A STUDY OF THE RABBIT LEUCOCYTE INTEGRINS AND THEIR ROLE IN CELL ADHESION

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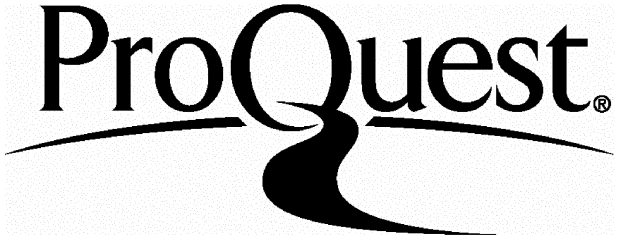

DEDICATION

To my Grandmother Kate, with love

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ABSTRACT

It is clear that the leucocyte integrins play an important role in many cell-cell interactions within the immune system. While such interactions may be necessary for an effective immune response, they must be tightly controlled since the inappropriate activation of leucocytes, as in autoimmune diseases, results in pathology. An understanding of the molecular interactions and activation states of the leucocyte integrins is required to allow development and evaluation of therapeutic strategies. The rabbit provides a suitable species in which to study these processes.

A new rabbit $CD8^+$ T cell line, BJ/873, has been characterised by flow cytometry and together with the rabbit $CD4^+$ T cell line, RL-5, has been used to produce a panel of mAb which recognise a variety of rabbit cell surface antigens. mAb which recognise rabbit CD18, CD11a and CD11c have been produced and characterised using cell surface immunofluorescence and flow cytometry, immunohistochemical staining of rabbit lymphoid tissues, Western blotting and immunostaining, metabolic and cell-surface labelling of cell lines followed by immunoprecipitation and N-terminal amino-acid sequence analysis of immunoaffinity purified proteins. Using these, and previously characterised mAb, the expression of the leucocyte integrins by rabbit lymphoid tissues was investigated by flow cytometry.

Following stimulation with phorbol-ester the RL-5 cells were found to homotypically aggregate and inhibition studies revealed that this aggregation is LFA-1 dependent. A number of the anti-LFA-1 mAb produced also stimulated LFA-1 dependent aggregation of the RL-5 cells. The ligand for rabbit LFA-1 in this system was not identified.

Three anti-CD11c mAb induced homotypic aggregation of BJ/873 cells. mAb blocking studies showed that anti-CD18 and anti-ICAM-1 mAb were inhibitory and suggested that in the rabbit, p150,95 acts as a ligand for ICAM-1. The BJ/873 cells co-express LFA-1 and p150,95 as well as other adhesion molecules. Before a definitive statement concerning the interaction of rabbit p150,95 with ICAM-1 can be made, these molecules must be expressed in isolation. A probe for rabbit CD18 was produced by PCR and used to screen a rabbit spleen cDNA library. A single clone containing a 2.3kb insert was selected and sequenced. The clone does not code for the N-terminal portion of CD18 and this region has been amplified by PCR and sequenced.

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LIST OF ABBREVIATIONS

A	Adenine
BSA	Bovine serum albumin
C	Cytosine
C5a	Complement factor fragment 5a
CHO	Chinese hamster ovary
CR3, CR4	Receptors for activated complement fragment C3
CD	Cluster of differentiation
cDNA	Complementary deoyribonucleic acid
DAB	3,3'-Diamino benzidine
dATP	Deoxyadenosine 5'-triphosphate
dCTP	Deoxycytidine 5'-triphosphate
dGTP	Deoxyguanosine 5'-triphosphate
dTTP	Deoxythymidine 5' triphosphate
DIG	Digoxigen
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetra-acetic potassium salt
ELISA	Enzyme linked immunoadsorbant assay
EtOH	Ethanol
FACS	Fluorescence activated cell sorter
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
G	Guanine
mAb-FITC	Fluoresceinated mAb
HRP	Horse-radish peroxidate
iC3b	Complement factor fragment 3b
Ig	Immunoglobulin
IgSF	Immunoglobulin supergene family
IL	Interleukin
LB	Lauri Broth
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MHC	Major histocompatability complex
M_r	Relative molecular mass
NP-40	Nonident P-40
OPD	O-phenylenediamine
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMA	Phorbol myristate acetate
PMSF	Phenyl-methyl-sulfonyl-fluoride
R_f	Relative migration value
RNA	Ribonucleic acid
SDS	Sodium dodecylsulphate
SLe^a	sialyl Lewis A
SLe^x	sialyl Lewis X
SSC	Sodium chloride/sodium citrate buffer

T	Thymine
TAE	Tris-acetate/EDTA buffer
TBE	Tris-borate/EDTA
TBS	Tris buffered saline
TE	Tris/EDTA
TCSN	Tissue culture supernatant
TEMED	Tetre-methyl-ethylene-diamine
x-gal	5-bromo-4-chloro-indolyl-β-D-galactoside

CHAPTER ONE

General Introduction

1.1. Overview

The immune system is made up of two major branches which act in concert to provide protection from the myriad of pathogenic micro-organisms which constantly challenge the human body, tumours and the development of autoimmunity. Natural or innate immune responses provide non-specific protection from any kind of pathogenic micro-organism; the intensity of the response is the same upon each contact with the pathogen. Adaptive immune responses are raised against a specific antigen displayed on a pathogenic micro-organism and rapidly cause destruction of the micro-organism; the intensity of the response is heightened following subsequent encounters with the antigen.

1.1.1. The natural immune system

The natural immune system includes mechanical factors such as the skin and the mucous membranes that provide a physical barrier to the entrance of micro-organisms, and chemical factors, such as gastric juice and the anti-bacterial enzyme lysosyme, which neutralise micro-organisms or their toxins. If pathogens bypass these systems and enter the tissues, a number of anti-microbial proteins and non-specific phagocytic cells are present to eliminate them. Anti-microbial proteins include interferons (INF), that appear to be the first line of defence against many different viruses, and the proteins of the complement system (alternative pathway), which promote phagocytosis or cell lysis of micro-organisms and contribute to the development of an inflammatory response. Non-specific phagocytic cells include granulocytes, monocytes and macrophages (figure 1.1).

Three different types of granulocytes have been described: neutrophils, eosinophils and basophils. Neutrophils, which constitute 60-70% of peripheral blood leucocytes, are short lived, highly phagocytic cells derived from pluripotent stem cells in the bone marrow. Neutrophils may phagocytose pathogenic micro-organisms following

attachment through carbohydrate receptors or via their cell-surface receptors for complement proteins (CR1 and CR3 receptors), or the Fc portion of IgG (FcγRII and FcγRIII) that becomes attached to the pathogen (opsoninisation). Phagocytosed material is degraded by a combination of enzymes and other anti-bacterial proteins which are stored in cytoplasmic granules and released into the phagosome.

Eosinophils, which account for about 2-5% of peripheral blood leucocytes, are weakly phagocytic and can ingest pathogenic micro-organisms although they are more effective at killing large parasites, such as schistosomes, by releasing the contents of a combination of toxic granules. Eosinophil degranulation may be contact-dependent or may simply require deposition of microbial toxins within the local tissues. Degranulation is also triggered by binding to IgG or IgE coated pathogens via FcγRII and FcεRII receptors, or by binding to pathogens coated with complement proteins via complement protein receptors. Although the released toxins are effective against certain pathogens, they can also cause tissue damage in a number of inflammatory diseases where eosinophil infiltration is prominent, particularly allergic disorders such as asthma. Basophils account for 2% of peripheral blood leucocytes. They are not phagocytic but they express high-affinity IgE receptors and, on contact with a pathogen coated with IgE, release a number of toxic granules and pharmacological agents including histamine which triggers an inflammatory response and so brings other cells of the immune system into the area.

Monocytes constitute approximately 10-15% of peripheral blood leucocytes. Like granulocytes they are derived from pluripotent stem cells located in the bone marrow. Following haematopoiesis, monocytes enter the blood and circulate for approximately two days. After this period they migrate into the tissues where they further differentiate to form macrophages. Macrophages are present in most tissues including the spleen, lungs and lymph nodes, in the liver they are known as Kupffer cells and those in the central nervous tissue as microglia. They are highly phagocytic, and phagocytosis is facilitated by the expression of receptors for the Fc region of antibody (FcγRII and FcγRIII) and complement proteins (CR1 and CR3). Monocytes and macrophages express MHC class-II molecules and can serve as antigen presenting cells.

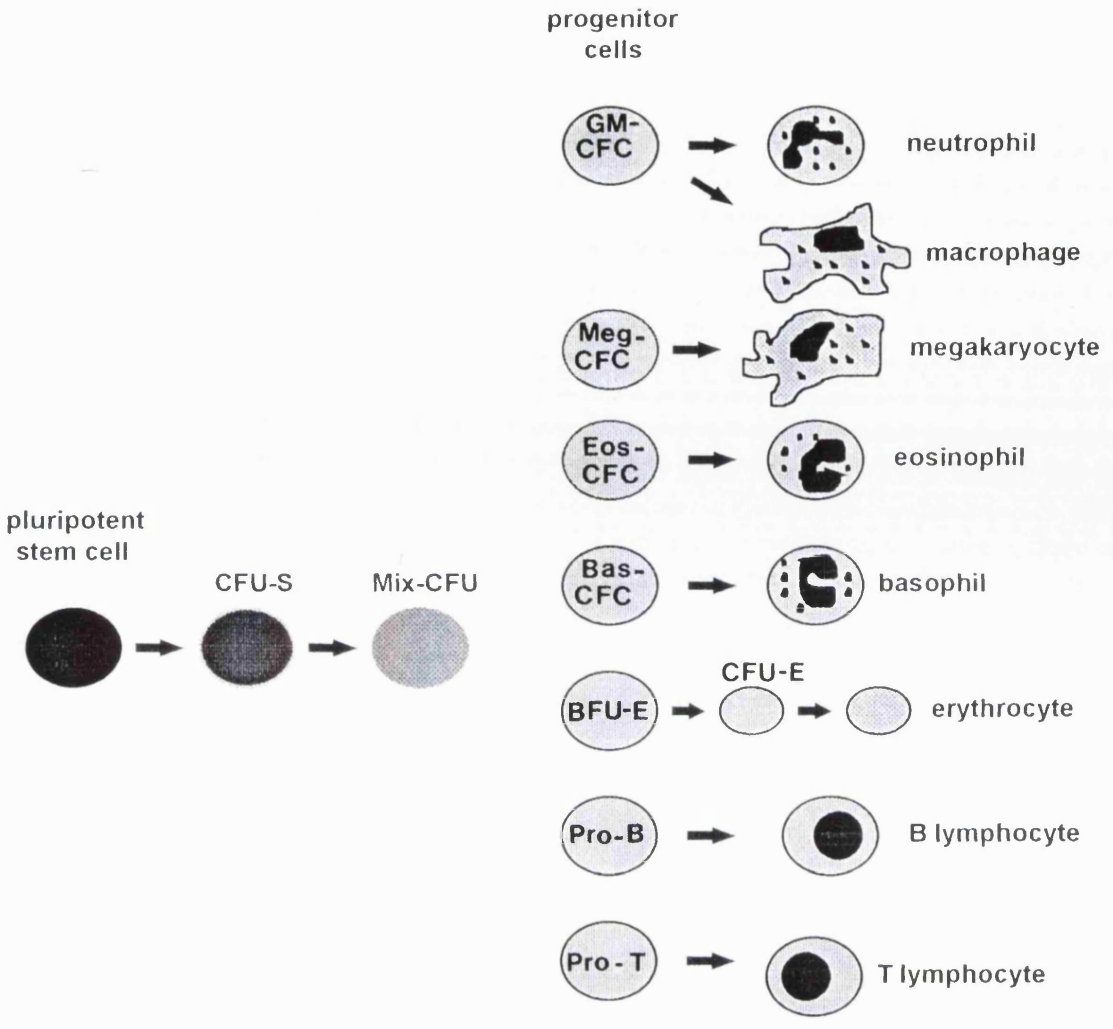

Figure 1.1. Haemopoiesis - cells of the immune system, adapted from Male et al., (1993) (CFC-S - early precursor cell defined by the spleen colony-forming assay, Mix-CFU - multipotential cells, GM-CFC - granulocyte-macrophage colony forming cells, Meg-CFC - megakaryocyte colony forming cells, Eos-CFC eosinophil colony forming cells, Bas-CFC - basophil colony forming cells, BFU-E - burst forming units-erythroid, CFU-E - colony forming units-erythroid).

The responses of phagocytic cells are expressed in the tissues during inflammatory responses that may be triggered by the entry of micro-organisms or chemical agents to the tissues or by physical injury to the tissues. Inflammatory responses are characterised by: (i) increased blood flow to the site, (ii) increased capillary permeability resulting in swelling and (iii) increased migration of leucocytes from the micro-circulation into the tissues. Neutrophils are the first cells to arrive at sites of inflammation followed later by monocytes and lymphocytes. Leucocyte migration is directed by chemotactic proteins released from cells already present at the site, for

example by mast cells or basophils, or possibly the pathogen itself. In response to these chemotactic proteins, leucocytes attach to the walls of the post capillary venules adjacent to the extravascular inflammatory site and roll along the vessel wall. Subsequently, some of these rolling leucocytes flatten against the vessel wall, and migrate through the inter-endothelial junctions and the sub-endothelial extracellular matrix, accumulating at the inflammatory site. Migration is dependent on the direct interaction of adhesion molecules on the surface of the leucocytes with adhesion molecules displayed on the post capillary endothelium. The adhesion molecules involved in the migration of neutrophils during an inflammatory response have been the focus of many studies over the last decade. Consequently, a multi-step model of neutrophil migration has been developed and is summarised in figure 1.2.

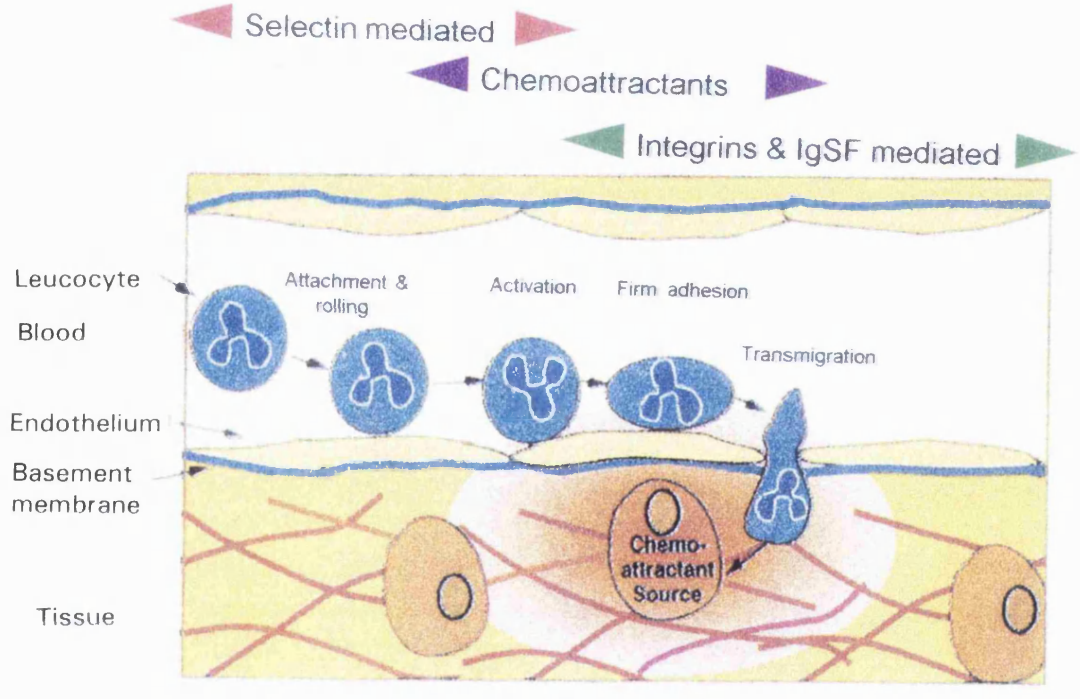

Figure 1.2. The three step model of neutrophil transmigration, adapted from Springer (1994)

1.1.2. The adaptive immune system

The components of the adaptive immune system recognise a specific antigen displayed on a potential pathogen and mount a specific response to eliminate the source of the antigen. The adaptive immune system can be sub-divided into two closely allied systems. The humoral immune response, or antibody-mediated immunity, is particularly effective against bacteria and extracellular virus and involves the recognition of antigens by B lymphocytes, while the cellular immune response, or cell-mediated immunity, is effective against fungi, parasites, intracellular viruses and foreign transplant tissue and involves the specific recognition of antigens by T lymphocytes.

Each B lymphocyte expresses a cell surface receptor, surface immunoglobulin, which is capable of recognising a specific antigenic site. When a B lymphocyte encounters its complementary antigen in the presence of T lymphocyte derived cytokines such as interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-5 (IL-5) and IFNγ, it becomes activated. Some activated B lymphocytes differentiate to form plasma cells which secrete antibody that is reactive with the initiating antigen. Binding of antibody to its antigen promotes phagocytosis by neutrophils and monocytes, antibody dependent-killing by large granular lymphocytes, or activation of the complement system which results in lysis of a microbial cell by the membrane attack complex. Following the interaction of a virgin, or naive, B lymphocyte with antigen, IgM antibodies are secreted. As the response develops, antibodies of increased affinity are produced (affinity maturation) and individual B lymphocytes can switch from producing IgM antibody to another more functionally relevant isotype (class switching). B lymphocytes which become activated but do not form antibody secreting plasma cells remain as memory B lymphocytes within germinal centres of lymphoid tissues. Memory B lymphocytes are more sensitive to antigenic stimulation and respond rapidly should the same antigen appear at a future time.

T lymphocytes also express a cell surface receptor, the TCR/CD3 complex, which recognises a particular antigen. T lymphocytes do not recognise free antigen but require antigenic peptides to be presented in association with molecules of the major histocompatibility complex (MHC). There are two major sub-populations of T

lymphocytes which are distinguished by their expression of CD4 or CD8 cell surface molecules. $CD4^+$ lymphocytes require peptide fragments of antigens to be presented by MHC class-II molecules and are generally known as T helper (Th) lymphocytes because they produce a number of cytokines which modulate the function of other leucocytes during an immune response. The expression of MHC class-II molecules is restricted to a limited number of cells which are collectively known as antigen presenting cells. These cells are able to internalise micro-organisms, degrade them and present antigenic peptides bound to MHC class-II at the cells surface. Macrophages, B lymphocytes, Langerhans cells in the skin and dendritic cells, found as interdigitating cells in T lymphocyte areas of lymphoid tissue and the thymus, all act as antigen presenting cells. $CD8^+$ lymphocytes, also referred to as cytotoxic T lymphocytes (Tc), recognise antigenic peptides derived from intracellular pathogens such as viruses in the context of MHC class-I molecules. MHC-class I molecules are expressed on the surface of most nucleated cells throughout the body. Following the interaction of $CD8^+$ T lymphocytes with their specific antigen bound to MHC class-I molecules, the lymphocyte kills the target cell thus preventing further replication of the intracellular pathogen.

The numbers of lymphocytes capable of recognising any one specific antigen are relatively small and so, to ensure continuous immunosurveillience, B and T lymphocytes constantly circulate around the body passing from the blood through the secondary lymphoid and other tissues, and back through the lymphatics to the blood. The secondary lymphoid tissues provide an appropriate micro-environment for lymphocyte stimulation by antigen and it is to these areas that antigen entering the tissues drain. They include the spleen and numerous lymph nodes randomly dispersed throughout the gastrointestinal tract, respiratory passageways, urinary tract and reproductive tract.

T lymphocytes which have not previously encountered antigen (naive lymphocytes) enter the secondary lymphoid tissues directly from the blood by migrating across specialised post-capillary venular sites called high endothelial venules (HEV) (Ager, 1987). HEV are characterised by a distinct cuboidal morphology and the expression of adhesion molecules which support lymphocyte transendothelial migration. HEV are

not found in the spleen and T lymphocytes migrate across the walls of vessels in the marginal zone. In the secondary lymphoid tissues, T lymphocytes may, or may not, come into contact with their antigen. In the absence of an activating signal, naive T lymphocytes drain into the efferent lymphatic vessels and re-enter the blood via the thoracic duct, and recirculate to another secondary lymphoid site. Naive lymphocytes do not show a preference for the lymphoid tissue they migrate or "home" to and they generally do not migrate to extra-lymphoid sites such as the skin.

When a lymphocyte encounters its antigen in the secondary lymphoid tissues it becomes activated and an immune response is initiated. The T lymphocyte clonally expands producing effector cells some of which then convert to memory lymphocytes. Memory T lymphocytes differ from naive cells in having an altered expression of adhesion molecules and a different circulatory pattern. They no longer leave the circulation through the HEV in the secondary lymphoid tissues, but transmigrate at post-capillary vessels into non-lymphoid tissues and gain entrance to the secondary lymphoid tissue via the draining afferent lymphatics. This migration is non-random and memory T lymphocytes preferentially "home" to the secondary lymphoid tissue where they first encountered antigen and thus provide immediate protection in an environment where antigen is likely to be re-encountered (Mackay *et al.*, 1992a: Mackay *et al.*, 1992b; Mackay, 1992c). The recirculation of memory T lymphocytes is modified during inflammation. A localised inflammatory response in a lymph node, initiated by the entry of an antigen, is associated with increase in blood flow and traffic of both naive and memory cells into the node. The entry of memory cells is associated with expression of adhesion molecules on non-HEV blood vessel endothelium within the lymph node (Mackay *et al.*, 1992a). The entry of a previously encountered antigen into the tissues induces localized accumulation of memory T lymphocytes. The phenotype of these cells is similar to the that of cells which recirculate through the tissues sites under basal conditions (Pitzalis *et al.*, 1988). This suggests that during inflammation in the tissues, the molecular interactions which control lymphocyte recirculation are up regulated.

To reach the secondary lymphoid tissues, recirculating T lymphocytes must interact with endothelial cells at the HEV and post-capillary sites. Early models proposed that lymphocyte recirculation would be controlled by interactions between tissue specific adhesion molecules on the vascular endothelium (vascular addressins) with differentially expressed adhesion molecules on lymphocytes (homing receptors). In its most basic form this would involve one specific receptor-ligand pair for each tissue site. However, functional blocking of different adhesion molecules *in vitro* and *in vivo* has revealed that several receptor-pairs are involved at each tissue site. Studies of neutrophil-endothelial cell interactions revealed that extravasation requires the successful completion of four steps: primary adhesion (attachment), secondary adhesion triggering, secondary adhesion (firm adhesion) and transmigration (figure 1.2.) (Springer, 1994). It is now recognised that the basic steps of the adhesion cascade, elucidated by studies of neutrophil migration (figure 1.2), are also involved in the recirculation of naive and memory T lymphocytes. Thus, initial attachment of lymphocytes to endothelial cells is mediated by the selectins and some members of the integrin superfamily, and stable arrest and subsequent transendothelial migration is mediated by the integrins. The ligands of these molecules are members of the immunoglobulin supergene family. Table 1.1 summarises the T lymphocyte - endothelial interactions which have been shown to be involved in lymphocyte recirculation and migration to inflamed tissues. The expression, structure and function of these molecules is discussed in the following sections.

