruffo A, Amiot M, Seed B (1991) The hematopoietic and epithelial forms of CD44 are distinct polypeptides with different adhesion potentials for hyaluronate-bearing cells. *EMBO J.* **10:** 343-348.

Stamper HB, Woodruff JJ (1976) Lymphocyte homing into lymph nodes: In vitro demonstration of selective affinity of recirculating lymphocytes for high endothelial venules. *J. Exp. Med.* **144:** 828-833.

Staunton DE, Dustin ML, Springer TA (1989) Functional cloning of ICAM-2, a cell adhesion ligand for LFA-1 homologous to ICAM-1. *Nature* **339:** 61-64.

Staunton DE, Dustin ML, Erickson HP, Springer TA (1990) The arrangement of the immunoglobulin-like domains of ICAM-1 and the binding sites for LFA-1 and rhinovirus. *Cell* **61:** 243-254.

Strauch UG, Lifka A, Gosslar U, Kilshaw PJ, Clements J, Holzmann B (1994) Distinct binding specificities of integrins α4β7 (LPAM-1), α4β1 (VLA-4), and αIELβ7. *Int. Immunol.* **6:** 263-275.

Streeter PR, Berg EL, Rouse BTN, Bargatze RF, Butcher EC. (1988) A tissue-specific endothelial cell molecule involved in lymphocyte homing. *Nature* **331:** 41-46.

Steinhoff G, Behrend M, Schrader B, Duijvestijn AM, Wonigeit K (1993) Expression patterns of leukocyte adhesion ligand molecules on human liver endothelia. *Am J Pathol* **142:** 481-488.

Strober S, Dilley J (1973) Maturation of B lymphocytes in the rat. I. Migration pattern, tissue distribution, and turnover rate of unprimed and primed B lymphocytes involved in the adoptive antidinitrophenyl response. *J. Exp. Med.* **138:** 1331-1344.

Strykmans PA, Debusscher L, Collard E (1977) Cell kinetics in chronic lymphocytic leukaemia (CLL). *Clin. Haematol.* **6:** 159-167.

Sugama Y, Tiruppathi C, Janakidevi K, Andersen TT, Fenton II JW, Malik AB (1992) Thrombin-induced expression of endothelial P-selectin and intercellular adhesion

molecule-1: a mechanism for stabilizing neutrophil adhesion. *J. Cell Biol.* **119:** 935-944.

Sweet DL, Golomb HM, Ultmann JE (1977) The clinical features of chronic lymphocytic leukaemia. *Clin. Haematol.* **6:** 185-202.

Swerlick RA, Lee KH, LI LJ, Sepp NT, Caughman SW Lawley TJ (1992) Regulation of vascular cell adhesion molecule-1 on human dermal microvascular endothelial cells. *J. Immunol.* **149:** 698-705.

Takeuchi H, Katayama I (1993) Surface phenotype and adhesion activity of B-cell chronic lymphoid leukemias. *Leuk Lymph* **10:** 209-216.

Tamaki T, Kanakura Y, Kuriu A, Ikeda H, Mitsui H, Yagura H, Matsumura I, Druker B, Griffin JD, Kanayama Y, Yonezawa T (1992) Surface immunoglobulin-mediated signal transduction involves rapid phosphorylation and activation of the protooncogene product Raf-1 in human B-cells. *Cancer. Res.* **52:** 566-570.

Tamatani T, Kitamura F, Kuida K, Shirao M, Mochizuki M, Suematsu M, Schmid-Schonbein GW, Watanabe K, Tsurufuji S, Miyasaki M (1993) Characterization of rat LECAM-1 (L-selectin) by the use of monoclonal antibodies and evidence for the presence of soluble LECAM-1 in rat sera. *Eur. J. Immunol.* **23:** 2181-2188.