	Expression	Ligands	Implications	Reference
L-selectin	naive T lymphocytes	GlyCAM-1, CD34, MAdCAM-1	PLN homing, PP homing inflammation	(Hamann *et al.*, 1994; Hamann *et al.*, 1991; Tedder *et al.*, 1995)
P-selectin	inflamed endothelium	PSGL-1, SLe^x decorated proteins, 120 kDa	inflammation	(Subramaniam *et al.*, 1995)
E-selectin	inflamed endothelium	ESL-1, SLe^x decorated proteins, CLA	inflammation, skin homing	(Silber *et al.*, 1994; Berg *et al.*, 1991; Picker *et al.*, 1991; Shimizu *et al.*, 1991)
$\alpha_4\beta_1$	T lymphocytes	VCAM-1, fibronectin	inflammation	(Silber *et al.*, 1994; Christensen *et al.*, 1995; Gorczynski and Wojcik, 1992; Ennis *et al.*, 1993)
$\alpha_4\beta_7$	T lymphocytes	MAdCAM-1 VCAM-1, fibronectin	PP homing, inflammation	(Hamann *et al.*, 1994)
CD11a/CD18	T lymphocytes	ICAM-1, ICAM-2	homing, inflammation	(Issekutz, 1992; Hamann *et al.*, 1988; Santamaria *et al.*, 1994)
PECAM-1	endothelium, naive T lymphocytes	PECAM-1, GAG, $\alpha_v\beta_3$	homing	(Piali *et al.*, 1995)
CD44	T lymphocytes endothelium	hyaluronic acid sulphated proteoglycan	homing	(Jalkanen *et al.*, 1986; Jalkanen *et al.*, 1987)

Table 1.1. Summary of the T lymphocyte - endothelium interactions during inflammation and T lymphocyte homing

1.2. THE SELECTINS

The selectins designated L-Selectin, P-selectin and E-selectin are expressed on leucocytes, platelets and endothelial cells. The members of this family share a common mosaic structure consisting of a C-type (Ca^{2+}-dependent) lectin-like binding domain at the N-terminus, followed by an epidermal growth factor like domain, several short consensus repeats (SCR) similar to those found in complement-regulatory proteins such as CR1 and C4b-binding protein, a membrane spanning region and a short cytoplasmic domain. The selectins share 40-60% overall homology at the nucleotide and amino-acid level. The main structural difference between the members of the selectin family is in the number of SCR present: E-selectin has six (Bevilacqua *et al.*, 1989) P-selectin has alternatively spliced forms with eight and nine (Johnston *et al.*, 1989; Johnston *et al.*, 1990) and L-selectin has two (Tedder *et al.*, 1989; Camerini *et al.*, 1989).

The discovery that anti-selectin mAb inhibit lymphocyte-endothelial cell interactions in static assays and under flow, but not stable arrest, spreading or lymphocyte transmigration (Shimizu *et al.*, 1991; Brady *et al.*, 1992; Kunzendorf *et al.*, 1993; Jones *et al.*, 1994; Yago *et al.*, 1995) led to the suggestion that the selectins also mediated the initial recognition of endothelium by T lymphocytes. The development of anti-selectin knockout mice has confirmed the importance of selectin-interactions in both the recirculation of T lymphocyte between lymphoid tissues and migration of T lymphocytes to inflammatory sites (Subramaniam *et al.*, 1995; Tedder *et al.*, 1995).

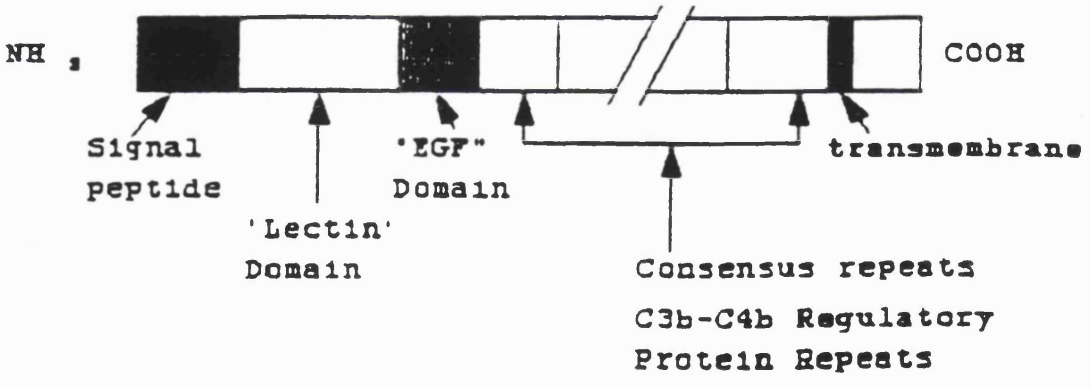

Figure 1.3. Domain organisation of the selectins

L-selectin is expressed on neutrophils, monocytes and approximately 60% of lymphocytes (Lewinsohn *et al.*, 1987). Expression of L-selectin on these cells is rapidly down-modulated following cell activation with chemokines by an unusual protease activity that cleaves L-selectin from the cell membrane (Kishimoto *et al.*, 1990; Griffin *et al.*, 1990). L-selectin was first described by Gallatin *et al.*, (1983), as a lymphocyte adhesion molecule which supported binding of lymphocytes to frozen sections of HEV from peripheral lymph nodes (PLN). It recognises carbohydrate ligands that are sialylated and fucosylated, like sialyl Lewis X (SLe^x) and sialyl Lewis A (SLe^a), but that are also sulphated (Rosen, 1993; Imai *et al.*, 1993). These carbohydrate structures are presented by proteins containing heavily O-glycosylated mucin-like domains (rich in serine and threonine residues). The protein ligands for L-selectin described to date include: Glycosylation-dependent adhesion molecule-1 (GlyCAM-1) (Lasky *et al.*, 1992), CD34 (Baumhueter *et al.*, 1993), and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) (Berg *et al.*, 1993) (section 1.3.6). GlyCAM-1 is expressed mainly on HEV of PLN (Lasky *et al.*, 1992; Dowbenko *et al.*, 1993). It is a secretory molecule and the mechanism by which it interacts with the cell surface to support lymphocyte adhesion is unclear. CD34 is universally expressed on vascular endothelium, but acts as a L-selectin ligand only when appropriately decorated, as in the HEVs of PLN (Baumhueter *et al.*, 1993).

P-selectin is expressed by activated platelets, megakaryocytes and endothelial cells (McEver and Martin, 1984; McEver *et al.*, 1989). Platelets store P-selectin in cytoplasmic α-granules, and endothelial cells in Wiebel-Palade bodies. On cellular activation with agonists such as thrombin, P-selectin is rapidly mobilised to the cell surface where its expression is short lived, peaking at 5-10 minutes and returning to basal levels within 30-60 minutes (Hattori *et al.*, 1989). P-selectin is internalised to vesicles from where it may recycle, although a proportion is directed to lysosomes where it is degraded (Green *et al.*, 1994). P-selectin mediates the adhesion of neutrophils, monocytes, a lymphocyte sub-population and NK cells to post-capillary endothelial cells through its interaction with sialylated, fucosylated carbohydrate structures such as SLe^x and SLe^a (Handa *et al.*, 1991). Carbohydrate P-selectin ligands are displayed by the recently identified P-selectin glycoprotein ligand-1 (PSGL-1), a

homodimer composed of two 120 kDa transmembrane glycoprotein subunits which are disulphide bonded (Moore *et al.*, 1992; Norgard *et al.*, 1993).

E-selectin is a 115 kDa inducible endothelial cell adhesion molecule (Bevilacqua *et al.*, 1987). Its expression is upregulated following stimulation of endothelial cells with the cytokines IL-1, IL-3 and tumour necrosis factor-α (TNFα), by bacterial endotoxins and by neuropeptides (Bevilacqua *et al.*, 1987; Shimizu *et al.*, 1991; Weller *et al.*, 1992; Smith *et al.*, 1993). Expression of newly synthesised E-selectin peaks 3-6 hours after stimulation, returning to basal levels by 48 hours. Like P-selectin, E-selectin is internalised and degraded in lysosomes. E-selectin supports the adhesion of granulocytes, monocytes, T lymphocyte subsets and natural killer cells to stimulated endothelial cells through its interaction with sialylated, fucosylated carbohydrate ligands notably SLe^x and SLe^a (Lowe *et al.*, 1990; Tiemeyer *et al.*, 1991). Using an E-selectin-Ig chimeric molecule, a major protein ligand for E-selectin has been identified in the mouse and designated E-selectin ligand-1 (ESL-1) (Steegmaier *et al.*, 1995). Expression of this 150 kDa ESL-1 molecule is restricted to myeloid cells.

In the human E-selectin has been shown to bind to the cutaneous lymphocyte antigen (CLA). CLA is expressed by memory T lymphocytes which recirculate through the skin, but not on memory T lymphocytes found in unrelated extra-lymphoid sites such as the lung or naive T lymphocytes. Thus it was proposed that this E-selectin-CLA interaction control the specific homing of T lymphocytes to the skin (Berg *et al.*, 1991; Picker *et al.*, 1991).

1.3. THE INTEGRIN SUPERGENE FAMILY

The integrins are a family of transmembrane glycoproteins that mediate cell adhesion events. They are non-covalently associated $\alpha\beta$ heterodimers expressed by a wide variety of cells. To date, sixteen α- and eight β-subunits have been described and these combine to form at least twenty-two distinct heterodimer receptors which, based on β-subunit usage, have been divided into three subfamilies commonly referred to as β_1, β_2 and β_3. The more recently described "alternative" β-subunits, β_4, β_5, β_6, β_7 and β_8, associate with α-subunits already assigned to a subfamily making their classification more difficult (figure 1.6).

The β_1, β_3 and some of the alternative integrins, generally act as extracellular matrix receptors and are involved in processes such as development and wound healing. The β_2 integrins, together with $\alpha_4\beta_1$, $\alpha_4\beta_7$, and $\alpha_E\beta_7$, bind to ligands expressed by endothelial cells and other leucocytes and are involved in the cell-cell adhesion required for leucocyte migration and leucocyte effector functions. The integrins are phylogenetically ancient and homologous molecules have been described in Drosophila (Wilcox and Leptin, 1985). Their fundamental importance is also emphasized by their conservation across species, for example the recently cloned chicken β_2 subunit shares 65% identity with human β_2 (Bilsland and Springer, 1994).

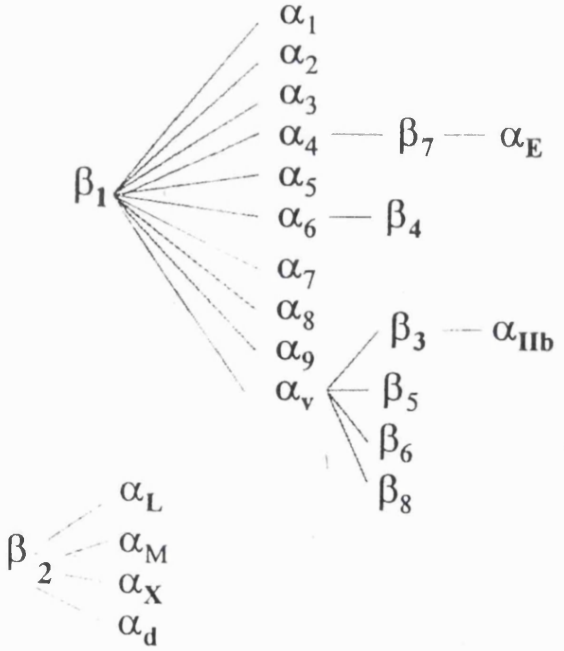

Figure 1.4. The integrin superfamily

1.3.1. Common Structural Features of Integrin α-subunits

The integrin α-subunits are transmembrane glycoproteins (130-210 kDa) which contain a large extracellular domain, a transmembrane domain and a short cytoplasmic domain of 30-50 amino-acids. Of the sixteen α-subunits identified to date, complete sequence data is available for at least thirteen of the human chains (α_1 (Briesewitz *et al.*, 1993), α_2 (Takada and Hemler, 1989), α_3 (Takada *et al.*, 1991), α_4 (Takada *et al.*, 1989), α_5 (Argraves *et al.*, 1987), α_6 (Tamura *et al.*, 1990), α_8 (Schnapp *et al.*, 1995), α_v (Suzuki *et al.*, 1986; Suzuki *et al.*, 1987), α_{IIb} (Poncz *et al.*, 1987), α_E (Shaw *et al.*, 1994), α_L (Larson, 1989), α_M (Corbi *et al.*, 1988) and α_X (Corbi *et al.*, 1987)) and overall sequence identity between them varies between 20-30%. The N-terminal portion of the extracellular region contains seven tandomly repeated homologous domains which are numbered I-VII. Each domain contains approximately 60 amino-acid residues. The last three or four repeated domains contain cation binding motifs and cation binding has been shown to be essential for integrin function (Dustin and Springer, 1989). Generally the transmembrane domains of the α-subunits are highly conserved which suggests that they have a functional role, possibly stabilisation in the membrane. The level of sequence identity between the different cytoplasmic domains of the α-subunits is low, although a highly conserved motif, GFFKR, is present in all of the cytoplasmic domains. The residues LGFFK were deleted from the cytoplasmic domain of the α_L-subunit. Co-expression of the α_L mutant in chinese hamster ovary (CHO) cells with β_3 resulted in high affinity ligand binding. This suggests that the GFFKR motif may be important in affinity regulation (O'Toole *et al.*, 1994).

The α_1, α_2, α_L, α_M and α_X subunits, have a insert of 180-200 amino-acids, known as the I domain, located between domains II and III. The I domain is homologous to the ligand binding domains of von Willibrand factor, cartilage matrix protein and the complement proteins C2 and factor B, and has been shown to be involved in integrin ligand binding (Diamond *et al.*, 1993; Randi and Hogg, 1994). The remaining integrin α-subunits, α_3, α_5, α_6, α_7, α_{IIb} and α_v also share a structural feature. These α-subunits undergo post-translational cleavage close to the C-terminus producing a heavy and light chain which are linked by a disulphide bond. The exceptions to these groupings are the α_E-subunit, which contains both a disulphide bond and an I domain (Shaw *et al.*, 1994),

and α_4 which does not contain an I domain or a C-terminal cleavage site (Hemler, 1990). However α_4 contains a putative cleavage site in the middle of the chain and fragments of 70 and 80 kDa, in addition to the 150 kDa protein, have been immunoprecipitated with anti-α_4 mAbs.

1.3.2. Common Structural Features of Integrin β-subunits

Like the α-subunits, the β-subunits are integral membrane proteins with a large extracellular domain containing several potential glycosylation sites, a transmembrane domain and a short cytoplasmic tail. The complete primary sequence for β_1 (Argaves *et al.*, 1987), β_2 (Kishimoto *et al.*, 1987; Law *et al.*, 1987), β_3 (Fitzgerald *et al.*, 1987; Rosa *et al.*, 1988), β_4 (Suzuki and Naitoh, 1990), β_5 (Ramaswamy and Hemler, 1990), β_6 (Suzuki and Naitoh, 1990), β_7 (Yuan *et al.*, 1990) and β_8 (Schnapp *et al.*, 1995) has been determined and overall sequence identity ranges from approximately 30-50%. Alignment of the β_1, β_2, β_3, β_5 and β_6 sequences showed that they all contain 56 conserved cysteine residues. Many of these are located within four cysteine-rich repeats in the C-terminal portion of the cytoplasmic domain. The β_7 subunit lacks the two most C-terminal cysteine residues in the extracellular domain (Yuan *et al.*, 1990) and the β_4 sequence contains only 47 of the 56 conserved cysteine residues (Suzuki and Naitoh, 1990).

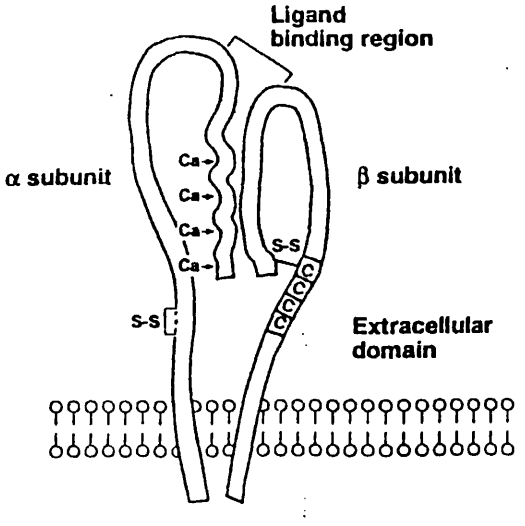

Figure 1.5. Schematic structure of an integrin. The subunits associated in a non-covalent manner. The α subunit shown belongs to the group which undergoes post-translational modification. (Ca - denotes metal binding domain, C - cysteine rich region and S-S donotes disulphide bond).

1.3.3. β_1 Integrin subfamily

With ten family members, the β_1 integrins form the largest integrin sub-family. The $\beta 1$ integrins are commonly referred to as VLA proteins (very late antigens) because the first two members were found to be expressed very late after activation of T lymphocytes by antigen or mitogen (Hemler *et al.*, 1985). This sub-family has a wide tissue distribution and family members generally act as receptors for extracellular matrix proteins including fibronectin, laminin and collagen. The β_1 integrins are differentially expressed by leucocyte populations in the human. Generally, resting T lymphocytes express very low amounts of $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_3\beta_1$, and higher levels of $\alpha_4\beta_1$, $\alpha_5\beta_1$ and $\alpha_6\beta_1$. B lymphocytes express $\alpha_4\beta_1$, low levels of $\alpha_2\beta_1$ and $\alpha_3\beta_1$, but not $\alpha_1\beta_1$, $\alpha_5\beta_1$ or $\alpha_6\beta_1$. Monocytes express all β_1 integrins in varying amounts, while neutrophils have been reported to lack cell surface β_1 expression and therefore expression of this family of molecules (Hemler, 1990).

The $\alpha_4\beta_1$ molecule differs from other β_1 integrin family members because it functions as both a cell-extracellular matrix receptor and a cell-cell receptor. $\alpha_4\beta_1$ is a ligand for fibronectin, binding an alternatively spliced domain, CS-1, within the heparin II binding fragment (Wayner *et al.*, 1989), VCAM-1 (Elices *et al.*, 1990) and the bacterial outer membrane protein invasin (Isberg and Leong, 1990). These different ligands bind to distinct sites in $\alpha_4\beta_1$ (Masumoto and Hemler, 1993). VCAM-1 has been shown to support $\alpha_4\beta_1$-dependent lymphocyte adhesion in static assays (Lange *et al.*, 1994) and lymphocyte rolling and firm adhesion under flow (Subramaniam *et al.*, 1995; Berlin *et al.*, 1995). Expression of the $\alpha_4\beta_1$ ligand, VCAM-1, is upregulated during inflammation suggesting that the $\alpha_4\beta_1$-VCAM-1 interaction is important in the recruitment of lymphocytes to inflammatory sites. This observation was confirmed by Pravsgaad Christensten *et al.*, (1995), who showed that blocking the $\alpha_4\beta_1$-VCAM-1 interaction prevented the accumulation of murine T lymphocytes at sites of inflammation.

The α_4 subunit can also associate with β_7 giving $\alpha_4\beta_7$, which binds to mucosal addressin molecule MAdCAM-1 (Berlin *et al.*, 1993), fibronectin and VCAM-1 (Ruegg *et al.*, 1992). Recently it has been suggested that $\alpha_4\beta_7$ may be able to bind to α_4 subunits on

adjacent cells, providing a novel mechanism of cell-cell adhesion (Altevogt *et al.*, 1995). MAdCAM-1 is exclusively expressed on Peyer's patches (PP) HEV and venules in the intestinal tract and mAb which functionally block $\alpha_4\beta_7$-MAdCAM-1 interactions significantly reduce lymphocyte recirculation to PP and the intestinal tract. Thus $\alpha_4\beta_7$ appears to support the adhesion of recirculating lymphocytes to the intestinal tract in the mouse and also possibly in sheep (Mackay *et al.*, 1992b) and humans (Erle *et al.*, 1994).

1.3.4. The β_2 Integrin subfamily

The β_2 integrin family also known as the leucocyte integrins or CD11/CD18 integrins has three well characterised family members, $\alpha_L\beta_2$ (180/95 kDa), $\alpha_M\beta_2$ (170/95 kDa) and $\alpha_X\beta_2$ (150/95 kDa). $\alpha_M\beta_2$ was the first member of the subfamily to be defined, initially in the mouse (Springer *et al.*, 1979) and later in the human (Springer *et al.*, 1982) as a marker for myeloid cells. The human and murine $\alpha_L\beta_2$ heterodimer were first identified by mAb which were able to inhibit cytotoxic T lymphocyte-target cell killing (Davignon *et al.*, 1981; Pierres *et al.*, 1982). Biochemical characterisation of $\alpha_M\beta_2$ and $\alpha_L\beta_2$ showed that the two heterodimers shared a common β-subunit, though the α-subunits were distinct (Trowbridge and Omary, 1981). Immunoprecipitation studies using anti-β subunit mAb identified $\alpha_X\beta_2$ as a third family member (Sanchez-Madrid *et al.*, 1983; Springer *et al.*, 1986). Alternative names for these molecules are given in table 1.2 and CD nomenclature will be used for the remainder of this thesis, regardless of species of origin.

Recently a fourth α-chain, designated α_d, which associates with β_2 has been described for the human and the dog (Danilenko *et al.*, 1992; Danilenko *et al.*, 1995). The human α_d molecule has been cloned from a cDNA library and sequenced. Isolated canine α_d has been sequenced at the N- and C-terminals and the two molecules are homologous in these regions. In the dog, α_d expression is restricted to macrophages and minor lymphocyte sub-populations. Human α_d has been shown to preferentially bind ICAM-3 and it has been proposed that this novel molecule may be functionally important in leucocyte-leucocyte interactions rather than leucocyte-endothelial interactions.

CD11a/CD18	CD11b/CD18	CD11c/CD18
LFA-1	Mac-1	p150,95
$\alpha_L\beta_2$	$\alpha_M\beta_2$	$\alpha_X\beta_2$
	CR3	CR4

Table 1.2 Common nomenclature used for the β_2 integrins

Expression of the CD11/CD18 integrins is restricted to leucocytes. CD11a/CD18 is expressed by all leucocytes and increased surface expression has been associated with cytokine stimulation by Rousset *et al.*, (1989), who showed that IL-4 treatment of B cell lymphoma lines resulted in increased CD11a/CD18 expression at the cell surface. The conversion of T lymphocytes from a naive to a memory phenotype was shown to result in an increase in the levels of CD11a/CD18 (Chatila *et al.*, 1988) at the cell surface, although this has recently been questioned since a population of naive $CD8^+$ T lymphocytes, which express high levels of CD11a/CD18, have been identified (Okumura *et al.*, 1993a; Okumura *et al.*, 1993b). CD11b/CD18 and CD11c/CD18 are expressed by granulocytes, monocytes, macrophages, natural killer cells and some activated lymphocytes. The level of expression of CD11b/CD18 and CD11c/CD18 can be rapidly increased at the cell surface of monocytes and neutrophils. These cells store intracellular pools of CD11b/CD18 and CD11c/CD18, but not CD11a/CD18, which can be mobilised to the cell surface in response to stimulation with chemoattractants including fMLP and C5a, or phorbol ester (Springer *et al.*, 1984; Lanier *et al.*, 1985; Miller *et al.*, 1986). Stimulation of human peripheral B lymphocytes with phorbol esters results in *de novo* synthesis and increased expression of CD11c/CD18, but not CD11b/CD18 (Postigo *et al.*, 1991). In resting monocytes the relative amounts of these antigens is CD11a≥CD11b>CD11c, while the relative amounts expressed by tissue macrophages are CD11c>CD11a>CD11b (Miller *et al.*, 1986; Freyer *et al.*, 1988).

cDNA clones have been isolated for all three human CD11 subunits and the amino-acid sequence of the mature proteins deduced. The CD11a (Larson *et al.*, 1989), CD11b (Corbi *et al.*, 1988) and CD11c (Corbi *et al.*, 1987) have extracellular domains of 1063, 1092 and 1081 amino-acids respectively, containing seven homologous tandem

repeats (I-VII), a ~200 residue I domain located between domains II and III and three cation binding sites between domains V and VII. The CD11a, CD11b and CD11c subunits contain 29, 19 and 10 potential glycosylation sites respectively and these are located just outside of the cation binding and I domains. The subunits have transmembrane domains of about 26 residues and cytoplasmic tails of 53 (CD11a), 19 (CD11b) and 26 (CD11c) amino-acids. Comparison of the amino-acid sequences shows that CD11b and CD11c are more homologous to each other (63%), than to CD11a (35.7% and 37.4% respectively). Regions of highest homology include the divalent cation binding and transmembrane domains; over these regions CD11b and CD11c share 88% identity.

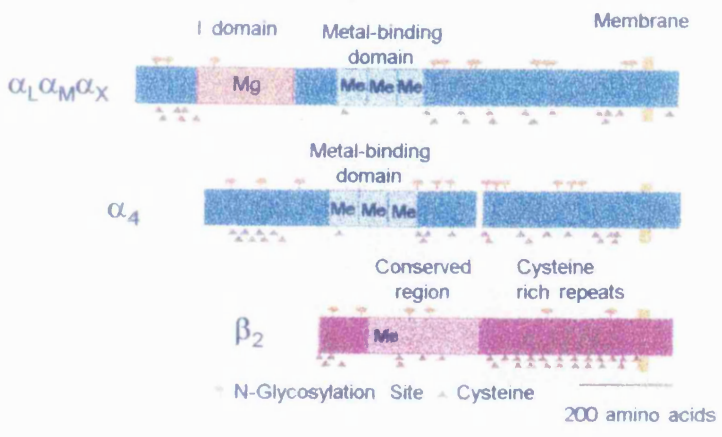

Figure 1.6. *Structural features of the CD11/CD18 integrin subunits, adapted from Springer (1994). The α_4 (CD49d) subunit is shown for comparison*

Molecular cloning of the CD18 subunit has shown that it is a 769 amino-acid glycoprotein with a 678 amino-acid extracellular domain and a 23 residue transmembrane domain which is followed by a cytoplasmic tail of 46 amino-acids (Kishimoto *et al.*, 1987; Law *et al.*, 1987). The sequence contains six potential glycosylation sites.

The complete sequence of murine (Wilson *et al.*, 1989), bovine (Shuster *et al.*, 1993) and avian CD18 (Bilsland and Springer, 1994) are available and share 82%, 83% and 64% identity with the human sequence. Areas of highest homology include a region of 245 amino-acids located towards the N-terminus of the extracellular domain and the cytoplasmic domains. The cytoplasmic domains of the β subunit have been found to be important in regulating the adhesive function of the integrins.

The CD11/CD18 integrins are able to interact with several cell-surface molecules and other soluble ligands (table 1.3). Studies using mAb, chimeric integrin molecules and soluble peptides have shown that the homologous I domain of the CD11/CD18 integrins and other I domain integrins acts as a major recognition site for ligand binding (Diamond *et al.*, 1993; Steegmaier *et al.*, 1995; Diamond *et al.*, 1995). The crystal structure of the I domain of CD11b/CD18 has been determined and the results show that a metal binding site is present at the surface of the I domain and this is thought to be involved in ligand interactions (Lee *et al.*, 1995).

CD11a/CD18	CD11b/CD18	CD11c/CD18
ICAM-1	ICAM-1	ICAM-1 ?
ICAM-2	iC3b	iC3b
ICAM-3	Fibrinogen	Fibrinogen
Bacterial LPS	Factor x	Bacterial LPS
	β-glucan	Heparin
	Heparin	Endothelium ?
	Bacterial LPS	
	NIF	

Table 1.3. Ligands known to interact with the CD11/CD18 integrins

The CD11/CD18 integrins must be activated before they are able to bind to their ligands. This activation step appears to promote a change in the 3-D conformation of the integrin molecule, as certain reporter mAb are able to bind to active CD11/CD18 molecules but not to the inactive molecules (Altieri and Edgington, 1988). Phorbol esters, cross-linking of the T cell receptor and CD3, or mAb binding to other molecules such as CD2 and CD45 trigger signals which promote CD11/CD18 integrin-ligand interactions, a process known as inside-out signalling. Also, specific anti-CD11 and anti-CD18 mAb have been described which are able to induce CD11a/CD18 and CD11b/CD18 adhesion in the absence of signalling events possibly by directly changing the 3-D conformation of the integrin molecule from an inactive to an active form (Keizer *et al.*, 1988; Andrew *et al.*, 1993; Petruzzelli *et al.*, 1995). CD11/CD18 integrin-mediated adhesion is dependent on the presence of the divalent cations Mg^{2+}, Ca^{2+} and Mn^{2+}, and cations have been shown to be involved in the regulation of integrin affinity. Purification of functionally active CD11/CD18 heterodimers seems to require the presence of cations during the isolation procedure which suggests that cations may be important in CD11 and CD18 subunit association (Stacker and Springer, 1991). Recently, Li *et al.*, (1995), demonstrated that a peptide derived from the CD11a/CD18 ligand, ICAM-2, induces CD11/CD18 integrin dependent adhesion which suggests that interaction with ligands may promote active CD11/CD18 conformation.

The CD11/CD18 integrins mediate a wide range of adhesion-dependent functions including: (i) conjugate formation between T lymphocytes during antigen presentation and cytotoxic killing (Krensky *et al.*, 1984; Keizer *et al.*, 1987), (ii) phorbol ester stimulated homotypic aggregation of leucocytes (Rothlein and Springer, 1986), (iii) binding of monocytes and neutrophils to iC3b (Wright *et al.*,1983; Myones *et al.*, 1988), (iv) phagocytosis of opsonized particles (Rothlein and Springer, 1985), (v) as receptors for bacteria (Rieu *et al.*, 1994) (vi) activation of eosinophils (Kaneko *et al.*, 1995) and (vii) adhesion of leucocytes to endothelial cells of post-capillary venules and HEV and migration of leucocytes to inflammatory sites (Anderson *et al.*, 1986; Keizer *et al.*, 1987; Hamann *et al.*, 1988; Stacker and Springer, 1991; Issekutz, 1992).

The functional importance of the CD11/CD18 integrins was highlighted when it was discovered that the leucocytes of patients suffering from an immunodeficiency disease

did not express CD11a/CD18, CD11b/CD18 or CD11c/CD18 (Anderson and Springer, 1987). The disease, termed Leucocyte Adhesion Deficiency syndrome or LAD, is the result of a mutation in the CD18 subunit, which consequently interferes with CD11 and CD18 subunit association preventing heterodimer expression at the cell surface. Molecular cloning of CD18 has shown that one of a number of mutations in CD18 can result in the disease. Patients suffer from recurrent bacterial infections which are associated with an inability of phagocytic cells to migrate to sites of infection. However, impaired lymphocyte functions are not generally seen and this is probably due to the expression of $\alpha_4\beta_1$ on lymphocytes, but not neutrophils, which can support lymphocyte-endothelial interactions (Kavanaugh *et al.*, 1991; Van Kooyk *et al.*, 1993). Patients who are moderately affected (3-10% of normal expression) may survive into adulthood if infections are treated, while those with less than 1% of normal CD11/CD18 expression rarely survive childhood unless they receive a bone marrow transplant.

1.4. IMMUNOGLOBULIN SUPERGENE FAMILY

Members of the immunoglobulin supergene family (IgSF) contain one or more immunoglobulin domains composed of 90-100 amino-acids arranged in a sandwich of two anti-parallel β-strands with a conserved disulphide bond between the sheets in most, but not all such domains (Williams and Barclay, 1988). The immunoglobulin domain structure appears as a common motif in many of the molecules involved in antigen recognition during the adaptive immune response including the TCR, surface immunoglobulin and MHC class I and II. Five members of the IgSF are involved in lymphocyte-endothelial cell interactions: ICAM-1, ICAM-2, VCAM-1, MAdCAM-1 and PECAM-1.

1.4.1. Intracellular adhesion molecule-1

ICAM-1, also known as CD54, was first identified by screening mAb for their ability to inhibit CD11a/CD18-dependent homotypic aggregation of lymphocytes (Rothlein *et al.*, 1986). Confirmation of the ICAM-1 and CD11a/CD18 interaction came from a series of studies in which purified ICAM-1-coated surfaces were shown to support CD11a/CD18-dependent lymphocyte adhesion, and from gene transfection studies (Makgoba *et al.*, 1988; Simmons *et al.*, 1988).

ICAM-1 is a 85-110 kDa transmembrane glycoprotein with a core protein backbone of 55 kDa. Molecular cloning showed that ICAM-1 contains 5 Ig-like domains (Simmons *et al.*, 1988; Staunton *et al.*, 1988), and these form an extended rod like structure in which a short hinge region separates the third and fourth Ig-like domains (Kirchhausen *et al.*, 1993). Eight potential N-linked glycosylation sites are contained in the sequence. ICAM-1 expression has been reported on endothelial cells, fibroblasts, lymphocytes, keratinocytes, synovial cells, monocytes, cells within the CNS, epithelial cells and hepatocytes. Generally ICAM-1 is expressed at low levels, though high levels of expression are induced following stimulation with inflammatory cytokines such as IL-1, TNF and INF-γ or LPS (Dustin *et al.*, 1986). Increased expression requires *de novo* protein synthesis. On cultured endothelial cells, increased expression is detected

after 4 hours and levels peak at 18-24 hours (Gamble *et al.*, 1985; Pohlman *et al.*, 1986). Soluble ICAM-1 is present in human serum. It contains most of the extracellular region of the membrane-bound form and is able to interact with LFA-1 (Rothlein *et al.*, 1991).

Deletion studies have identified discontinuous amino-acids in the first Ig-like domain of ICAM-1 as the primary CD11a/CD18 binding site, while CD11b/CD18 binds to a site in the third Ig-like domain (Staunton *et al.*, 1988). In addition, ICAM-1 acts as a receptor for the major group of rhinoviruses (Staunton *et al.*, 1989), the etiological agent in 40-50% of common colds. Virus binds to a region in the first Ig-like domain at a site distinct from the CD11a/CD18 binding site (Staunton *et al.*, 1988). ICAM-1 has also been shown to be a receptor for *Plasmodium falciparium*-infected erythrocytes (Berendt *et al.*, 1989). This may play a role in the pathogenesis of malaria, where binding of infected erythrocytes to endothelium in the liver is the primary step leading to complications in severe and cerebral malaria. The ICAM-1 binding site of infected erythrocytes is located in Ig-like domain 1, but is distinct from the CD11a/CD18 and rhinovirus binding sites (Ockenhouse *et al.*, 1992). Recent reports suggest that native ICAM-1 on the cell membrane and soluble ICAM-1 self associate to form a 200-220 kDa homodimer (Reilly *et al.*, 1995; Miller *et al.*, 1995). The transmembrane domain, the cytoplasmic tail and Ig-like domain 3 may be involved in dimer formation. ICAM-1 homodimers were found to support greater levels of CD11a/CD18-dependent cell adhesion than chimeric ICAM-1 monomers suggesting that dimerisation may play a role in the regulation of adhesion.

The gene encoding ICAM-1 is located on chromosome 19 and is composed of 7 exons. Recently, the production of ICAM-1-deficient mice was reported (Sligh *et al.*, 1993). The ICAM-1 gene in these mice was mutated in exon 5. mAb reactive with ICAM-1 were shown not to react with tissues from these animals and reverse transcription-polymerase chain reaction (RT-PCR) analysis showed that the normal cell surface form of ICAM-1 was not produced. Animals exhibited abnormal immune responses including decreased neutrophil migration in response to chemical peritonitis. Further studies however, detected some ICAM-1 expression in the lung and thymus of these animals and this was shown to result from alternative RNA splicing of the ICAM-1

gene. Three novel isoforms of ICAM-1 have been detected in both mutant and wild type mice and 2 of these are able to interact with CD11a/CD18 (King *et al.*, 1995). The biological significance of these novel ICAM-1 molecules is not clear.

1.4.2. Intercellular adhesion molecule-2

ICAM-2, or CD102, was identified by transfecting COS cells with an endothelial cDNA library and selecting for binding to CD11a/CD18 coated plates in the presence of a ICAM-1 blocking mAb (Staunton *et al.*, 1989). Subsequently, anti-ICAM-2 mAb were shown to inhibit adhesion of ICAM-2^+ COS cells to purified CD11a/CD18, and reduced the level of adhesion in CD11a/CD18-dependent leucocyte homotypic aggregation studies and leucocyte-endothelial cell interactions. The affinity of ICAM-2 for CD11a/CD18 seems to be lower than that for ICAM-1 (de Fougerolles *et al.*, 1991)

ICAM-2 has two extracellular Ig-like domains which share 34% homology to the two N-terminal domains of ICAM-1 and CD11a/CD18 is believed to interact with the first Ig-like domain of ICAM-2 (Nortamo *et al.*, 1991; Li *et al.*, 1993). The mature protein has a molecular mass of 55-65 kDa under reducing conditions (Nortamo *et al.*, 1991) and its tissue distribution is more restricted than that of ICAM-1. It is constitutively expressed by endothelial cells and mononuclear cells, unlike ICAM-1, expression is not increased by inflammatory cytokines (Nortamo *et al.*, 1991; de Fougerolles *et al.*, 1991). Recently ICAM-2 expression was detected on platelets (Diacovo *et al.*, 1994).

ICAM-1 and ICAM-2 are expressed by HEV in PLN and PP and on inflamed endothelial cells and binding of CD11a/CD18 to ICAM-1 and ICAM-2 appears to be an essential step for extravasation of lymphocytes across post-capillary vessels in the tissues and across HEV in the secondary lymphoid tissues.

1.4.3. Intercellular adhesion molecule-3

Anti-ICAM-1 and ICAM-2 mAb did not fully inhibit CD11a/CD18-dependent homotypic aggregation of some leucocyte cell lines suggesting that other CD11a/CD18 ligands existed (de Fougerolles *et al.*, 1991). ICAM-3 (CD103) was identified by specifically screening for mAb that completely inhibited adhesion of cell lines to purified CD11a/CD18 in combination with anti-ICAM-1 and ICAM-2 (Van Seventer *et al.*, 1992). Immunoprecipitation studies showed that it is a highly glycosylated protein of 124 kDa and cell type glycosylation differences are evident (de Fougerolles *et al.*, 1995). It is not expressed on resting or stimulated endothelium, but is highly expressed on resting lymphocytes, monocytes and neutrophils. Activation of lymphocytes with phorbol esters results in a two to threefold increase in ICAM-3 expression. Molecular cloning of ICAM-3 showed that it contains 5 Ig-like domains (Fawcett *et al,* 1992; Vazeux *et al.*, 1992). At the protein sequence level ICAM-3 is most like ICAM-1 sharing 47% identity overall and this rises to 77% in domain 2. Like ICAM-2, ICAM-3 binds to CD11a/CD18 but not CD11b/CD18 or CD11c/CD18 (de Fougerolles *et al.*, 1995) and site directed mutagenesis studies have shown that a cluster of 4 residues within domain 1 of ICAM-3 are important in this interaction (Holness *et al.*, 1995).

1.4.4. Vascular cell adhesion molecule-1 (VCAM-1)

VCAM-1, or CD106, was originally identified as a cytokine-inducible adhesion molecule on human endothelial cells which supported leucocyte binding (Osborn *et al.*, 1989). Expression is induced following stimulation of endothelial cells with cytokines including IL-1, IL-4, IL-13 and TNF and requires *de novo* protein synthesis with mRNA first seen at 2 hours (Thornhill *et al.*, 1990; Bochner *et al.*, 1995). Increased expression of VCAM-1 is maintained for 72 hours (Osborn *et al.*, 1989). In addition to endothelial cells, VCAM-1 is expressed by populations of dendritic cells in lymph nodes and skin (Freedman *et al.*, 1990) and cells within the inflamed joints of RA patients (Wilkinson *et al.*, 1993).

In the human, alternative splicing of a single VCAM-1 gene gives rise to two distinct isoforms. The predominant isoform is a 100-110 kDa protein containing seven Ig-like domains in the extracellular region, domains 1-3 are homologous to domains 4-6, a transmembrane region and a short cytoplasmic tail (Hession *et al.*, 1991). The second isoform lacks domain 4 (Osborn *et al.*, 1989). A variant form of VCAM-1 containing 8-domains has been described in the rabbit.

VCAM-1 is a ligand for $\alpha_4\beta_1$ and $\alpha_4\beta_7$ (section 1.3.3.) (Elices *et al.*, 1990; Ruegg *et al.*, 1992). Recent studies, using VCAM-1 deletion mutants and VCAM-1/ICAM-1 constructs, have shown that $\alpha_4\beta_1$ binds distinctly to domains 1 and 4 of VCAM-1 (Osborn *et al.*, 1994) and that a linear sequence of six amino-acids, identical in both of these domains, is involved in the $\alpha_4\beta_1$ binding site. This six amino-acid sequence may be a conserved integrin binding motif, as homologous sequences are present in other members of the IgSF (Vonderheide *et al.*, 1994).

1.4.5. Platelet/endothelial cell adhesion molecule-1 (PECAM-1)

PECAM-1, or CD31, is an adhesion molecule constitutively expressed by endothelial cells, and also on platelets, monocytes and neutrophils. PECAM-1 has been cloned (Newman *et al.*, 1990) and the translated sequence contains six Ig-like domains. The core protein molecular weight is 80 kDa, though the mature protein is highly glycosylated with an apparent molecular weight of 130 kDa. Alternatively spliced variants have been detected (DeLisser *et al.*, 1994).

PECAM-1 mediates neutrophil and monocyte-endothelial cell adhesion and transendothelial migration, and inter-endothelial cell adhesion. It may also be involved in migration of leucocytes through the extracellular matrix following transendothelial migration (Vaporciyan *et al.*, 1993; Muller *et al.*, 1993). The adhesion mediated by PECAM-1 involves both homophilic interactions (CD31-CD31) and heterophilic interactions (Muller *et al.*, 1992; DeLisser *et al.*, 1994). It has been proposed that homophilic binding sites are located within domains 2-3 and 5-6, though the loss of any one domain completely inhibits homophilic interactions possibly due to incorrect spacing

(Fawcett *et al.*, 1995). Heterophilic ligands for PECAM-1 have been identified as cell surface glycosaminoglycans (GAGs) and the integrin $\alpha_v\beta_3$ (DeLisser *et al.*, 1993; Piali *et al.*, 1995). PECAM-1-$\alpha_v\beta_3$ interactions, which are cation dependent, support leucocyte-endothelial interactions (Piali *et al.*, 1995). PECAM-1 is expressed on naive T cells and it may be involved in recirculation through secondary lymphoid tissues.

1.4.6. Mucosal addressin cell adhesion molecule-1 (MAdCAM-1)

MAdCAM-1 was first described as a 58-66 kDa antigen expressed by murine HEV in PP and on venules in the small intestine that was involved in lymphocyte adhesion (Streeter *et al.*, 1988). Molecular cloning showed that MAdCAM-1 contains 2 N-terminal Ig-like domains, which share homology with ICAM-1 and VCAM-1, followed by a mucin-like domain, an Ig-like domain, a transmembrane domain and a cytoplasmic tail (Briskin *et al.*, 1993). Purified MAdCAM-1 has been shown to support the adhesion of peripheral lymphocytes and cell lines (Nakache *et al.*, 1989). It serves as a receptor for both L-selectin, through its mucin-like domain, and $\alpha_4\beta_7$ which binds to the N-terminal Ig-domains (Berg *et al.*, 1993; Berlin *et al.*, 1993). The $\alpha_4\beta_7$/MAdCAM-1 interaction, together with L-selectin interactions, mediate most of the recirculation of lymphocytes through the intestinal tract.

1.5. OTHER MOLECULES INVOLVED IN LYMPHOCYTE-ENDOTHELIAL INTERACTIONS

1.5.1. CD44

CD44 is a family of cell surface glycoproteins which are highly expressed on T lymphocytes. It has been shown that CD44 supports the binding of lymphocytes to HEV in PP *in vitro* (Jalkanen *et al.*, 1986; Jalkanen *et al.*, 1987). Recently, Camp *et al.*, (1993) showed that mAb induced shedding of CD44 from the cell surface of leucocytes in mice reduced the level of oedema and leucocyte infiltration at sites of delayed-type hypersensitivity 24 hours after challenge. However, the number of lymphocytes in draining lymph nodes was not affected. These authors suggest that CD44 may be involved in leucocyte recruitment to inflammatory sites, but not in the normal recirculation of lymphocytes through lymphoid tissues. A possible ligand for CD44 in leucocyte migration to inflammatory sites is HA which has been shown to support CD44-dependent adhesion *in vitro* (Miyake *et al.*, 1990). A novel CD44 ligand, gp600, has recently been described and this may be important in CD44-dependent lymphocyte adhesion at inflammatory sites (Toyama-Sorimachi and Miyasaka, 1994; Toyama-Sorimachi *et al.*, 1995).