Tanaka Y, Albeda S, Horgan KJ, van Seventer GA, Shimizu Y, Newman W, Hallam J, Newman PJ, Buck CA, Shaw S (1992) CD31 expressed on distinctive T cell subsets is a preferential amplifier of β1 integrin-mediated adhesion. *J. Exp. Med.* **176:** 245-253.

Thorbecke GJ, Amin AR, Tsiagbe VK (1994) Biology of germinal centers in lymphoid tissue. *FASEB J.* **8:** 832-840.

Till KJ and Cawley JC (1994) Accessory cells mediate hairy-cell proliferation by mechanism(s) involving both adhesion and TNFα secretion. *Brit J Haematol* **87:** 687-694.

Trowbridge IS, Omary MB (1981) Human cell surface glycoprotein related to cell proliferation is the receptor for transferrin. *Proc. Natl. Acad. Sci USA* **78:** 3039-3043.

Turunen JP (1994) Effect of lymphocyte activation status on binding to endothelium. *APMIS* **102:** 597-602.

Visser L, Shaw A, Slupsky J (1989) Monoclonal antibodies reactive with hairy cell leukaemia. *Blood* **74:** 320-325.

Walcheck B, Watts G, Jutila MA. (1993) Bovine gamma/delta T cells bind E-selectin via a noval glycoprotein receptor: first characterization of a lymphocyte/E-selectin interaction in an animal model. *J. Exp. Med.* **178:** 853-863.

Waldmann TA (1989) The multi-subunit interleukin-2 receptor. *Annu. Rev. Biochem.* **58:** 875-911.

Weller A, Isenmann S, Vestweber D (1992) Cloning of the mouse endothelial selectins: expression of both E- and P-selectin is inducible by tumor necrosis factor-α. *J. Biol. Chem* **267:** 15176-15183.

Wellicome RL, Kapahi P, Mason JC, Lebranchu Y, Yarwood H, Haskard DO (1993) Detection of a circulating form of vascular cell adhesion molecule-1: raised levels in rheumatoid arthritis and systemic lupus erythematosus. *Clin. Exp. Immunol.* **92:** 412-418.

Wilkinson PC (1986). The locomotor capacity of human lymphocytes and its enhancement by cell growth. *Immunology* **57:** 281-289.

Wilson GL, Fox CH, Fauci AS, Kehrl JH (1991) cDNA cloning of the B cell membrane protein CD22: a mediator of B-B cell interactions. *J. Exp. Med.* **173:** 137-146.

Woessner S, Asensio A, Florensa L, Pedro C, Besses C, Sans-Sabrafen J (1994) Expression of lymphocyte function-associated antigen (LFA) -1 in B-cell chronic lymphocytic leukemia. *Leuk. Lymphoma* **13:** 457-461.

Wortis HH (1992) Surface markers, heavy chain sequenced and B cell lineages. *Internat. Rev. Immunol.* **8:** 235-246.

Wortis HH, Teutsch M, Higer M, Zheng J, Parker DC (1995) B-cell activation by crosslinking of surface IgM or ligation of CD40 involves alternative signal pathways and results in different B-cell phenotypes. *Proc. Natl. Acad. Sci. USA.* **92:** 3348-3352.

Xie Y, Muller WA (1993) Molecular cloning and adhesive properties of murine platelet/endothelial cell adhesion molecule-1 (PECAM-1). *Proc. Natl. Acad. Sci. USA* **90:** 5569-5573.

Yellin MJ, Sinning J, Covey LR, Sherman W, Lee JJ, Glickman-Nir E, Sippel KC, Rogers J, Cleary AM, Parker M, Chess L, Lederman S (1994) T lymphocyte T cell-B cell-activating molecule/CD40-L molecules induce normal B cells or chronic lymphocytic leukaemia B cells to express CD80 (B7/BB-1) and enhance their costimulatory activity. *J.Immunol* **153:** 666-674.

Zauli D, Gobbi M, Crespi C, Tazzari PL, Miserocchi F, Tassinari A (1988) Cytoskeleton organisation of normal and neoplastic lymphocytes and lymphoid cell lines of T and B origin. *Brit. J. Haem.* **68:** 405-409.