A more interesting role for CD44 in cell adhesion has recently been suggested. During extravasation, the integrin-dependent attachment step is essential for transendothelial migration. However, the integrin molecules are not constitutively active for their endothelial ligands and it is believed that chemoattractants are presented to rolling leucocytes at the site of extravasation by surface proteoglycans such as CD44. Tanaka *et al.*, (1993) showed that the chemoattractant macrophage inflammatory protein-1β, bound to CD44, stimulated $\alpha_4\beta_1$-dependent T lymphocyte adhesion to VCAM-1.

1.5.2. VAP-1 and VAP-2

Vascular adhesion protein-1 (VAP-1) is a 90 kDa endothelial adhesion protein that is expressed by HEV in most lymphoid tissues, although only low levels of expression are detected in the appendix and lamina propia of the intestinal tract, reviewed (Jalkanen and Salmi, 1993). Some expression is seen on the endothelium in non-lymphoid tissues including: brain, skin, kidney, heart and liver. Anti-VAP-1 mAb blocked lymphocyte binding to HEV *in vitro*. VAP-2 has also been identified and described as a 70 kDa endothelial molecule constitutively expressed on HUVECs and on sub-populations of endothelial cells in lymphoid and non-lymphoid tissues. It is also expressed on 20% of peripheral lymphocytes. Lymphocyte binding to HUVECs is inhibited by anti-VAP-2 mAb (Airas *et al.*, 1993).

1.6. THE USE OF THE RABBIT AS AN EXPERIMENTAL MODEL OF HUMAN DISEASE

The rabbit is a widely used experimental animal in biomedical research. This is attributable in part to its size, being small enough for handling and economical housing, but large enough to allow adequate blood volumes to be collected for analysis and for surgical manipulations which would prove to be too difficult in smaller mammals.

The rabbit has been used as a model of a number of human diseases including diabetes (Roth and Conaway, 1982), leprosy (Mshana *et al.*, 1983), bacterial meningitis (Tuomanen *et al.*, 1989), lung disease (Thompson and Myrcik, 1985) and syphilis (Wicher *et al.*, 1983). The Dumonde and Glynn rabbit model of rheumatoid arthritis closely resembles the human disease (Dumonde and Glynn, 1962). In this animal model, the injection of a soluble antigen into the knee joint of sensitised rabbits gives rise to an acute arthritis that persists for about one week followed by a chronic synovitis that generally lasts for several months. The rabbit rheumatoid arthritis model has been used to investigate novel therapeutic approaches to disease management (Jasin *et al.*, 1992). Atherosclerosis has been studied using the rabbit, of particular value is the Watanabe heritable hyperlipidemic rabbit as a model of familial hypercholesterolemia (Watanabe, 1975; Buja *et al.*, 1983).

The laboratory rabbit is readily infected with the human T lymphotropic virus type I (HTLV-1), the cause of Adult T-cell leukaemia which is endemic in southwestern Japan, the Caribbean islands and central Africa (Seto *et al.*, 1988; Akagi *et al.*, 1985). Studying this model has led to an increased understanding of viral transmission and disease progression and the rabbit model is being used to test drug and vaccine strategies (Uemura *et al.*, 1987). Rabbits also support persistent infection with the human immunodeficiency virus type I (HIV-1), although the usefulness of this model for testing anti-viral agents or vaccines is questionable since rabbits do not overtly show clinical signs of disease (Kindt *et al.*, 1992). Recently the construction of a rabbit with a human CD4 transgene has been described and this may be useful as a HIV-I infection model (Hague *et al.*, 1992).

Many of the diseases which have been studied in the rabbit have an immunological basis involving direct infection of leucocytes, as in HTLV-I infection, or inappropriate activation of the immune response, as in rheumatoid arthritis. A thorough knowledge of the rabbit immune system is necessary to allow the results of these investigations to be related to human disease. The structure of rabbit immunoglobulins has been studied in considerable depth (Kindt, 1975), as has the organisation of the MHC of the rabbit which appears to be similar to the human. mAb 2C4, which inhibits mixed lymphocyte reactions, recognises rabbit DQ class II antigens and has been used in this study (De Smet *et al.*, 1983). The isolation of rabbit leucocyte populations has received considerable attention and distinct populations of T and B lymphocytes have been described (Bast *et al.*, 1979). Further, the T lymphocyte population has been divided into cells which function as cytotoxic T lymphocytes or cells which function as helper T lymphocytes (Watkins *et al.*, 1984). mAb 12.C7 is believed to recognise CD8, though this has not been confirmed by the isolation of the molecule (De Smet *et al.*, 1983). Other defined leucocyte populations include granulocytes and monocytes.

Some anti-human mAb have been found to be cross-reactive with rabbit cells. One of these species cross-reactive mAb, 60.3, has been particularly useful for studying cell migration during inflammation in rabbit models. mAb 60.3 recognises CD18 and inhibits the recruitment of neutrophils to inflammatory sites *in vivo* (Price *et al.*, 1987; June *et al.*, 1990; Argenbright *et al.*, 1991), and the tissue damage associated with leucocyte activity following ischemia and reperfusion (Vedder *et al.*, 1990). Recently mAb L13/64 has been shown to recognise rabbit CD18. As in the human, rabbit CD18 associates with three distinct α-subunits presumably CD11a, CD11b and CD11c (Galea-Lauri *et al.*, 1993). mAb 198 recognises rabbit CD11b and has been shown to inhibit phagocytosis of opsonised particulate antigens (Smet *et al.*, 1986). Rabbit CD11a and CD11c have not been characterised. The CD11/CD18 integrin ligand, ICAM-1, has been detected in the rabbit using the anti-human ICAM-1 mAb R6.5. R6.5 inhibits neutrophil infiltration into lungs during a phorbol ester-induced inflammatory lung model in rabbits (Barton *et al.*, 1989).

Inappropriate activation, migration and accumulation of leucocytes is a feature of graft rejection, atherosclerosis and autoimmune diseases such as rheumatoid arthritis and multiple sclerosis. Investigators have used mAb recognising a variety of cell adhesion molecules to identify those involved in this inappropriate leucocyte accumulation and the results of these studies suggest that therapy which interferes with cellular adhesion may be useful in treating a number of diseases. To allow the development of possible therapeutic agents, further investigations are required to gain a thorough understanding of the nature of leucocyte-leucocyte and leucocyte-endothelial cell interactions in the human, and in species used as models of human disease. Although the rabbit continues to be the experimental model of choice for many research groups and may be valuable in the development of novel vaccines and anti-inflammatory agents, its use is becoming limited because of a lack of mAb reactive with cell surface antigens. The aims of this project are:

(i) Screen panels of anti-human mAb for reactivity with rabbit leucocyte antigens and determine the cellular distribution of these antigens on rabbit leucocytes.

(ii) Produce and characterise mAb that distinguish rabbit leucocyte sub-populations.

(iii) Develop and characterise mAb that react with the CD11/CD18 integrins and determine whether these mAb can functionally inhibit cell adhesion *in vitro*.

(iv) Evaluate the potential of anti-adhesive mAb in preventing induced inflammatory diseases *in vivo*.

(iv) Determine whether the rabbit CD11/CD18 proteins are homologous to their human counterparts.

These studies have resulted in the development of mAb which recognise the rabbit equivalents of CD4, CD28, CD43, CD44 and CD45. Anti-rabbit CD11a, CD11c and CD18 mAb have also been generated and used to investigate the expression of the CD11/CD18 integrins on rabbit leucocyte cell populations and the role of the CD11/CD18 integrins in cell adhesion processes *in vitro* and *in vivo*. Amino-acid sequencing of purified rabbit CD11c and molecular cloning of rabbit CD18 has shown that the rabbit molecules share a high level of homology with their human counterparts. Publications which have arisen from this work are given at the end of this thesis.

mAb	Anitgen	Reference
Ken-4	CD4	Kotani *et al.*, 1993
12.C7	CD8	De Smet *et al.*, 1983
Ken-5	CD5 (p)	Kotani *et al.*, 1993
RCN1/21	CD18	Galea-Lauri *et al.*, 1993
198	CD11b	Smet *et al.*, 1986
L13/64	CD18	Jackson *et al.*, 1983,
L13/74	CD18	Jackson *et al.*, 1983, Galea-Lauri, 1992
L13/103	CD18	Jackson *et al.*, 1983, Galea-Lauri, 1992
Kei-α-1	CD25	Kotani *et al.*, 1993
2C4	Class II (RD-Q)	Lobel *et al.*, 1984
RPN3/57	n/d	Galea-Lauri, 1992
W4/28	n/d	Jackson *et al.*, 1983
L11/135	CD43	Jackson *et al.*, 1983, Wilkinson *et al.*1984
ACM-1	n/d	Chen *et al.*, 1984
9AE10	20-25 kDa	McNicholas *et al.*, 1981, Chen *et al.*, 1984

Table 1.4. Anti-rabbit monoclonal antibodies used during this investigation

(n/d = not determined, p = putative)

mAb	Antigen	Reference
T1/7	chicken troponin T	Bird *et al.*, 1985
T1/61	chicken troponin T	Bird *et al.*, 1985
R7.1	CD11a	Argenbright *et al.*, 1991
R15.7	CD18 (canine)	Entman *et al.*, 1990 Argenbright *et al.*, 1991
60.3	CD18	Beatty *et al.*,1983
IB4	CD18	Wright *et al.*, 1983
7E4	CD18	Lindbom *et al.*, 1990
RR1/1	ICAM-1	Rothlein *et al.*, 1986
AIIB2	CD29 (β_1)	Hemler, 1990
HP1/2	CD49d (α_d)	Sanchez-Madrid *et al.*, 1986
BIIG2	CD49e (α_e)	Hemler, 1990
GoH3	CD49f (α_f)	Hemler, 1990

Table 1.5. Species cross reactive mAb used during this investigation

CHAPTER TWO

MATERIALS AND METHODS

2.1. Maintaining Cell Lines in Culture and Isolation of Rabbit Lymphoid Cell Populations

2.1.1. Maintenance of rabbit T cell lines in culture

Three rabbit T cell lines, RL-5 (Daniel *et al.*, 1974) BJ-610 (Wilkinson *et al.*, 1992) and BJ/873 (Blackford *et al.*, 1996) were maintained in culture at a density of 5 x 10^5/ ml in RPMI-1640 containing 10% heat inactivated FCS, 100U/ml penicillin, 100μg/ml streptomycin, 2mM L-glutamine and 20ng/ml IL-2, supplied as human recombinant IL-2. Cells were cultured at 37°C in a humidified incubator with 5% CO_2.

2.1.2. Freezing for long term storage

Cells were counted, washed in sterile PBS and resuspended at a density of 5 x 10^6 to 1 x 10^7/ml in RPMI-1640 containing 20% FCS and 10% DMSO. Cells were aliquoted (1ml) into sterile cryo-vials, stored at -80°C in a polystyrene box overnight and transferred to liquid nitrogen storage tanks.

2.1.3. Thawing cells from liquid nitrogen storage

Vials were thawed in a 37°C water bath, the cell suspension was transferred to a 15ml tube, diluted with 10ml of medium and centrifuged at 160g for 10 minutes. Cells were cultured at 5 x 10^5 overnight in RPMI-1640/20% FCS and antibiotics.

2.1.4. Isolation of rabbit peripheral mononuclear cells

Peripheral mononuclear cells were isolated from whole blood according to a method adapted from Boyum, (1968). Blood, 9ml, was collected from the marginal ear vein of New Zealand White or ½ Sandlop rabbits into syringes containing 1ml of acid citrate dextrose as anti-coagulant (trisodium citrate 85mM, citric acid 70mM and glucose 110mM). Blood was transferred to a 50ml tube, diluted with 10ml PBS pre-warmed to 37°C and 6ml of this was carefully layered over 3ml of NycoPrep Animal separation media in a 15ml centrifuge tube. Tubes were spun at 600g for 15 minutes at 22°C in a Beckman J-6 centrifuge (brake off) and the mononuclear cells were harvested from the interface between the upper plasma layer and the lower NycoPrep layer using a plastic Pasteur pipette. The cell layer was transferred to a fresh 15ml tube, PBS was added to 15ml and the cells pelleted by centrifugation at 400g for 15 minutes at 22°C. Cells were resuspended in PBS, counted, pelleted and resuspended at 2 x 10^6 for flow cytometry.

2.1.5. Isolation of rabbit peripheral neutrophils

Neutrophils were isolated from the pellet produced during density gradient centrifugation of whole blood. Following isolation of the mononuclear rich interface layer (2.1.4), plasma and Ficoll layers were removed and the remaining red cell pellet was diluted with 9ml of warmed PBS. To remove erythrocytes, 1ml of 6% dextran/PBS was added and the tube was incubated at 37°C for 30 minutes. The neutrophil rich supernatant was collected, transferred to a fresh 50ml tube, PBS was added to 50ml and the cells pelleted by centrifugation at 400g for 15 minutes at 22°C. Cells were resuspended in PBS, counted, washed again and resuspended at 2 x 10^6 before staining for flow cytometry.

2.1.6. Isolation of cells from lymphoid tissues

Rabbit thymus, spleen, lymph node and bone marrow were collected and placed into 50ml tubes containing 10ml RPMI-1640. The contents of a tube were transferred to a sterile petri dish and the cells released by disrupting the organ using the flat top of the plunger from a sterile, 1ml syringe while holding the tissue with forceps. Cells were resuspended and passed through sterile gauze into a 50ml tube and 30ml of PBS was added. Cells were pelleted by centrifugation at 200g for 10 minutes, resuspended and washed with PBS. Finally, cells were resuspended in PBS, counted and diluted to a density of 2×10^6 ml.

2.1.7. Cell counting and viability testing

From a cell suspension a 25μl sample was taken and added to 25μl of 0.1% trypan blue in a microfuge tube. The coverslip was placed over the counting chambers of a Neubauer haemocytometer and a drop of the cell/trypan blue suspension was drawn under the coverslip. The cells which settled in the 25 square grid of the counting chamber were counted using the 40x objective lens, cells which had taken up the dye (dead cells) were excluded from the total. Two counts were taken for each cell population and an average count calculated. The square represents 1×10^{-4} ml, thus the number of cells/ml can be determined with the following formula:

$$\text{Average cell count} \times 10^4 \times 2 \text{ (dilution factor)} = \text{N}^\circ \text{ cells/ml}$$

$$\frac{\text{Live cell count}}{\text{Total cell count}} \times 100 = \% \text{ viability}$$

2.2. Production of Monoclonal Antibodies

2.2.1. Immunisation Strategy

Female Balb/C mice were immunised with RL-5 or BJ/873 cells. Cell lines were grown in continuous culture as described in 2.1.1, washed twice with sterile PBS and resuspended at 1 x 10^7/ml. The mice were injected, intraperitoneally, with 0.5ml of the cell suspension. Four similar immunisations were given at 14 day intervals. A final tail vein injection of 5 x 10^6 cells in 0.5ml PBS was given 3 days prior to fusion. To ensure that mice had mounted an immune response a small amount of blood was taken from the periorbital cavity using a glass Pasteur pipette and transferred to a microfuge tube. The blood was left to coagulate at room temperature for 1 hour and incubated at 4°C for 2-3 hours to allow the clot to retract. Serum was collected and the presence of antibody determined by immunofluorescence staining of the cell line originally used to immunise the animal.

2.2.2. Preparation of splenocytes

On the day of fusion a second sample of blood was taken using the method described in 2.2.1. and serum prepared and stored at 4°C to be used as a positive control in hybridoma screening assays. Mice were sacrificed by cervical dislocation, laid with left side uppermost and the abdominal area was covered with 70% ethanol. Using autoclaved instruments, the abdominal skin was snipped, pulled apart and the exposed area sprayed with 70% ethanol. The spleen was removed and placed into a 50ml tube containing 10ml of warmed RPMI-1640/10% FCS. Under sterile conditions, the spleen was transferred to a petri dish, excess connective tissue was removed and the organ capsule gently punctured, using the plunger of a 1ml syringe, to release splenocytes. The splenocytes were resuspended, transferred to a 50ml tube and 30ml of warmed RPMI-1640 was added. The cell suspension was passed through a cell strainer into a fresh 50ml tube and centrifuged at 200g for 10 minutes. The cell pellet was resuspended to 20ml RPMI-1640/10% FCS and transferred to a petri dish which was placed in a 37°C incubator for 2 hours to remove monocytes and fibroblasts, by plastic

adherence, from the cell population. After this incubation, splenocytes were transferred to a 50ml tube containing 30ml RPMI-1640, counted, pelleted and resuspended at 5 x 10^6/ml.

2.2.3. Preparation of the fusion partner cells

The plasmacytoma line SP2/0 was the fusion partner used throughout this study. Ten days before fusion, cells were thawed from frozen stocks and grown in culture at a cell density of 5 x 10^5/ml in RPMI-1640/10% FCS. On the day of fusion cells were counted, and resuspended at 1 x 10^6/ml RPMI-1640/10% FCS.

2.2.4. Fusion of splenocytes with plasmacytoma cells

The procedure used for the production of hybridoma cell lines was adapted from the original method described by Kohler and Milstein, (1975). Splenocytes and SP2/0 cells suspensions were combined at a ratio of 1:10, the cells were pelleted, resuspended in 50ml of warmed RPMI-1640 and centrifuged again at 600g. Fusion was induced by the addition of a 50% solution of polyethanol glycol (PEG). Previously, 2g aliquots of PEG were weighed into universal bottles and sterilised by autoclaving. On the day of fusion, an aliquot was diluted with 2ml RPMI-1640, heated to 50°C to allow the PEG to dissolve and kept at 37°C until required. Using a 1ml disposable glass pipette, 1ml of PEG solution was mixed with the pelleted cells over 1 minute. The cells were stirred for a further minute and the PEG diluted on the addition of warm RPMI-1640/10% FCS; 1ml over the first minute followed by 20ml over the next 5 minutes. The cell suspension was centrifuged at 200g for 10 minutes. Hybridomas were selected by one of the following two methods:

Method A: Cell were resuspended in 150ml RPMI-1640 containing 10% FCS, antibiotics, 5.8μM 8-azaserine and 100μM hypoxanthine (Foung *et al.*, 1982). The suspension was plated at 300μl/well of 5, 96-well plates and incubated at 37°C in a humidified incubator with 5%CO_2. Wells were aspirated at day 4 and fed with the same medium.

Method B: Cells were resuspended into 150ml RPMI-1640 containing 15% FCS, 10% H-1 Condimed, 2mM glutamine, 1mM sodium pyruvate, 5μM 2-mercaptoethanol and hybridoma selection was made using HAT selection media (final concentration of 100μM hypoxanthine, 0.4μM aminopterin and 16μM thymidine) (Littlefield, 1964). The cell suspension was plated into 10, 96-well tissue culture plates which were incubated at 37°C in 5% CO_2. On day 4, wells were fed with 150μl/well of the same media.

2.2.5. Preparation of cellular ELISA plates for primary screening

Flat bottomed, 96 well microtitre plates were coated with 50μl poly-L-lysine (1mg/1ml) for 30 minutes at room temperature (Landsdorp *et al.*, 1980). RL-5 or BJ/873 cells were washed and resuspended in PBS at 2 x 10^6/ml. Excess poly-L-lysine was removed by flicking and 50μl of the cell suspension was added to each well. Plates were centrifuged at 200g for 5 minutes and the cell monolayer fixed by adding 50μl of 0.05% glutaraldehyde/PBS followed by incubation for 15 minutes at room temperature. Plates were washed 3 times with PBS, 150μl of 0.02% gelatin/PBS (type II, swine skin) was added and the plates were incubated for 1 hour on the bench. Plates were either used immediately or wrapped in cling film and stored at -20°C for a maximum of 3 months.

2.2.6. Screening of hybridoma supernatant

Approximately 10 days after fusion, the yellowed supernatant from hybridoma-containing wells was screened for activity by cellular ELISA using glutaraldehyde-fixed cells as the target antigen (2.2.5). All stages were performed at room temperature and the wash buffer used was PBS/0.05% Tween 20. Cellular ELISA plates were thawed, washed twice, flicked to remove excess wash buffer and 50μl of TCSN from a single well of a fusion plate was added to 2 wells of the ELISA plate and left to react for 1 hour. TCSN was flicked out and plates were washed 3 times. Polyclonal goat anti-mouse HRP mAb was diluted 1:2000 in wash buffer and 100μl of this was added to each well. Plates were incubated for 1 hour. After 4 washes, the specifically bound

HRP was detected on the addition of enzyme substrate (H_2O_2, 3.52mM) and chromogen (O-phenylene diamine, 2.21mM) in a phosphate-citrate buffer. OPD was dissolved in HPLC grade methanol at 10mg/ml, 1ml of this and 10μl of H_2O_2 was added to 24ml of phosphate citrate buffer (0.1M citric acid, 0.2M Na_2HPO_4 pH 5.0) and 100μl of this substrate solution was added to the plates which were incubated, in the dark, for 20 minutes. The reaction was inhibited by the addition of 50μl of 2.5M H_2SO_4 and plates were read at 492nm using a MINIREADER-II (Dynatec) plate reader.

Supernatant from positive wells was re-screened by surface immunofluorescence of RL-5 and BJ/873 cells and cells from wells positive by both assays were transferred to 24 well plates and the culture expanded overnight. Selected wells were cloned (2.2.7), stocks frozen, and TCSN grown to be used as controls in later screening stages.

2.2.7. Cloning of selected hybridomas

To obtain a monoclonal cell line, hybridomas were cloned a minimum of 3 times or until all the wells in a cloning plate tested positive. Hybridomas were resuspended, counted and a sample taken and diluted to 1 x 10^3 cells/ml RPMI-1640/10% FCS (1 cell/μl). Generally, hybridomas were cloned in 3 plates at 1, 2 and 4 cells/well using one of the following methods:

Method A: The spleen was removed from a Balb/C mouse, using the method described in 2.2.4, and disrupted to release the cells. The cells were resuspended into 40ml RPMI 16-40 medium and passed through a cell strainer. To clone at 1 cell/well, 100μl of the hybridoma cell suspension was added to 5ml of spleen cells, cells were diluted with 10ml RPMI16-40 and this suspension was plated over a 96-well plate. Plates were incubated at 37°C in a humidified incubator with 5% CO_2 for 10 days.

Method B: To clone at 1 cell/well, 100μl of the diluted sample was added to 30ml of RPMI-1640 containing 15% FCS and 10% H-1 Condimed and this was plated into 1, 96 well plate at 300μl/well. Plates were incubated as above.

Once yellowed, the TCSN from wells containing single growth foci were re-screened by cellular ELISA as described in 2.2.6. Cells from 3 positive wells were transferred to a 24 well plate, expanded and stocks frozen. The cells from a fourth well were recloned and the remaining cells from that well were cultured in a 24 well plate and frozen. After the final cloning, the new hybridoma cell line was expanded, stocks established and TCSN grown up.

2.2.8. Production of Tissue Culture Supernatant

Hybridoma cell lines were grown in RPMI-1640 containing 5-10% FCS at a density of 5 x 10^5 cell/ml. Cultures were gradually expanded into 200ml flasks, transferred to spinner culture flasks and grown to a volume of 800ml. The cultures were allowed to "yellow out" and when the majority of cells had died, the culture was centrifuged at 3000g for 30 minutes at 4°C in a J-6 centrifuge (Beckman). The supernatant was carefully collected and, if to be used as TCSN only, filtered using a Milipore syringe filtration unit (Swinnex-25) through Whatman glass microfibre filters. Sodium azide was added to 3mM and the TCSN stored at 4°C short term, or -80°C long term.

For mAb purification, supernatant was made up to a final concentration of 1.5M glycine and 3M NaCl, and the pH was adjusted to 8.9 with 5M NaOH. This was filtered through glass fibre filter paper ready to be passed down a protein A-column (2.2.9).

2.2.9. Purification of TCSN by affinity chromatography

Mouse mAb were purified from tissue culture supernatant by affinity chromatography using protein A-Sepharose CL-4B beads which were packed into a column. The method, originally described by Ey *et al.*, (1978), was taken from Pharmacia Separation News, 13.5. Protein A-Sepharose beads, 0.5g, were swollen in 10ml PBS and left to rotate at 4°C for 1 hour. The gel was centrifuged at 400g for 5 minutes, washed with 10ml of PBS, pelleted and resuspended into 10ml of PBS. This was transferred to a 1 x 10 cm BIORAD glass column and allowed to settle under gravity. The column was

used repeatedly, being washed after each use with 100mM citric acid, pH 3.0, followed by PBS and stored in PBS/0.05% sodium azide at 4°C.

Before use, the column was removed from storage, stirred and allowed to settle. A peristaltic pump was attached and set to run at 1ml/minute. Storage PBS/sodium azide was removed by passing 50ml PBS through the column. It was then equilibrated with 50ml of 1.5M glycine, 3M NaCl titrated to pH 8.9 with 5M NaOH (binding buffer). The sample was run through overnight and, the following morning, the column was washed extensively with binding buffer. Bound mAb was eluted with 100mM citric acid adjusted to either pH 5.0 for IgG1 antibodies or, to pH 3.5 for IgG2b antibodies. Fractions were collected, 10 x 1ml, and the absorbance of each read at 280nm, those with a reading above 0.4 were pooled and dialysed against PBS overnight. The absorbance of the dialysed sample was read at 280nm and the protein concentration calculated using an extinction coefficient:

$$E^{1\%,\ 1cm}_{280} \ of\ 14$$

Purified mAb was either filter sterilized or made to 3mM sodium azide, aliquoted into 1ml vials and stored at 4°C or -80°C.

2.2.10. Determination of mAb purity

The purity of mAb was assessed by SDS-PAGE as described in 2.4.3. mAb, 5μg, was added to 5μl of 2x SDS-sample buffer, the solution was boiled for 5 minutes and run on a 10% SDS-PAGE gel. The gel was stained for protein with Coomassie Blue R250 and destained overnight as described in 2.4.4.

2.2.11. Determination of mouse immunoglobulin class and sub-class

The class/sub-class of mAb was determined using an isotyping kit (Serotec) which was specifically designed, in collaboration with Coombs *et al.*, (1987), for the identification of mouse monoclonal antibodies in TCSN. It is based upon red cell agglutination and gives positive results in 1 hour. The kit has 8 reagent vials containing a suspension of sheep red blood cells (SRBC) coated with rat anti-mouse-Ig mAb, negative control SRBC are coated with irrelevant mAb and the positive control SRBC are coated with polyclonal rat anti-mouse-Ig. TCSN was diluted 1:50 with PBS and 30μl was added to 8 wells across a round bottomed microtitre 96 well plate. From each reagent vial, 30μl was added to a single well, the plate was gently mixed and left on a flat surface to develop for 1 hour. Negative reactions appear as a button of cells at the bottom of the well, the cells having settled as single cells. Positive reactions, in which the rat-anti-mouse Ig binds to the isotype to which it was directed producing a lattice of cells, give a cloudy appearance in the well.

2.2.12. Preparation of Fab and F(ab')$_2$ fragments of mAb BJ3/22

mAb BJ3/22 was purified from tissue culture supernatant by affinity chromatography on protein A-Sepharose as described in section 2.2.9.

Fab fragments: Fab fragments were produced by digestion with immobilised papain. Papain slurry, 0.5ml, was washed 2 times with 4ml of PBS containing 20mM cysteine-HCl, 10mM EDTA, at pH 7.0. The slurry was resuspended in 0.5ml of the same buffer and this was added to 1ml of purified protein at 2.75mg/ml and the digestion was left to proceed on a roller, overnight at 37°C. The following morning, 1.5ml of buffer (10mM Tris-HCl, pH 7.5) was added and the sample was centrifuged at 400g for 5 minutes. The supernatant was transferred to a fresh tube and made up to 3.5M NaCl, pH 8.8 and passed through a column of protein A-Sepharose to remove uncleaved IgG and Fc. Unbound material containing Fab fragments was dialysed extensively against PBS at 4°C.

$F(ab')_2$ Fragments: $F(ab')_2$ fragments were obtained following digestion with bromelain. Bromelain was activated in buffer containing 1.0M Na acetate, 3mM EDTA, 50mM cysteine, pH 5.5, at 37°C, according to the manufacturer's instructions. 100μl of activated bromelain was added to 10mg mAb BJ3/22 and the digestion was left to proceed on a roller at 37°C. Digestion was inhibited after 2 hours by the addition of protease inhibitors (0.5mg/ml each of antipain and leupeptin) and incubation at 4°C. The sample was made to 3.5M NaCl, pH 8.8 and passed through a protein A-Sepharose column to remove uncleaved IgG and Fc. Bromelain was removed from the $F(ab')_2$ sample by chromatography on DEAE Sepharose, equilibrated with 20mM Tris-HCl, pH 7.8. $F(ab')_2$ was eluted with the same buffer containing 0.1M NaCl and dialysed against PBS.

The absorbance of the dialysed $F(ab')_2$ and Fab samples was measured and the protein concentration of the samples calculated. Samples were analysed by SDS-PAGE and shown to be free from contaminating Fc and whole IgG.

2.3. Immunofluorescence Staining for Flow Cytometry

2.3.1. Conjugation of fluorescein isothiocyanate to antibody

mAb were conjugated to fluorescein isothiocyanate according to the method of Wood *et al.*, (1965). Purified mAb was dialysed for 18 hours against borate saline buffer (1.035M $Na_2B_4O_7$ and 0.08M NaCl, pH 9.2), protein concentration was determined spectrophotometrically and the sample was diluted to 0.5mg/ml in PBS. FITC was dissolved in 100% ethanol, diluted to 1mg/ml with borate saline buffer and 50μl was added to 1ml of diluted mAb. The reaction was allowed to proceed for 2 hours at 37°C. mAb was dialysed extensively against PBS to remove unconjugated FITC, filter sterilised and stored in the dark at 4°C.

2.3.2. Conjugation of biotin to antibody

Biotinylation of mAb was performed according to the method of Bayer and Wilchek, (1980). Purified mAb was dialysed for 18 hours against 0.1M $NaHCO_3$ buffer, protein concentration was determined and mAb was diluted to 1mg/ml. To 1ml of the sample, 0.2mg N-hydroxysuccinimidobiotin, from a stock of 1mg/ml in DMSO, was added and left to react for 4 hours at room temperature. Unconjugated biotin was removed by extensive dialysis against PBS and the conjugated mAb was filter sterilised and stored at 4°C.

2.3.3. Single colour analysis

Cultured cells were washed in PBS and resuspended at 10^6/ml in PBS containing 1% BSA and 0.05% NaN_3 (FACS-buffer). Isolated rabbit cell populations were resuspended at $1x10^6$ into FACS-buffer supplemented with 1% heat inactivated normal rabbit serum. Cells, 10^6, were dispensed into Falcon 12 x 75mm round-bottomed polystyrene tubes and pelleted in a Beckman J-6B centrifuge at 200g for 5 minutes at 4°C. Tubes were inverted to remove wash buffer. The cells were then gently resuspended in 100μl of FACS-buffer containing 10μg purified mAb or 100μl of TCSN and incubated on ice for 30 minutes. Cells were washed twice with 2ml of FACS-buffer as before, resuspended in 100μl of FACS-buffer containing 1:100 dilution of $F(Ab')_2$ rabbit anti-mouse Ig FITC-conjugate and incubated on ice for 30 minutes. Finally, cells were washed twice and resuspended in 0.5ml PBS/0.05% NaN_3 for immediate data collection, or were fixed by resuspending into PBS containing 0.05% NaN_3 and 1% paraformaldehyde and stored at 4°C in the dark to be analysed the next day. Flow cytometry was performed on a FACScan machine (Becton Dickinson, Immunoctometry systems, Mt. View, CA.) and data was collected and analysed using the consort 30 program supplied by Becton Dickinson.

2.3.4. Dual colour analysis

Cell populations were washed, resuspended at 10^6/ml in FACS-buffer and incubated on ice for 30 minutes with 10-20μg FITC-conjugated primary mAb. Alternatively, if insufficient mAb was available for fluorescination, cells were stained with unlabelled primary mAb, washed and incubated with FITC-conjugated mAb as in single colour analysis 2.3.4. After washing, cells were incubated with 10-20μg of primary biotinylated mAb as before. Cells were washed, resuspended in 100μl of FACS-buffer containing streptavidin-phycoerythrin (PE) conjugate, incubated for 10 minutes and washed twice prior to analysis.

2.3.5. Inhibition of direct immunofluorescence

To determine if mAb recognising the same molecule have related epitopes, inhibition of direct labelling was assessed. Cells were prepared and resuspended in increasing amounts of the first mAb as described for single colour analysis (2.2.3) and incubated on ice for 30 minutes. Following two washing steps, cells were incubated with 20μg of a second competitive mAb which had been conjugated to FITC. The cells were washed again and resuspended for analysis.

2.3.6. Staining of cultured cells for cell sorting

All procedures for staining of cells and sorting by FACS were carried out under sterile conditions. Cells, 2 x 10^7, were washed twice with RPMI-1640, resuspended into 100μl of RPMI-1640/10% FCS and 100μl of azide free mAb TCSN and incubated on ice for 30 minutes. Cells were washed twice, resuspended into 1.5ml RPMI-1640/10% FCS containing FITC-conjugated secondary mAb as described (2.3.3.) and incubated on ice for 30 minutes. Finally, cells were washed twice, resuspended at 1 x 10^6 in RPMI-1640/0.5% FCS and sorted using a Becton Dickinson Cell Sorter and Lysis II software. Sorted cells were cultured in RPMI-1640 containing 15% FCS, antibiotics and Fungizone.

2.4. Separation of Proteins by SDS-polyacrylamide Gel Electrophoresis (SDS-PAGE)

Cell proteins were separated by SDS-PAGE according to the method of Laemmli (1970), transferred to nitrocellulose membranes and analysed by immunostaining. Proteins immunoprecipitated from cell lysates were also resolved by SDS-PAGE.

2.4.1 Reagents used in SDS-PAGE

2x SDS sample buffer

reduced samples	*non-reduced samples*
125mM Tris, pH 6.8	125mM Tris, pH 6.8
4% SDS	4% SDS
20% glycerol	20% glycerol
50mM iodoacetamide	10% β-mercaptoethanol
0.025% bromophenol blue	0.025% bromophenol blue

Polyacrylamide gel stock solutions

Bis-acrylamide	29.2% acrylamide, 0.8% bis-acrylamide, stored 4°C
Stacking gel buffer	0.5M Tris-HCl, pH 6.8, stored 4°C
Resolving gel buffer	2M Tris-HCl, pH 8.8, stored at 4°C
SDS	10%
Ammonium persulphate	10% freshly prepared solution
Electrophoresis buffer	10x, 0.25M Tris, 0.192M glycine, 0.1% SDS

2.4.2. Preparation of proteins for SDS-PAGE

Cells were washed twice in sterile PBS and resuspended at 1 x 10^8/ml in distilled H_2O. The cell suspension was mixed with an equal volume of 2x SDS sample buffer and immediately boiled for 5 minutes. Next, the sample was centrifuged at 100 000g in a MSE ultra-centrifuge for 60 minutes at 4°C to remove released DNA and the supernatant was collected and stored at -20°C.

2.4.3. Resolution of proteins using the discontinuous system

Gels were prepared as follows:

Stacking gel	*Resolving gel*
4.5% acrylamide	7, 8, 10, 15% acrylamide
0.125M Tris-HCl, pH 6.8	0.38M Tris HCl, pH 8.8
0.1% SDS	0.1% SDS

Polymerisation of the gels was induced on the addition of TEMED (final concentration of 6.67mM) and ammonium persulphate (final concentration 2.19mM). Gels were cast and run in the BioRad Mini-protean II system (8 x 7cm, 0.75mm thickness) or the Protean II xi system (16 x 20cm, thickness 1.5mm) according to the manufacturer's instructions. For mini-gels, wells were filled with 8-10μl of SDS-cell extracts using a Hamilton syringe and the gel was run at a constant current of 19mA until the bromophenol blue tracking dye reached 0.5cm from the end of the gel. The wells of large gels were filled with 60μl of sample and run at a constant current of 25mA.

2.4.4. Staining of proteins with Coomassie Blue

Coomassie Blue staining solution	*Destain solution*
0.2% Coomassie brilliant blue R250	7% acetic acid
45% methanol	20% methanol
10% acetic acid	73% H_2O
45% H_2O	

Gels were immersed in Coomassie blue staining solution and left to stain for 30 minutes. Next, the gels were destained for a minimum of 2 hours in several changes of destain solution. The gels were stored in destain solution until the proteins were transferred to nitrocellulose.

2.4.5. Transfer of separated proteins to a membrane support for immunoblotting

Proteins were transferred from Commassie stained gels to nitrocellulose according to method described by Jackson and Thompson (1984). Before transfer, gels were equilibrated in 25mM Tris, 190mM glycine and 1% SDS, for 20 minutes at room temperature. Electroblotting was performed using the semi-dry transfer method described by Kyhse-Anderson (1984), using a 'Sartoblot' apparatus. Three sheets of Whatman 3MM paper (9 x 6cm) soaked in buffer 1 (25mM Tris, 40mM capronic acid, 20% methanol, pH 9.4) were placed on the cathode of the sartoblot. The equilibrated gel was placed on top and covered with a sheet of nitrocellulose membrane which had been soaked in methanol and then buffer 2 (25mM Tris, 20% methanol, pH 10.4). Air bubbles were removed by rolling a glass rod across the surface of the membrane. The membrane was covered with 2 sheets of Whatman 3MM paper soaked in buffer 2 and a third sheet soaked in buffer 3 (300mM Tris, 20% methanol, pH 10.4). Proteins were transferred for 2 hours at 40mA/mini-gel and 80mA/large-gel. Following transfer, gels were re-stained with Coomassie blue to ensure efficient transfer had occurred. Before immunoblotting, non-specific mAb binding sites were blocked by incubating membranes for 18 hours at 4°C in PBS containing 1.5% gelatin (swine skin, type II). Blots were stored under these conditions until required.

2.4.6. Detection of Proteins by Immunoblotting

Gelatin was liquified by gently heating the storage Petri dish, the membrane was transferred to a clean tray and washed in PBS/0.3% Tween 20 (wash buffer) for 5 minutes at room temperature on a shaking platform. The membrane was cut into lane strips, each strip was placed into a well of a multi-slot tray and 3ml of wash buffer was added. After 2 x 10 minutes washes, strips were incubated with 2ml primary mAb solution, purified mAb diluted to 0.1mg/ml in wash buffer or neat TCSN, for 1 hour at room temperature on a rocking platform. Strips were washed three times to remove unbound primary mAb and incubated in 3ml of wash buffer containing goat anti-mouse IgG-HRP secondary mAb (diluted 1:2000 in wash buffer) for 1 hour. Strips were washed, twice in wash buffer and twice in PBS, and the enzyme reaction developed on

the addition of 3ml of PBS containing 8.82mM H_2O_2 as the substrate and 1.5mg of diaminobenzidine as the chromogen. Once the colour had developed, seen as a brown precipitate, the reaction was stopped by flooding the wells with distilled H_2O.

2.4.7. Calculating the relative molecular weight of proteins

The relative molecular weight of specific proteins was calculated from a calibration curve constructed from a set of molecular weight standards (SDS-PAGE protein weight standards). The relative migration value (R_f) was calculated for each of the standard proteins and these values were plotted against the log molecular weight:

$$\frac{\text{distance migrated by protein}}{\text{distance migrated by dye}} = R_f$$

2.5. Characterisation of Antigen by Immunoprecipitation

2.5.1. Metabolic labelling with ^{35}S-methionine

RL-5 or BJ/873 cells (5 x 10^7) were harvested, washed twice with methionine-free DMEM and resuspended at 1 x 10^7/ml into 5ml of the same media containing 500μCi ^{35}S-methionine and 10% dialysed FCS. Cells were cultured for 16 hours as described in section 2.1.1., pelleted, washed twice in PBS and lysed as described below.

2.5.2. Surface labelling with biotin

The method used for the biotinylation of cell surface proteins was developed during this study from the methods described by Nesbit *et al.*, (1992). Cultured RL-5 or BJ/873 cells (1 x 10^8) were washed twice in PBS and resuspended at 1 x 10^7/ml in biotinylation buffer, (20mM $NaHCO_3$, 150mM NaCl, 0.1mg/ml NHS-LC-biotin titrated to pH 8.0). The cells were mixed at room temperature for 30 minutes and then for 90 minutes at

4°C. Residual biotin was removed on the addition of lysine to a final concentration of 10 mM (added from 100x stock) and the cells were washed twice with PBS and lysed as described an 2.5.3.

2.5.3. Lysis of labelled cells under mild conditions

Metabolic or surface labelled cells were resuspended in ice cold lysis buffer (10mM Tris, 150mM NaCl and 1.5mM MgCl) at 1 x 10^8/ml and lysed in the same buffer containing 1% NP-40 and 2mM phenyl-methyl-sulphonyl-fluoride. The cells were vortexed and placed on ice for 1 hour. Nuclei and other cell debris were removed by centrifugation at 100 000g in an MSE ultracentrifuge for 1 hour at 4°C. Lysate was pre-cleared and stored as described in 2.5.5.

2.5.4. Preparation of immunoadsorbant

The molecular weight of antigen recognised by uncharacterised mAb was determined by immunoprecipitation from labelled cell lysates. Protein A-Sepharose CL-4B beads, 0.25g, were swollen in 10ml immunoprecipitation wash buffer (PBSA containing 0.1% BSA, 0.5% NP-40, 0.1% SDS and 3mM sodium azide) at 4°C with continual mixing for 2 hours. The gel was pelleted at 200g for 5 minutes, washed in 10ml buffer and resuspended in 1ml PBSA. To this, 1ml of rabbit anti-mouse serum was added and the gel was incubated at 4°C for 1 hour with continual mixing. Finally, the gel was washed twice, to remove unconjugated rabbit anti-mouse immunoglobulin, resuspended as a 10% solution in wash buffer and stored at 4°C. To confirm that rabbit antibodies had bound to the protein A-Sepharose, a 50μl aliquot was boiled with 50μl of 2 x SDS sample buffer and analysed by SDS-PAGE.

2.5.5. Immunoprecipitation of antigen

To prevent non-specific binding of material to the rabbit anti-mouse Ig/Protein-A immunoadsorbant complex during immunoprecipitation, lysate was first pre-cleared. Lysate from 1 x 10^8 cells (2.5.3.) was added to 20μl of packed immunoadsorbant beads

and incubated overnight at 4°C. The beads were pelleted and the lysate divided into aliquots containing lysate from 5 x 10^6 ^{35}S-labelled cells, or 1 x 10^7 biotinylated cells, these were used immediately or stored at -80°C.

For immunoprecipitation, 10μg of mouse anti-rabbit mAb was incubated with 10μl of packed anti-mouse immunoadsorbant beads in a screw-capped microfuge tube for 2 hours at 4°C by rotation. The beads were washed 3 times with 1ml immunoprecipitation wash buffer and incubated with an aliquot of labelled cell lysate for 4 hours at 4°C. The beads were pelleted and washed a minimum of 8 times with immunoprecipitation wash buffer and twice with PBS containing 0.5% NP-40, 0.1% SDS and 3mM sodium azide. The beads were pelleted, wash buffer was carefully removed and the beads were boiled in 50μl of 1x SDS sample buffer for 5 minutes. Finally, the beads were pelleted, the supernatant was collected and the proteins separated by SDS-PAGE on 16 x 20 cm sized, 1.5mm thick gels (2.4.3).

2.5.6. Detection of ^{35}S-labelled proteins by autoradiography

After separation of proteins by SDS-PAGE, gels were stained for 1 hour with Coomassie blue, destained overnight and dried onto 3MM Whatman filter paper under vacuum at 80°C for 2 hours. Dried gels were exposed to Fuji X-ray film in a light tight cassette and stored at -80°C for 2-7 days before developing.

2.5.7. Detection of biotinylated proteins by Enhanced Chemiluminescence (ECL)

Proteins were separated by SDS-PAGE and transferred to Hybond™-ECL nitrocellulose using the semi-dry method described in 2.4.5. Following transfer, membranes were incubated overnight in 1.5% gelatin/PBS at 4°C. The next morning, the blot was washed vigorously for 60 minutes at room temperature in PBS containing 0.3% Tween-20 and incubated for 60 minutes in 30ml of wash buffer containing streptavidin-biotinylated horseradish peroxidase (HRP) antibody complex (diluted 1:3000). The blot was washed for 60 minutes in wash buffer and rinsed with PBS. In a dark room, excess buffer was drained and the ECL detection reagents were applied (0.125ml/cm^2)

and left to react with the blot for 1 minute. The blot was removed from the detection reagents, drained on paper towels, placed on a glass plate, protein side up, and covered with plastic film. The blot was exposed to a sheet of Hyperfilm-ECL for 30 seconds and the film was developed. If necessary a second piece of film was exposed. If the background was particularly high, the blot was washed for a further 60 minutes and re-developed.