Zola H, Neoh SH, Mantzioris BX, Webster J, Loughnan MS. (1990) Detection by immunoflourescence of surface molecules present in low copy number. *J Immunol Meth* **135:** 247-255.

# Abbreviations and Alternative Nomenclature

**CLL=chronic lymphocytic leukaemia**

**HCL=hairy-cell leukaemia: HCs**=the abnormal clonal lymphoid cells of HCL

***Adhesion molecules***

| Name used in this Thesis | CD N$^{0.}$ | Other names |
|---|---|---|
| α2β1 | CD49b/CD29 | VLA-2, platelet glycoprotein Ia-IIa, ECMRI, Coll R |
| α3β1 | CD49c/CD29 | VLA-3, ECMRII, laminin receptor |
| α4β1 | CD49d/CD29 | VLA-4, LPAM-2 (VLA= Very Late Antigen) |
| α5β1 | CD49e/CD29 | VLA-5, GPIc-IIa, fibronectin receptor, αFβ1 |
| α6β1 | CD49f/CD29 | VLA-6, IcIIa, laminin receptor |
| α4β7 | CD49d/- | α4βP, LPAM-1 |
| αHβ7 | - | human mucosal lymphocyte-(HML-)1, αHβ7, αEβ7 |
| αLβ2 | CD11a/CD18 | leukocyte function associated molecule- (LFA-)1 |
| αMβ2 | CD11b/CD18 | Mac-1, leukocyte adhesion receptor Mo1 , CR3 |
| αXβ2 | CD11c/CD18 | p150,95, Leu M5, CR4, LeuCAMc |
| αVβ3 | CD51/CD61 | vitronectin receptor |
| **CD31** | CD31 | platelet endothelial cell adhesion molecule-1 (PECAM-1), endoCAM |
| **VCAM-1** | CD106 | (vascular cell adhesion molecule), INCAM-110 |
| **ICAM-1** | CD54 | (intercellular adhesion molecule-1) |
| **ICAM-2** | CD102 | (intercellular adhesion molecule-2) |
| **ICAM-3** | CD50 | (intercellular adhesion molecule-3) |
| **L-selectin** | CD62L | LAM-1, Leu-8, TQ1, gp90$^{mel-14}$, LECAM-1 |
| **P-selectin** | CD62P | GMP-140, LECAM-3, PADGEM |
| **E-selectin** | CD62E | ELAM-1 (endothelial leukocyte adhesion molecule), LECAM-2 |

***Cell types:***

**HUVEC,** human umbilical vein endothelial cells

**HEV,** high endothelial venules

**ECs,** endothelial cells

***Extracellular matrix (ECM) proteins***

**FN,** fibronectin

**VN,** vitronectin

**Coll,** collagen

**ECL,** entactin,collagen,laminin

***Activating agents***

**IL-10, (-4, -1),** interleukin-10 (-4, -1)

**TNFα,** tumour necrosing factor α

**PMA,** phorbol mysteric acid

**SAC,** Staphylococcus aureus Cowan-1

***Miscellaneous***

**Ab, mAb,** antibody, monoclonal Ab

**Ig, sIg,** immunglobulin, surface Ig

**F-actin,** filamentous(polymerised)actin

***Buffer solutions***

**PBS,** phosphate-buffered saline, pH7.4

**RPMI,** synthetic culture medium, balanced salts, amino acids, vitamins and glucose

**TBS,** Tris-buffered saline, pH7.4

***Techniques***

**APAAP,** alkaline phosphatase, anti-alkaline phosphatase: immunocytochemistry

**FACS,** fluorescence-activated cell sorting: immunocytochemistry

**ELISA,** enzyme-linked immunosorbent assay

**SDS-PAGE,** sodium dodecyl sulphate-polyacrylamide gel electrophoresis