2.5.8. Sequential immunoprecipitation analysis

To determine whether two mAb detected the same antigen, sequential immunoprecipitation studies were carried out. Immunoadsorbants were prepared as described in 2.5.4. An aliquot of labelled cell lysate was pre-cleared 3 times with immunoadsorbant for the first test mAb. At the same time, a second aliquot of labelled cell lysate was pre-cleared 3 times with the second test mAb. The pre-cleared lysates were re-precipitated overnight with immunoadsorbant of the competitive mAb. The following morning, immunoadsorbants were washed extensively and the proteins eluted, separated by SDS-PAGE and detected as described 2.5.5-6.

2.6. Immunoprecipitation of Antigen for Amino-Acid Sequencing and Peptide-Mass Fingerprint Analysis

2.6.1. Coupling of mAb to Sepharose beads

Purified mAb were coupled to cyanogen-bromide activated Sepharose. For each mAb, 0.3g of dry Sepharose was mixed for 10 minutes in 10ml of 1mM HCl at room temperature. The gel was transferred to a sintered glass funnel and washed with 50ml of 1mM HCl followed by 100ml of coupling buffer (0.1M $NaHCO_3$ and 0.5M NaCl, pH 8.3). The gel was transferred to a 15ml tube and washed in 10ml coupling buffer. The gel was resuspended in 15ml of coupling buffer containing 5mg of purified mAb and incubated for 2 hours on a rotor at room temperature. After two washing steps, any unbound reactive sites on the activated Sepharose were blocked by incubating in

20ml of 0.1M Tris-HCl pH 8.0, for 2 hours. The gel was washed three times with with the following sequence of buffers:

i) 0.1M Na acetate, 0.5M NaCl pH 4.0
ii) 0.1M Tris, 0.5M NaCl pH 8.0

Finally, the immunoadsorbant was washed twice with PBS and stored as a 10% suspension in PBS containing 3mM NaN_3 at 4°C. To check that mAb had efficiently bound to the Sepharose beads, the absorbance of diluted mAb was measured before and after coupling. As a further test, 100μl of the immunoadsorbant was boiled in 25μl of 2x sample buffer and run on mini-SDS-PAGE 7% gels which were stained and destained as described in section 2.4.3.

2.6.2. Preparation of cell lysates for protein purification

To immunoaffinity purify cell surface antigens, cells were lysed at 1 x 10^8/ml in lysis buffer as described in 2.5.3. Lysates were pre-cleared with protein A-Sepharose-rabbit anti-mouse Ig complex overnight at 4°C and stored as 1 x 10^8 aliquots at -80°C.

2.6.3. Immunoprecipitation and separation of proteins for mass-fingerprint analysis or amino-acid sequencing

Immunoadsorbants for mAb NR185 and BJ3/22 were prepared. Packed immunoadsorbant, 30μl, was incubated, in a microfuge tube, with 1ml of lysate from 1 x 10^8 cells, for 3 hours at 4°C with rotation. The immunoprecipitates were washed sequentially with PBS, PBS/0.5M NaCl and PBS/0.1% Tween 20, for 10 minutes at 4°C and then overnight in PBS/0.5% NP-40. The next morning, the immunoprecipitates were washed again in PBS, boiled in 25μl of 2x SDS reducing sample buffer and run on a 7% SDS-PAGE mini-gel, which had polymerised overnight to quench polymer free-radicals, in Tris/glycine buffer containing 0.025% mercaptoacetic acid. Electrophoresis was stopped when the dye front reached 1 cm from the bottom of the gel.

Packed R9/17 immunoadsorbant, 150μl, was incubated with lysate from 3 x 10^8 RL-5 cells for three hours at 4° C by rotation. The immunoprecipitate was washed as above, boiled in 50μl of 2x SDS reducing buffer and proteins were separated on a 15%, 20 x 16 cm SDS-PAGE gel prepared and run as described above.

2.6.4. Transfer of separated proteins to nitrocellulose

Gels were equilibrated by soaking for 30 minutes in electroblotting buffer containing 0.1% MeOH and 10mM 3-cyclohexylamino-1-propanesulphonic acid (CAPS) titrated to pH 11.0 with NaOH. Proteins were transferred to Immobilon-P membrane using the Sartoblot II system. Before transfer, membranes were soaked in MeOH for 1 minute and rinsed in electroblotting buffer twice. The apparatus was assembled as described:

Cathode
3 layers Whatman 3 MM paper soaked in buffer
equilibrated gel
membrane
3 layers Whatman 3 MM paper soaked in buffer
Anode

Electroblotting proceeded for 2 hours at 150mA.

Following transfer, the membrane was rinsed several times with distilled water and dried thoroughly before staining for protein with Sulphorhodamine B using the method first described by Reig and Klein (1988) and modified by Pappin *et al.*, (1990). The staining process is dependent on the fact that the membrane only becomes wet in solutions containing greater than 50% MeOH. Proteins alter the local charge of the membrane and allow it to become wet by solutions of less than 50% MeOH. The membrane was immersed in 30% methanol, 0.2% v/v acetic acid and 0.005% w/v sulphorhodamine B for a few minutes, rinsed in deionised water and dried, under vacuum, for 20 minutes. Stained proteins bands were visualised under uv illumination and bands of interest were cut from the membrane for further analysis.

2.6.5. Peptide-mass fingerprinting

This technique, developed at the ICRF laboratories, was kindly performed by D.J.C. Pappin (Pappin *et al.*, 1993). Sample proteins are digested with trypsin and the mass of the peptides produced is determined by mass spectrometry (Robins *et al.*, 1994). This peptide-mass fingerprint and the molecular weight of the protein, if known, are used to screen the MOWSE database which contains the peptide-mass fingerprints generated from theoretical trypsin digests of approximately 72 000 proteins. The program produces a ranked list of the 50 best matched protein entries.

Protein bands were digested with trypsin, 2% w/w in 50mM NH_4HCO_3, pH 8.2 for 4 hours at 37°C. Samples were lyophilized and dissolved in 50% MeCN/1%TFA. Aliquots of 0.5-1.0μl (1-5pmol protein), mixed with 0.5μl α-cyano-4-hydroxycinnamic acid, were analysed directly by matrix-assisted laser desorption (MALD) time of flight mass spectrometry on a LaserMAT mass spectrometer.

2.6.6. Amino acid sequencing

Dr D.C. Pappin, based at the ICRF laboratories, also kindly performed the N-terminal amino-acid sequencing (Pappin *et al.*, 1990). Prior to sequencing, the isolated protein band was moistened with 10μl of 0.1% PITC in ethyl acetate and allowed to dry for 15-20 seconds. The membrane was placed on a mylar sheet lying on a heating block (55°C) and wetted with 30μl of 50% MeOH containing 2% triethylamine which evaporated off over 7-8 minutes. While being held with forceps, 5μl of 0.1% DITC in ethyl acetate was added to both sides of the membrane. As this evaporated a thin film of DITC was left behind. The membrane was covered with 30μl of 50% MeOH containing 2% triethylamine and 0.1 poly(allylamine) hydrochloride, allowed to dry and 20μl of 50% MeOH containing 2% triethlyamine was pipetted onto the surface. This treatment results in the proteins being entrapped with a polymer network at the surface of the membrane. The polymer-coated membrane was washed vigorously with MeOH, deionized water and MeOH. Proteins were subjected to automated solid-phase Edman degregation in a MilliGen/Biosearch 6600 ProSequencer.

2.6.7. Internal peptide sequencing

Internal peptide sequencing data was kindly produced by Mr. A. C. Willis at the MRC Immunochemistry Unit, Department of Biochemistry, Oxford University. Material from the immunoadsorbant (section 2.6.3) was run on a 10% SDS-PAGE gel and electroblotted onto Problott membrane. The membrane was stained, as described in section 2.6.5, and the band of interest was excised. The protein band was digested with trypsin, and eluted peptides were separated by reverse-phase HPLC. HPLC fractions were analysed by matrix assisted laser desorption time of flight MS (MALDI-TOF) using a Laser-MAT (Finnigan-MAT, San Jose, CA) and an α-cyano-4-hydroxycinnamic acid matrix. HPLC fractions that appeared to contain a single dominant peptide species were then sequenced by automated Edman microsequencing using a pulsed liquid protein sequencer with on-line HPLC (model 477A: Applied Biosystems).

2.7. Homotypic Aggregation of Rabbit T Cell Lines

2.7.1. Treatment of RL-5 cells with phorbol ester

Aggregation of the RL-5 cells was induced following stimulation with PMA as described by Rothlein and Springer (1986). RL-5 cells were washed in RPMI-1640/10% FCS containing 5mM Hepes buffer and resuspended at 2 x 10^6 cells/ml in medium and 100μl of this was added to the wells of 96-well tissue culture plates. Aggregation was brought about by the addition of 50μl of PMA from a stock of 200ng/ml to the wells. The volume in the wells was made up to 200μl and the plates were incubated at 37°C in 5% CO_2. Aggregation was assessed after 2 hours and scored by eye on a scale of 0-5.

2.7.2. Aggregation induced by incubation with monoclonal antibody

RL-5 and BJ/873 cells were washed, resuspended and plated as above (2.7.1.). mAb induced aggregation was initiated by the addition of purified mAb to a final concentration of 25μg/ml. The volume in the well was made up to 200μl and the plates were incubated and assessed as described above.

2.7.3. Inhibition of homotypic aggregation

To assess the inhibitory effects of mAb on cell aggregation, cells were plated as above, purified mAb were added to assay wells at a final concentration of 25μg/ml and the plates were incubated as described. After 1 hour, PMA or stimulatory mAb was added to the wells and the plates were incubated for a further 2 hours .

Temperature dependence was assessed by incubating cells at 5 x 10^5/ml in 25mm Petri dishes. These were incubated on an orbital shaker at 4°C, room temperature or 37°C for two hours.

Cation dependence was assessed by pre-incubating cells at 5 x 10^5/ml in RPMI-1640 containing 5mM EDTA for 30 minutes at 4°C. Cells were washed in medium containing EDTA and aggregation assays were carried out in Ca^{2+} and Mg^{2+} free media.

Requirement for metabolic energy was assessed by pre-treating cells at 5 x 10^5/ml in medium containing 0.2% sodium azide for 60 minutes at 4°C. Cells were washed in medium and resuspended and plated into medium containing 10% FCS.

Cells were also pre-treated with the protein kinase inhibitor Staurosporine (200mM) for 20 minutes at 37°C at a density of 5 x 10^5/ml. Cells were washed in medium and resuspended and plated as described.

2.8. Basic Molecular Biology

2.8.1. Small scale isolation of plasmid DNA

Methods used for minipreparations of DNA were adapted from the method of Sambrook *et al.*, (1989). A single bacterial colony, or a loop of frozen stock, was transferred into a 15ml Falcon tube containing 3ml of LB plus 25μg/ml ampicillin, the tube was loosely capped and incubated overnight at 37°C with vigorous shaking. Bacteria from 1.5ml of the culture were pelleted in a 1.5ml microfuge tube for 2 minutes in a Stratagene Profuge microfuge at top speed (10 000g). The supernatant was removed and the pellet resuspended into 100μl of solution I (50mM glucose, 25mM Tris-HCl pH 8.0, 10mM EDTA and 0.1μg DNAase-free RNase) and incubated for 5-10 minutes at room temperature. Next, 200μl of freshly prepared solution II (0.2M NaOH, 1% SDS) was added to induce cell lysis and tubes were inverted several times and incubated on ice for 5 minutes. Following this, 150μl of solution 3 (3M potassium acetate, pH 4.8) was added, the tubes were vortexed gently and cell debris and genomic DNA pelleted by spinning, as above, for 5 minutes. The supernatant was transferred to a second tube and combined with 225μl of TE saturated phenol (pH 7.6) and 225μl of chloroform isoamyl alcohol and the tube was spun for 2 minutes. The upper aqueous phase was collected, transferred to a fresh tube and the phenol/chloroform extraction repeated. The aqueous phase was transferred to a fresh tube, an equal volume of chloroform was added, the tube was spun and the aqueous phase transferred to a fresh tube. The plasmid DNA was precipitated by adding 0.2 volumes ammonium acetate, 2 volumes cold EtOH and incubation at -80°C for 30 minutes. The tube was spun at 13 000rpm in a bench top Eppendorf 5402 centrifuge for 15 minutes at 4°C, supernatant was poured away and the pellet was washed with 70% ethanol, centrifuged for 10 minutes as before and resuspended into 25μl of sterile TE or d.H_2O.

2.8.2. Restriction digests of plasmid DNA

Inserts were cut from plasmid vectors using restriction enzymes (*Eco*RI, *Not*I, *Hin*dIII, and *BstX*I). Plasmid DNA was isolated as described (2.8.1.). A 5μl sample of the isolated DNA was diluted with 5.3μl of sterile d.H_2O. To this, 0.5μl of restriction enzyme and 1.2μl of the appropriate 10x buffer solution was added, tubes were vortexed gently, microfuged briefly and incubated for 2 hours at the specified temperature. Digests were combined with 3μl of loading buffer (45 glycerol, 1% ficoll 2.5mM EDTA, 0.025% xylene cyanol, 0.025% bromophenol blue) and analysed by agarose gel electrophoresis.

2.8.3. Gel electrophoresis

DNA samples were analysed on 0.8, 1.0 or 1.2% horizontal agarose mini-gels containing 0.5μg/ml of ethidium bromide (Sharp *et al.*, 1973) and run in 1x TBE or 1x TAE buffers at 60v for 1-1.5 hours. λ DNA cut with *Hin*dIII or *Eco*R1 and *Hin*d III was used to estimate product size. Gels were photographed on Polaroid 667 black and white film under ultra-violet illumination.

2.8.4. Isolation of DNA bands from agarose gels

Samples were run on 1% low-melting point agarose gels, in 1x TAE as described in 2.7.3. Under ultra-violet illumination, DNA bands of interest were excised using sterile scalpels, transferred to sterile 1.5ml microfuge tubes and the DNA was purified using Geneclean II® Kit according to the manufacturer's instructions. The DNA was eluted into 30μl of TE buffer and 4μl, mixed with 1μl of loading buffer, was analysed by gel electrophoresis.

2.9. Production of Anti-Rabbit CD11c/CD18 DNA Probes

2.9.1. Production of oligonucleotides

cDNA sequences encoding, mouse, bovine and chicken CD18 were collected from Genbank and aligned using the MacVector program. Oligonucleotides were designed from regions shown to be conserved between species and are shown below:

Oligonucleotide CD18 1: 5' CCTGGTGCCAGAAGCTGAACTTC 3'
Oligonucleotide CD18 2: 3' GACCAGTAGACCTTCCGGGGACT 5'
Oligonucleotide CD18 3: 5' GCGTTCAACGTGACCTTCCGG 3'
Oligonucleotide CD18 4: 3' GGACTTTCAGTGGATACTGAGGAAGG 5'

For rabbit CD11c, oligonucleotides were designed from the transmembrane and metal-binding domains of the human CD11c sequence and from tryptic peptides of the rabbit CD11c sequence:

Oligonucleotide CD11c 1: 5' ATTGTCATCACTGATGG 3'
Oligonucleotide CD11c 2: 3' GTCATAAAACCCGTCCG 5'
Oligonucleotide CD11c 3: 5' TGGAGAAGGTGGTGGTGGTGAT 3'
Oligonucleotide CD11c 4: 3' CTCACGGTTCGTCCTTGTCTGG 5'

Oligonucleotide primers were dissolved in 1ml of DEPC-treated H_2O, the OD was determined spectrophotometrically by measuring absorbance at 260nm and a 20μM working solution was prepared.

2.9.2. Amplification of rabbit cDNA fragments using Polymerase Chain Reaction

PCR reactions (Saiki *et al.*, 1985; Mullis *et al.*, 1986; Saiki *et al.*, 1988) were performed in an Eppendorf Matercycler 5330. A 1:100 dilution of a rabbit spleen cDNA library was heated to 95°C for 3 minutes and cooled rapidly on ice for use as template in PCR. Reaction mixtures consisted of 2μl of library template, 200μM of each deoxynucleotide triphosphate, 1.0μM of forward and reverse primers, 1x reaction buffer (10mM Tris-HCl, 1.5mM $MgCl_2$, 50mM KCl, pH 8.3), 2.5 units of *Taq* DNA polymerase and DEPC-treated autoclaved H_2O was added to a final volume of 100μl.

Reaction mixtures were gently vortexed, microfuged briefly and overlaid with 30μl of sterile light mineral oil before being subjected to PCR which consists of the following stages:

Denaturation	95°C for 45 seconds
Annealing	52-56°C for 1 minute
Extension	72°C for 1 minute

After 30 cycles, samples were cooled to 4°C and 90μl PCR product carefully recovered from below the oil layer; 8μl of product mixed with 2μl of loading buffer was analysed by gel electrophoresis.

2.9.3. Ligation of PCR products into a plasmid vector

PCR reactions were performed as described above with a final extension step of 7 minutes to ensure that all DNA was double stranded with 3' A-overhangs. The concentration of DNA was estimated by running 10μl of product on a mini-gel next to a known standard. Remaining product was run, in a cold room, on a 1% low melting point agarose gel, bands of interest were purified as described in 2.7.2. and the product ligated into the pCR™II vector (TA Cloning®Kit) overnight at 14°C according to the manufacturers instructions. Ligation reactions were microfuged and placed on ice before transformation of competent cells.

2.9.4. Transformation of One Shot™ cells (INVαF')

For each ligation reaction, 2 LB plates containing 50μg/ml ampicillin were warmed to 37°C and 25μl of X-gal, from a 40mg/ml dimethylformamide stock, was spread over the surface and allowed to diffuse into the agar for 1 hour. One Shot™ cells (INVαF') were transformed according to the manufacturers instructions. Briefly, for each ligation a 50μl vial of competent cells were thawed slowly on ice. To this, 2μl of 0.5M β-mercaptoethanol plus 2μl of ligation reaction was added, the cells were mixed by stirring and incubated on ice for 30 minutes. The cells were heat shocked for 30

seconds at 42°C, placed on ice for 2 minutes and 450μl of warmed SOC medium was added before the vials were shaken for 1 hour at 225 rpm at 37°C. Following transformation, the 2 LB plates were spread with 100μl and 200μl from each transformation and placed at 37°C overnight. The next morning, the plates were transferred to a fridge to allow colour development. Several white colonies were selected and grown overnight for plasmid isolation and analysis as described in 2.8.1. A sample from the same colonies was streaked onto a section of a LB plate for short term storage.

2.9.5. Sequencing of DNA inserts

Inserts within the pCR™II vector were sequenced using both T7 and SP6 primers and the Sequenase®Version 2.0 or ^{T7}Sequencing™ kits both of which utilize the dideoxy-mediated chain termination method first described by Sanger *et al.*, (1977). Briefly, clones were grown overnight and plasmid DNA was collected using standard methods (2.8.1). Of this, 5μl was diluted with 13μl of sterile d.H_2O and denatured following the addition of 2μl of 2M NaOH. Samples were mixed, incubated at room temperature for 5 minutes and the single stranded DNA precipitated by adding 8μl of 5M ammonium acetate and 100μl of cold ethanol followed by incubation on dry ice for 10 minutes. The samples were spun at 13 000 rpm for 15 minutes at 4°C, washed with 500μl 70% cold ethanol and spun again for 10 minutes at 4°C at 13 000rpm. Pellets were dried and used immediately in sequencing reactions.

Sequencing using Sequenase® Version 2.0: DNA pellets were resuspended in 7μl of sterile H_2O and 2μl of sequencing buffer (200mM Tris-HCl pH 7.5, 100mM $MgCl_2$, 250mM NaCl) and 1μl of primer (5ng) were added. The tube was vortexed, microfuged briefly and heated to 65°C in a water bath for 2 minutes. The samples were cooled slowly at room temperature to 35°C to allow primer annealing and placed on ice. During this time, 2.5μl of termination mixtures A, C, G and T, from the kit, were aliquoted into labelled tubes and warmed to 37°C.

To the cooled DNA/primer tubes 1μl of 0.1M dithiothreitol (6.45mM), 2μl labelling mix (7.5μM dGTP, dCTP, dTTP) diluted 1:5 with H_2O, 0.5μl [^{35}S] dATPαS (Ci/mmol >1000) and 3.25 units sequenase polymerase diluted from 13 units/μl in (10mM Tris-HCl pH7.5, 5mM DTT, 0.5mg/ml BSA) was added. The reactions were mixed and incubated at room temperature for 3 minutes. After this, 3.5μl of the labelled DNA was added to each of the termination mixtures (A, C, G, T) and the reaction were left to proceed for 5 minutes at 37°C. Next, the reactions were inhibited by adding 4μl stop solution (95% formamide, 20mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol). Finally, reactions were heated to 75°C for 2 minutes and rapidly cooled on ice for 5 minutes before loading onto the gel.

Sequencing using ^{T7}Sequencing^{TM} Kit: DNA pellets were resuspended in 10μl of sterile H_2O and to this 2μl of universal primer (5pmol/μl) and 2μl of annealing buffer (1M Tri-HCl pH7.6, 100mM $MgCl_2$, 160mM DTT) were added. The tubes were vortexed, microfuged briefly and incubated at 65°C for 5 minutes, 37°C for 10 minutes and at room temperature for 5 minutes. To the annealed DNA template/primer 3μl of labelling mix (1.375μM each dCTP, dGTP and dTTP and 333.5mM NaCl), 1μl [^{35}S] dATPαS (Ci/mmol >1000) and 3.2 units of T7 DNA polymerase diluted in enzyme dilution buffer (20mM Tris-HCl pH 7.5, 5mM DTT, 100μg BSA/ml) were added. The reagents were mixed by pipetting, centrifuged briefly and incubated at room temperature for 5 minutes. Then 4.5μl of this labelling reaction was transferred to labelled tubes containing 2.5μl of termination reactions (A, C, G, T), which had been preheated to 37°C, the components were mixed and incubated at 37°C for 5 minutes. The tubes were microfuged and 5μl of stop solution was added to each. Finally 3μl was taken from each reaction, heated to 80°C, cooled rapidly on ice and loaded onto a gel.

2.9.6. Resolution of sequencing reactions

Sequencing reactions were resolved on 6% polyacrylamide denaturing gels (Sequagel^{TM}-6) 34 x 40cm and 0.25mm width cast in the Poker Face^{TM} II 1600 system (Hoefer) and run in 1x TBE buffer at 55W. For gels, 80ml of sequencing gel solution was mixed

with 20ml Sequagel complete buffer reagent and 250μl of 10% ammonium persulphate. The gel was poured immediately and left to polymerise for 1 hour. The casting comb was removed and any unpolymerised gel rinsed away before a sharks tooth comb was positioned. The gel was pre-run for 1 hour at 55W, the wells were rinsed with buffer and the samples loaded. Samples were run for 1.5 hours and then 3μl of the same samples were loaded and run for a further 1.5 hours. The gel was picked up onto a sheet of blotting paper 33 x 56cm, dried under vacuum at 80°C for 1 hour and exposed to Fuji RX medical film overnight.

2.10. Screening of Rabbit a Spleen cDNA for CD11c and CD18 Encoding Clones

2.10.1. Plating of library and transfer of colonies to nitrocellulose membranes

A rabbit cDNA library was diluted 1:10^6 in LB containing 25μg/ml ampicillin, this was spread over 40 LB agar plates (50μl/plate) which were incubated overnight at 37°C. The following morning, plates were incubated at 4°C for 1 hour. Nitrocellulose membrane filters (82mm disc) were labelled 1-40 and using blunt forceps placed face down over the corresponding plate. Orientation was marked in three locations by stabbing through the membrane into the agar using a needle dipped in waterproof ink. Membranes were left in place for 30 seconds and then placed onto filter paper. Plates were incubated at 37°C for 6 hours and stored inverted at 4°C.

2.10.2. Lysis of colonies and binding of DNA to nitrocellulose membranes

The method used to lyse bacterial colonies and bind released DNA to nitrocellulose was adapted from the method first described by Grunstein and Hogness (1975). Three large trays were filled with 1l of denaturing solution (0.5M NaOH, 1.5M NaCl), neutralisation solution (0.5M Tris, 1.5M NaCl, pH 7.5), or rinse buffer (0.5M NaCl, 2x SSC, 0.1% SDS). Membranes were floated, colony side up, on the surface of denaturing solution for 2 minutes. Next, they were carefully lifted using blunt forceps and floated on neutralisation solution for 2 minutes and then wash buffer for 5 minutes.

They were then transferred to sheets of 3MM paper, covered with a second sheet of 3MM paper and baked for 30 minutes at 80°C. Membranes were washed for 75 minutes in 2x SSC buffer with at least 3 buffer changes, to remove all bacterial debris and finally rinsed in the same buffer.

2.10.3. Prehybridisation and hybridisation

Membranes were divided between two heat-sealable bags and prehybridised in warmed hybrisol solution for 2 hours at 42°C. Probes were labelled using the ^{T7}QuickPrime Kit. Plasmid DNA was isolated from transformed colonies and digested with *Hind* III and *Not* I restriction enzymes to release the insert which was isolated from agarose gels as described in 2.7.3. Between 10-50ng of linearised DNA was labelled with [^{32}P]. Labelled probe was heated to 95°C, chilled rapidly and added to the membranes which were incubated at 42°C overnight. The following morning, bags were drained and the contents frozen to be reused. Membranes were washed repeatedly in 2x SSC at 45°C for a total of 2 hours, air dried and taped to sheets of 3MM paper. Orientation was marked using ^{14}C ink, the sheets were covered with plastic film and the membranes were exposed to Kodak XAR-5 film for autoradiography overnight at -70°C with intensifying screens.

2.10.4. Identification of positive colonies and secondary screening

Films were developed, positive hybridisation signals were identified and aligned with the appropriate plates. Areas giving positive hybridisation signals were identified and, using a sterile toothpick, a sample from a single colony was diluted into 50μl of sterile LB medium and this was spread over the surface of a LB agar plate. If a single hybridising colony could not be selected, a plug of agar was removed from the area giving the positive signal, the bacteria were dispersed into 100μl of LB medium and this was spread over a LB agar plate. Colonies were grown overnight at 37°C. Plates were rescreened following the same protocol as for primary screening of the rabbit spleen library and single colonies giving positive signals were selected and replated. A single, well isolated, colony from the third round of screening was selected for further study.

2.10.5. Analysis of cDNA clone

A single colony was picked up with a flamed loop and transferred to 10ml of LB medium containing 50μg/ml of ampicillin. Cultures were grown overnight at 37°C with shaking, plasmid DNA was isolated from 1.5ml of the culture (2.7.1) and digested with *Bst*X I to release the insert cDNA. The remaining culture was frozen for storage. The insert was sequenced at the Laboratory of Immunogenics, NIAID, NIH, Bethesda, MD.

2.11. Southern Blotting

2.11.1. Random labelling of DNA with Digoxigen-11-dUTP to produce a probe

Plasmid DNA was isolated from transformed cells and digested with restriction enzymes to release the insert (section 2.8.1 and 2.8.2). The insert was gel purified (section 2.8.4) and was randomly labelled with Digoxigen-11-dUTP (DIG) using the DIG-DNA labelling kit. Briefly, 10ng-3μg of isolated DNA insert was added to 2μl of hexanucleotide mixture, 2μl of dNTP labelling mixture, 19μl of H_2O and 1μl of Klenow enzyme in a sterile microfuge tube. The reaction was incubated at 37°C for 20 hours. After this period, the reaction was terminated by the addition of 2μl of EDTA (200mM, pH 8.0). DNA was precipitated using standard methods described in section 2.8.1. The DNA probe was resuspended in 50μl of TE buffer and the probe was stored at -20°C.

2.11.2. Gel electrophoresis and transfer of DNA to nitrocellulose

Restriction digests of DNA of interest were run on 1.0% TAE agarose gels using standard methods (2.8.3). The gel was placed in a sterile plastic container and covered with 250mM HCl, for 5 minutes while shaking at room temperature. The gel was rinsed with DEPC treated H_2O and submerged in buffer containing 0.5M NaOH, 1.5M NaCl, twice, for 5 minutes while shaking gently at room temperature. The gel was

rinsed with DEPC treated H_2O and submerged in buffer containing 0.5M Tris-HCl, 3M NaCl, pH 7.5, twice, for 15 minutes with shaking at room temperature. Next, the gel was placed onto a tray covered with 3MM paper soaking in 20x SSC buffer and covered with a sheet of nitrocellulose membrane which was slightly smaller than the gel itself, orientation marks were made and membrane was covered with three sheets of 3MM paper and a stack of absorbant paper. A 500g weight was placed on top and the DNA was transferred to the nitrocellulose overnight by capillary action. The following morning, the DNA was fixed to the membrane by baking at 120°C for 30 minutes.

2.11.3. Prehybridisation and hybridisation

The membrane was placed in a heat-sealable plastic bag containing 20ml standard prehybridisation solution (5x SSC, 1.0% blocking reagent, 0.1% N-lauroylsarcosine, 0.02% SDS) per 100cm^2 of membrane surface area and incubated at the hybridisation temperature (50-68°C) for 1 hour. After this incubation period, the prehybridisation solution was discarded and the membrane was incubated in prehybridisation solution containing (10ng/ml) of single stranded DNA probe at the hybridisation temperature, overnight. The following morning, the membrane was washed twice, 5 minutes per wash, in 0.1% wash solution (0.1% SSC, 0.1% SDS) at room temperature and then twice, 10 minutes per wash, in 0.1% wash solution at the hybridisation temperature.

2.11.4. Detection of DIG-labelled nucleic acids

All incubations were performed at room temperature with shaking. After the post-hybridisation washes, the membrane was soaked in buffer 1 (100mM maleic acid, 150mM NaCl, pH 7.5), for 1 minute and then in buffer 2 (in 1% blocking reagent dissolved in buffer 1) for 30 minutes. In a fresh container, the membrane was incubated for 30 minutes in buffer 2 containing anti-DIG-alkaline phophatase mAb diluted 1:5 000. The membrane was transferred to a fresh container and washed twice, 15 minutes per wash, in buffer 1. The membrane was then equilibrated with buffer 3 (100mM Tris, 100mM NaCl, 50mM $MgCl_2$, pH 9.5) for 2 minutes and incubated with

10ml buffer 3 containing 45μl NBT (75mg/ml nitroblue tetrazolium salt in 70% dimethylformamide) and 35μl X-phosphate (50mg/ml 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt in 100% dimethylformamide), in the dark for 12 hours. The colour development reaction was inhibited by washing the membrane in buffer 1 for 5 minutes.

CHAPTER THREE

Characterisation of a Virally Transformed Rabbit Lymphoid Cell Line that Expresses CD8

3.1. Introduction

Although the rabbit has proved to be a useful model for studying a number of human diseases including rheumatoid arthritis (Dumonde and Glynn, 1962), atherosclerosis (Buja *et al.*, 1983) and adult T-cell leukaemia (Miyoshi *et al.*, 1983), studies have been hampered by a lack of mAb which recognise rabbit leucocyte surface antigens and mAb which are able interfere with cell function *in vitro* or *in vivo*. One of the aims of this project was to produce and characterise mAb that could be used to distinguish different rabbit T cell sub-populations. Of particular interest were the rabbit homologues of CD4 and CD8, defining helper and cytotoxic T lymphocytes respectively.

Previously, anti-rabbit mAb have been raised against the rabbit T cell line RL-5 (Wilkinson *et al.*, 1984). This line, transformed with herpes virus ateles, is thought to belong to the CD4 subset (Daniel *et al.*, 1977; Wilkinson *et al.*, 1992). RL-5 cells have recently been compared with another rabbit T cell line BJ610, derived from a New Zealand White rabbit infected with Alcelaphine herpes virus, which was shown to belong to the CD8 subset (Wilkinson *et al.*, 1992).

A third rabbit cell line, transformed with ovine herpes-virus-2 and designated BJ/873, has now been obtained and compared with RL-5 cells by immunostaining and flow cytometric analysis.

3.2. Results

3.2.1. Derivation of the BJ/873 cell line

The BJ/873 cell line was a kind gift from Dr. Hugh Reid at the Mordun Institute, Edinburgh. The cells were prepared in his laboratory using the following methods. A New Zealand white rabbit was injected intravenously with a suspension of lymph node cells derived from a cow showing symptoms of malignant catarrhal fever (MCF) following infection with ovine herpes-virus-2. After twelve days the rabbit was sacrificed and the lymph nodes were isolated. A cell suspension was prepared and 10^8 cells were intravenously injected into a second, three month old, male, New Zealand White rabbit. After a twelve day period, this animal showed signs of MCF. On day thirteen the animal was killed and the mesenteric lymph nodes were cultured in Iscove's medium containing 10% FCS and 10 IU/ml rhIL-2 for several months. The cells were shown to contain viral DNA by the transmission of MCF to rabbits and amplification of the viral genome by PCR (Baxter *et al.*, 1993).

BJ/873 cells were grown in culture as described in section 2.1.1. In culture some 60% of the BJ/873 cells adhere to the surface of the tissue culture flask with the remaining cells growing as a single cell suspension. Adherent cells could be released by scraping or by brief incubation with EDTA (0.27mM). Flow cytometry of the adherent and suspended cell populations revealed no difference in their phenotypes and suspended cells adhere to the surface of a fresh tissue culture flask after overnight culture.

3.2.2. Characterisation of the BJ/873 cell line by flow cytometry

BJ/873 cells were compared with the RL-5 cell line by indirect immunofluorescence staining and flow cytometry. Both cell lines were stained with a panel of mAb reactive with a variety of rabbit leucocyte surface antigens. The experiment was repeated three times and the histograms from a typical experiment are shown in figure 3.1 and the mean fluorescence intensity of staining is given in table 3.1.

Staining with mAb L11/135 (anti-CD43) demonstrates that BJ/873, like RL-5, is of T cell origin. RL-5 cells stained with the newly generated anti-rabbit CD4 mAb, Ken-4, and therefore belong to the T helper subset, while the BJ/873 cells stained with 12.C7 (anti-CD8) which strongly suggests that it belongs to the cytotoxic subset. As expected for T cells, both cell lines express CD18, but not CD11b. The cell lines also expressed similar levels of CD44, CD45, CD58, MHC class-I molecules and the IL-2 receptor (CD25).

The cell lines were stained with mAb HP1/2 (Sanchez-Madrid *et al.*, 1986), BIIGII and GoH3 (Hemler, 1990) which cross-react with rabbit α_4, α_5 and α_6 respectively. While both cell lines express intermediate levels of α_6, α_5 is expressed only by RL-5 cells and α_4 is expressed only by the BJ/873 cells. The level of expression of the antigens recognised by mAb RR1/1, RPN3/57 and Ken-5 also differed between the two cell lines. mAb RR1/1 is an anti-human ICAM-1 mAb which has been shown to be cross reactive with rabbit ICAM-1 (Barton *et al.*, 1989). BJ/873 cells express intermediate levels of ICAM-1, while RL-5 cells express low levels (figure 3.1.N). mAb RPN3/57, raised against rabbit peritoneal neutrophils, recognises an antigen expressed on neutrophils, a sub-population of peripheral T lymphocytes and platelets. The same antigen is recognised by mAb ACM-1 and 9AE10 and is reported to be a 20-25 kDa protein (McNicholas *et al.*, 1981; Chen *et al.*, 1984). The RPN3/57 antigen is not expressed on RL-5 cells, while BJ/873 cells, and another rabbit $CD8^+$ cell line, BJ610, express it at high levels (figure 3.1.E). Although the molecular nature of the antigen recognised by these mAb is not known, the cells expressing it are found mainly within the $CD8^+$ T lymphocyte subset.

mAb Ken-5, raised against rabbit thymocytes was reported to recognise the rabbit CD5 homologue. It is reactive with the majority of peripheral T lymphocytes, but not B lymphocytes, and precipitates a single band of 67 kDa from ^{125}I-labelled thymocytes (Kotani *et al.*, 1993). Raman and Knight (1992), have cloned rabbit CD5 and raised a number of mAbs against the expressed rabbit CD5 protein. Unlike Ken-5, these mAbs are reactive with rabbit B lymphocytes and thus the specificity of mAb Ken-5 has been questioned. RL-5 cells were not labelled by mAb Ken-5, while the BJ/873 cells showed a bimodal staining pattern with approximately 75% of cells expressing

intermediate levels of the antigen (figure 3.1.F).

Staining of the BJ/873 cell line for expression of CD8 with mAb 12.C7 showed that there were two distinct cell populations (figure 3.1.D). Approximately 70% of the cells were $CD8^{HIGH}$, while the remaining cells were $CD8^{LOW}$. The percentage of $CD8^{HIGH}$ cells decreased over time in culture. An attempt was made to obtain a uniform culture of $CD8^{HIGH}$ cells by fluorescence-activated sorting of cells stained with 12.C7 (section 2.3.7). The results of this experiment are shown in figure 3.2. Cells expressing high levels of CD8 were selected and re-cultured, however after 35 days in culture 25% were found to be $CD8^{LOW}$. It is possible that further rounds of sorting and selection, or sub-cloning the cells by limiting dilution, may result in a stable $CD8^{HIGH}$ culture being obtained.

Antibody	Subclass	Specificity	Mean Fluorescence intensity	
			RL-5	BJ/873
T1/7	IgG1	-ve control	22	22
L11/135	IgG1	CD43	182	182
Ken-4	IgG2a	CD4	153	31
12.C7	IgG1	CD8	51	32% = 31 68% =147
RPN3/57	IgG1	n.d.	63% = 26 37% = 83	164
Ken-5	IgG1	n.d.	29	23% = 35 75% = 121
2C4	IgG1	Class II RD-Q	31	31
W4/86	IgG1	CD44	119	127
RPN3/24	IgG1	CD44	176	171
W4/28	IgG2b	n.d.	165	155
Kei-α-1	IgG2b	CD25	72	95
L13/64	IgG2b	CD18	150	156
198	IgG1	CD11b	26	22
RR1/1	IgG1	ICAM-1	61	125
NRBM	IgG1	anti-μ	30	29
VC21	IgG1	CD58	143	159
L12/201	IgG1	CD45	170	171
L12/27	IgG1	CD45	154	136
AIIB2	IgG	CD29	22	31
BIIG2	IgG	CDw49e	78	24
GoH3	IgG2a	CDw49f	104	84
HP1/2	IgG1	CDw49d	23	70

Table 3.1. Mean Fluorescence intensity, by flow cytometry, of two rabbit T cell lines stained with a panel of mAb. n.d. = not determined.

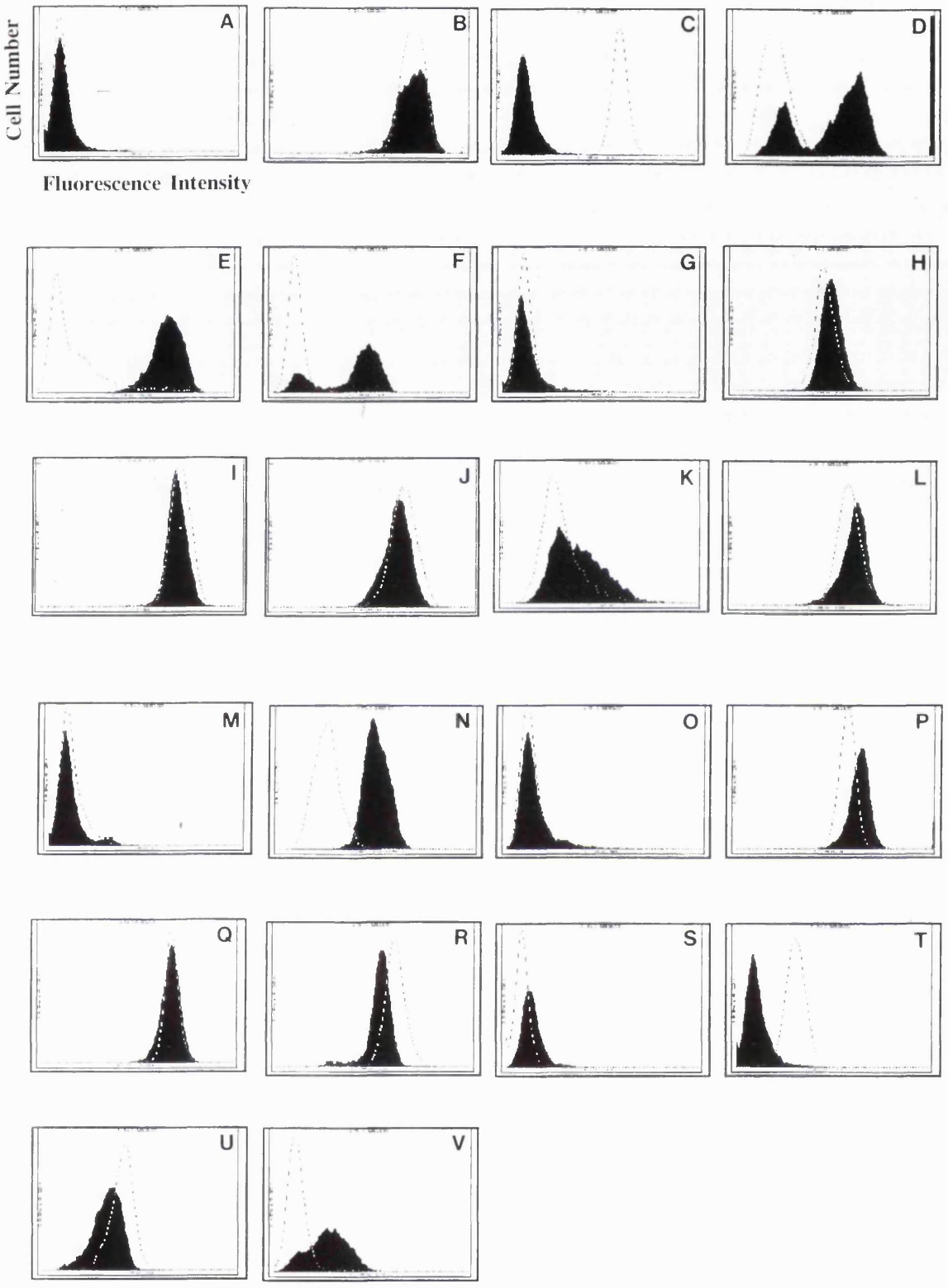

Figure 3.1. Flow cytometric analysis of rabbit T cell lines BJ/873 (full histogram) and RL-5 cells (open histogram) immunolabelled with mAb: (A) T1/7, (B) L11/135, (C) Ken-4, (D) 12.C7, (E) RPN3/57, (F) Ken-5, (G)2C4, (H) W4/86 (I) RPN3/24, (J) W4/28, (K)Kei-α-1, (L) L13/64, (M) 198, (N) RR1/1, (O) NRBM, (P)VC21, (Q) L12/201, (R) L12/27, (S) AIIG2, (T) BIIG2, (U) GoH3, (V) HP1/2. Details of mAb are given in table 3.1.

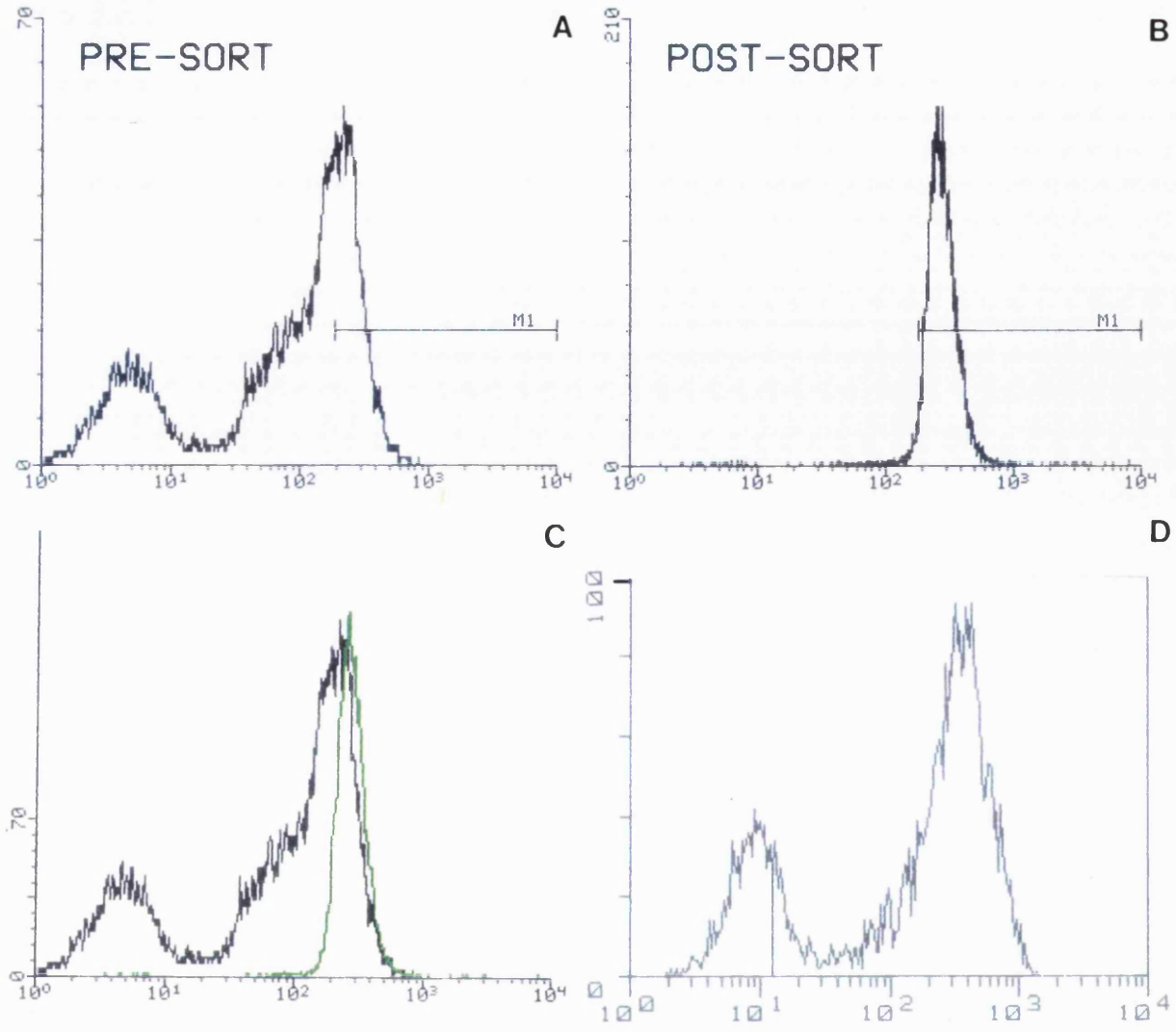

Figure 3.2. BJ/873 cells were stained with 12.C7 for flow cytometry under sterile conditions and cells which stained brightly were sorted and cultured: (A) shows the histogram produced from analysis of the whole BJ/873 population, (B) shows the sorted population, (C) compares the pre- and post-sorted populations and (D) shows the result of staining the sorted population with 12.C7 after 35 days in culture.

3.3. Discussion

The expression of leucocyte surface antigens by BJ/873 cells has been investigated and compared with the previously characterised rabbit T cell line, RL-5 (Daniel *et al.*, 1977). The results obtained are similar to those reported for RL-5 cells and another rabbit cell line, BJ610 (Wilkinson *et al.*, 1992). Preliminary characterisation shows that BJ/873 is derived from the CD8, cytotoxic subset, based on reactivity with mAb L11/135 and 12.C7.

Staining of the BJ/873 cells with mAb Ken-5 produced similar histograms to those obtained from staining with mAb 12.C7 and presumably the $CD8^{HI}$ BJ/873 cells are Ken-5 positive. This hypothesis may be confirmed by staining the BJ/873 cells with 12.C7 and Ken-5 for two colour flow cytometric analysis. Although identification of mAb Ken-5 as CD5 is controversial, these data suggest that this mAb may prove be a useful tool for labelling sub-populations of T lymphocytes.

Prior to this investigation, the RL-5 cell line was believed to be derived from the CD4, T helper subset since it stained strongly with L11/135, but was not reactive with 12.C7. Staining with the anti-rabbit CD4 mAb, Ken-4, confirms this hypothesis.

These cell lines have been used during this study to produce a panel of mAb which recognise rabbit leucocyte surface antigens and to investigate the adhesive interactions of rabbit T cells in homotypic aggregation assays.

CHAPTER FOUR

Identification of mAb which Recognise the Rabbit Analogues of CD43, CD44 and CD45

4.1. Introduction

In 1975 Kohler and Milstein described a method for the production of monoclonal antibodies. Soon after, mAb against novel cell surface antigens were described (Williams *et al.*, 1977; Springer *et al.*, 1978) and the study of leucocyte cell surface antigens was revolutionised. The production of mAb against leucocyte antigens has been of great importance for defining leucocyte subsets, for functional studies and for biochemical characterisation of antigens. The naming of human leucocyte cell surface antigens has been formalised in a series of international workshops. mAb which give the same labelling pattern with different populations of haemopoietic cells by flow cytometry are considered to recognise the same antigen and this antigen is given a CD number (Cluster of Differentiation) (Bernard and Boumsell, 1984). CD nomenclature has been extended to include homologous antigens from other species. During this investigation, mAb which recognise the rabbit equivalents of CD43, CD44 and CD45 have been produced and their characterisation is described in this chapter. In addition, a number of mAb which recognise novel rabbit cell surface molecules have been produced and these are also discussed.

CD43, also known as leucosialin (Carlsson and Fukuda, 1986) or sialophorin (Borche *et al.*, 1987), is a major membrane glycoprotein of leucocytes. CD43 appears to have a number of functions acting as a ligand for ICAM-1 (Rosenstein *et al.*, 1991) and as a molecule involved in T cell activation (Park *et al.*, 1991). Anti-CD43 mAb have been shown to induce CD18-dependent homotypic aggregation of T cells (Axelsson *et al.*, 1988; De Smet *et al.*, 1993) and neutrophils (De Smet *et al.*, 1993). CD43 is heavily glycosylated by *O*-linked carbohydrate units and differential glycosylation of the common CD43 protein core gives rise to two distinct CD43 isoforms. A 115 kDa isoform is expressed by thymocytes, lymphocytes and monocytes and a 135 kDa isoform is expressed by neutrophils and platelets (Remold-O'Donnell *et al.*, 1987).

mAb L11/135 is thought to recognise rabbit CD43 (Wilkinson *et al.*, 1992). L11/135 reacts with T lymphocytes, thymocytes, monocytes and macrophages, but not B lymphocytes, neutrophils or platelets, and precipitates a protein with an apparent molecular mass of 120 kDa from the rabbit T cell line, RL-5.

CD44 defines a family of transmembrane glycoproteins widely expressed on haemapoietic and non-haemapoietic cells reviewed by (Underhill, 1992; Lesley *et al.*, 1993; Imhof and Dunon, 1995). CD44 is a cell adhesion molecule which is thought to be involved in extracellular-matrix binding, lymphopoiesis, thymocyte development and lymphocyte migration and homing. Certain anti-CD44 mAb promote T lymphocyte homotypic aggregation and binding to cultured keratinocytes *in vitro,* which is LFA-1 dependent (Koopman *et al.*, 1990; Bruynzeel *et al.*, 1993). Multiple isoforms of CD44 have been described which are generated by alternative splicing of 12 of the 19 exons encoded by the CD44 gene (Screaton *et al.*, 1992). CD44 is highly glycosylated and cell-type specific glycosylation has been reported (Carter and Wayner, 1988; Brown *et al.*, 1991; Lokeshwar and Bourguignon, 1991; Camp *et al.*, 1991). The major CD44 isoform expressed on lymphocytes, CD44H, has an apparent molecular mass of 85-95 kDa by SDS-PAGE (Trowbridge *et al.*, 1982; Stamenkovic *et al.*, 1991). A 180-200 kDa form of CD44, expressed only on lymphocytes, has also been described (Jalkanen *et al.*, 1988) and shown to result from the covalent association of chondroitin sulphate side chains to the CD44 core protein. CD44 is a receptor for hyaluronan (HA) (Aruffo *et al.*, 1990; Peach *et al.*, 1993) and may interact with other ECM components including collagen type I and type VI (Wayner and Carter, 1987; Carter and Wayner, 1988). Chondroitin sulphate modified CD44 on lymphocytes has been shown to bind to fibronectin, laminin and collagen type I *in vitro* (Jalkanen and Jalkanen, 1992). mAb W4/86 and RPN3/24 have been shown to recognise the rabbit equivalent of CD44 (Wilkinson *et al.*, 1992). Both mAb immunoprecipitate a protein of 90 kDa from ^{35}S-labelled RL-5 cell lysates, and RPN3/24 immunostains a diffuse band of 90 kDa from SDS-extracts of RL-5 cells, separated by SDS-PAGE under non-reducing conditions.

CD45 is a high molecular weight transmembrane glycoprotein which is expressed in abundance on all leucocytes and their precursor cells in the human, rat and mouse. CD45 belongs to a large family of enzymes, the protein tyrosine phosphatases, and

recent studies have demonstrated a requirement for CD45 in lymphocyte activation and in thymocyte development reviewed by (Thomas, 1989; Trowbridge and Thomas, 1994). Different isoforms of CD44 are generated by alternative splicing of exons which encode an extracellular region of the molecule, and these isoforms are expressed in cell-type specific patterns on functional sub-populations of leucocytes (Streuli *et al.*, 1987; Thomas *et al.*, 1987; Saga *et al.*, 1987; Trowbridge and Thomas, 1994). Consequently, two classes of anti-CD45 mAb exist, those which recognise all isoforms, binding to constant regions of CD45, and those which recognise restricted epitopes, arising from expression of alternative exons, the CD45R mAbs (Trowbridge and Thomas, 1994). Cell type specific post-translation modifications, such as glycosylation, also give rise to restricted epitopes (Conzelmann and Lefrancois, 1988). The rabbit equivalent of CD45 has been shown to be recognised by mAb 1.24 (De Smet *et al.*1983) and L12/201 (J.M. Wilkinson and M.R. Gorden, unpublished). mAb 1.24, raised against rabbit leucocytes, precipitates a 200 kDa band from lysates of ^{35}S-labelled RL-5 cells and inhibits proliferation of T lymphocytes in response to a number of stimuli. It has been shown to immunolabel the majority of cells in the thymus, lymph nodes, spleen, peripheral blood and 60% of cells in the bone marrow (De Smet *et al.*, 1983). mAb L12/201 was raised against cell surface glycoproteins from the RL-5 cell line (Wilkinson *et al.*, 1984). The distribution of the antigen recognised by L12/201 on peripheral lymphocytes is similar to that of the 1.24 antigen (Wilkinson, 1988). mAb L12/201 was compared with 1.24 by immunoprecipitation following cross-linking of mAb to ^{35}S-labelled RL-5 cells. The results of this experiment showed that both mAb recognised a 200 kDa antigen which was presumed to be same (M.R. Gorden and J.M. Wilkinson, unpublished).

A panel of anti-rabbit leucocyte antigens have been raised against either rabbit peripheral neutrophils, RL-5 cells (Daniel *et al.*, 1977) or BJ/873 cells (this study). The molecular mass and cellular distribution of the antigens recognised by these mAb has been determined and the results of these experiments are presented in this, and subsequent, chapters.

4.2 Results

4.2.1. Production of mAb

Female Balb/c mice were immunized with rabbit peripheral blood neutrophils, RL-5 cells or BJ/873 cells according to the strategy outlined in section 2.2.1. Splenocytes from these animals were fused with SP2/0 plasmacytoma cells, as described in section 2.2.4, and cells were plated into 96 well tissue culture plates for hybridoma selection. After the fusion reaction unfused spleen cells die naturally in culture, while SP2/0 cells and hybridomas are immortal and would grow continually. It is therefore necessary to culture the cells in conditions which promote the growth of the hybridomas but inhibit the growth of SP2/0 cells. This is achieved by culturing the cells in media containing agents which inhibit *de novo* synthesis of nucleic acids. Hybridoma cells can survive in this media by using the salvage pathway of nucleic acid synthesis inherited from the parental spleen cell. However, SP2/0 cells have been selected to be deficient in an enzyme of the salvage pathway, hypoxanthine-guanidine phosphoribosyl transferase, and fail to produce the nucleic acids adenine and guanine, and die.

Media containing azaserine and hypoxanthine (Foung *et al.,* 1982), or aminopterin, hypoxanthine and thymidine (Littlefield, 1964), were compared for their ability to inhibit the growth of SP2/0 cells while allowing the L13/64 hybridoma cell line to divide. On this basis, for the first nine fusions, hybridomas were selected in media containing azaserine and hypoxanthine (method A). Selection occurred two to three days after fusion leaving hybridoma growth foci in many of the wells. At day ten, the hybridoma supernatant was assayed by cellular ELISA, as described in section 2.2.6, and positive wells were re-screened by immunofluorescence staining of the rabbit T cell lines (section 2.3.3). Cells secreting antibody which showed strong reactivity with the rabbit cell lines were cloned as described in section 2.2.7. In some of the fusion wells, the media became acidic (yellowed) at day four-five and the cells died. It appeared that in these wells, the selection process was inefficient allowing SP2/0 cells to grow and limit the growth of hybridomas. As a result, only 56% of wells were screened in this series of fusions and, of these, only 0.6% contained hybridomas producing antibody which were positive by both screening assays.

The development of a second protocol (method B), in which selection medium contained aminopterin, hypoxanthine and additional growth supplements (section 2.2.4), improved fusion efficiency. Typically 53% of wells were screened by cellular ELISA (described in section 2.2.6) and of these, 6.0% of wells contained cells secreting antibody against cell surface antigens as determined by immunofluorescence staining of RL-5 and BJ/873 cells (described in section 2.3.3). In practice, it was not possible to clone all of the positive wells from this series of fusions and thus wells were also screened by immunofluorescence staining of rabbit peripheral blood leucocytes followed by flow cytometry and for inhibition of PMA-induced RL-5 cell aggregation (described in section 2.7). Only wells containing cells secreting antibody which recognised leucocyte sub-populations or which inhibited/stimulated RL-5 cell aggregation (section 2.7) were cloned. Data for the production and characterisation of mAb against rabbit surface antigens is shown in tables 4.1. and 4.2

Fusion	Antigen	Selection protocol	No. wells screened	ELISA +ve	FITC +ve	Wells cloned
NR1	*Neu/RL-5	A	377	22	2	1
NR2	Neu/RL-5	A	62	0	0	0
NR3	Neu/RL-5	A	271	3	1	1
RL-3	RL-5	A	73	2	0	0
RL-4	RL-5	A	198	2	0	0
RL-5	RL-5	A	468	21	5	3
RL-6	RL-5	A	226	8	3	0
RL-7	RL-5	A	457	12	1	0
RL-8	RL-5	A	0	0	0	0
RL-9	RL-5	B	635	89	17	4
RL-10	RL-5	B	280	23	5	0
BJ-2	BJ/873	B	526	77	24	1
BJ-3	BJ/873	B	434	71	12	3
BJ-4	BJ/873	B	608	n/d	45	1
BJ-5	BJ/873	B	600	n/d	42	2
BJ-6	BJ/873	B	607	141	41	1
BJ-7	BJ/873	B	346	90	48	1

*Table 4.1. Table showing the type of cells used as antigen in each of the fusions and the protocol used to select hybridomas. The number of wells screened is shown out of a total of 480 for protocol A and 960 for B. The number of wells positive by ELISA and FITC-staining of rabbit cell lines is also shown, as is the number of wells cloned from each fusion. *Neu = rabbit peritoneal neutrophils.*

mAb	Subclass	Antigen	Distribution
BJ4/55	IgM	CD43	T, M
R5/23	IgG1	CD44	T, B, M, N
R9/380	IgG1	CD44	T, B, M, N
R9/483	IgG1	CD44	T, B, M, N
R5/151	IgM	CD45	T, B, M, N
R5/443	IgM	120/45 kDa	B (low) T, M
R9/17	IgG1	23 kDa	T, B, M
BJ5/69	IgG2b	n/d	T, B, M, N (low)
BJ5/74	IgG3	n/d	T (subpopulation)

Table 4.2. Summary of the mAb characterised in this chapter.
n/d = not determined, T = T lymphocytes, B = B lymphocytes, M = monocytes, N = neutrophils.

4.2.2. The antigen recognised by mAb BJ4/55 is CD43

mAb BJ4/55, raised against BJ/873 cells (fusion BJ-4), reacted strongly with both RL-5 and BJ/873 cells by cellular ELISA and surface immunofluorescence staining and was cloned. Tissue supernatant was grown up, filter sterilised and used in the following studies to determine the nature of the antigen recognised.

Proteins from RL-5 cells were immunoblotted with mAb BJ4/55. mAb L11/135 (anti-CD43) and W4/86 (anti-CD44) were used as positive controls and the anti-chicken troponin T mAb, T1/7, was used as a negative control (Bird *et al.*, 1985). The results (figure 4.1), show that mAb BJ4/55 stained a band at 120 kDa, which is analogous to the band stained by L11/135.

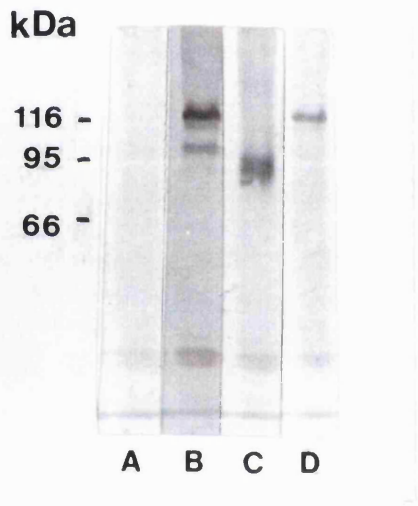

Figure 4.1. Immunolabelling of RL-5 cell proteins separated by SDS-PAGE on 7% gels under reducing conditions and transferred to nitrocellulose. Strips were incubated with (lane A) T1/7, (lane B) L11/135, (lane C) W4/86 and (lane D) BJ4/55 and mAb binding was detected as described in section 2.4.6.

Peripheral blood mononuclear cells were stained with BJ4/55 and L11/135 for flow cytometry (figure 4.2). The differences in their light scattering properties enabled lymphocytes, which have low forward scatter (FSC) and low SSC (SSC), and monocytes, which have high FSC and high SSC, to be identified (figure 4.2 A). The

histograms produced from labelling lymphocytes with mAb BJ4/55 and L11/135 were virtually identical. L11/135 and BJ4/55 stained T lymphocytes brightly ($MFI_{L11/135}=199$, $_{BJ4/55}=188$) (figure 4.2.B) and B lymphocytes weakly ($MFI_{neg}=40$, $_{L11/135}=49$, $_{BJ4/55}=53$) (figure 4.2.C). Monocytes stained at intermediate levels with BJ4/55 (MFI=179) which is comparable to L11/135 staining (MFI=197) (figure 4.3.D).

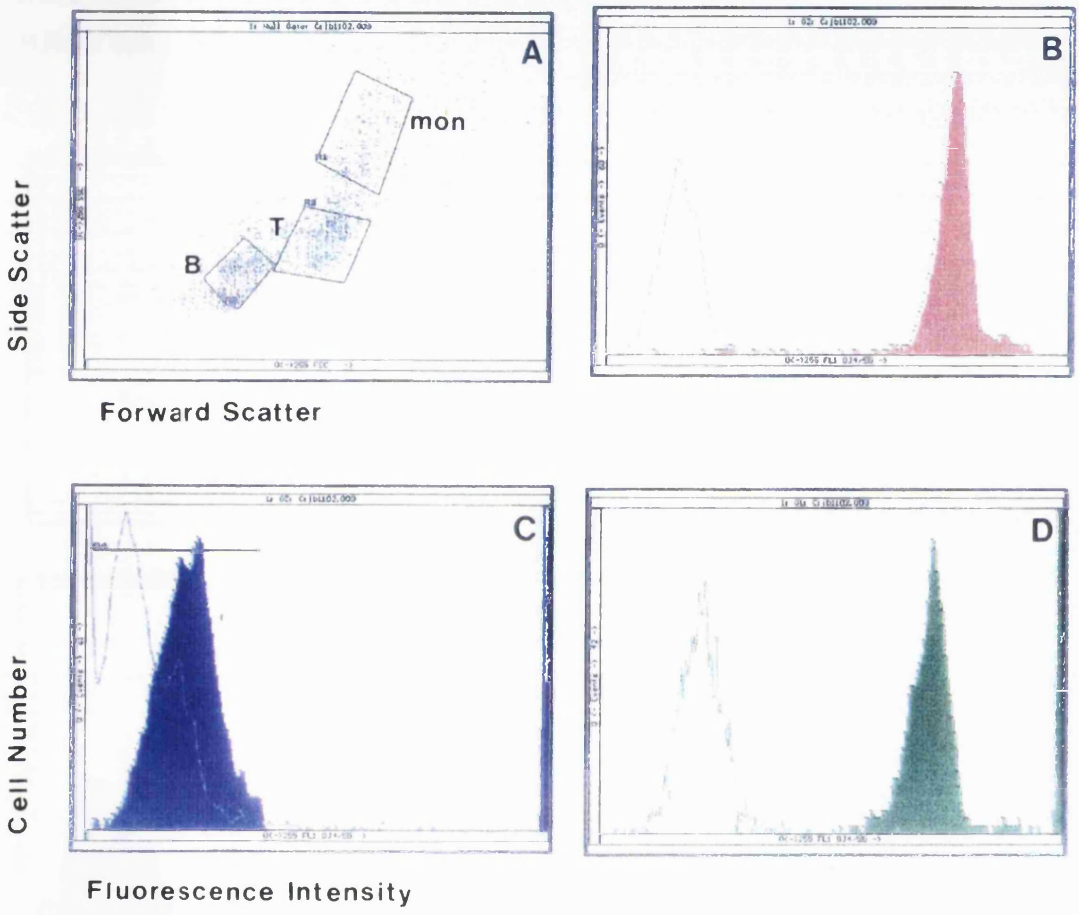

Figure 4.2. Flow cytometric analysis of rabbit peripheral mononuclear cells stained with BJ4/55: (A) scatter plot of peripheral blood mononuclear cells defining B and T lymphocytes and (mon) monocytes, (B) T lymphocytes (C) B lymphocytes and (D) monocytes, cells were also stained with T1/7 (negative control) shown as open histogram. Cells were stained according to methods described in section 2.3.3.

Rabbit T lymphocyte cell lines were immunolabelled with mAb L11/135 and BJ4/55 (figure 4.3). Like L11/135, mAb BJ4/55 stained both the RL-5 ($MFI_{L11/135}$=173, $_{BJ4/55}$=102) and BJ/873 ($MFI_{L11/135}$=166, $_{BJ4/55}$=110) cell lines (figures 4.3. A and B), although the mean fluorescence intensity from labelling with BJ4/55 was lower than with L11/135. The expression of the BJ4/55 antigen on two other rabbit T cell lines BJ610 (Wilkinson, 1992) and BJ880 (H. Reid, unpublished) was examined. L11/135 labelled BJ610 (MFI=186) and BJ880 (MFI=168) cell lines brightly, while the BJ4/55 antigen was not expressed by BJ610 cells (MFI=15) and was only weakly expressed by BJ880 cells (MFI_{neg} =15, $_{BJ4/55}$=38) (figures 4.2. C and D).

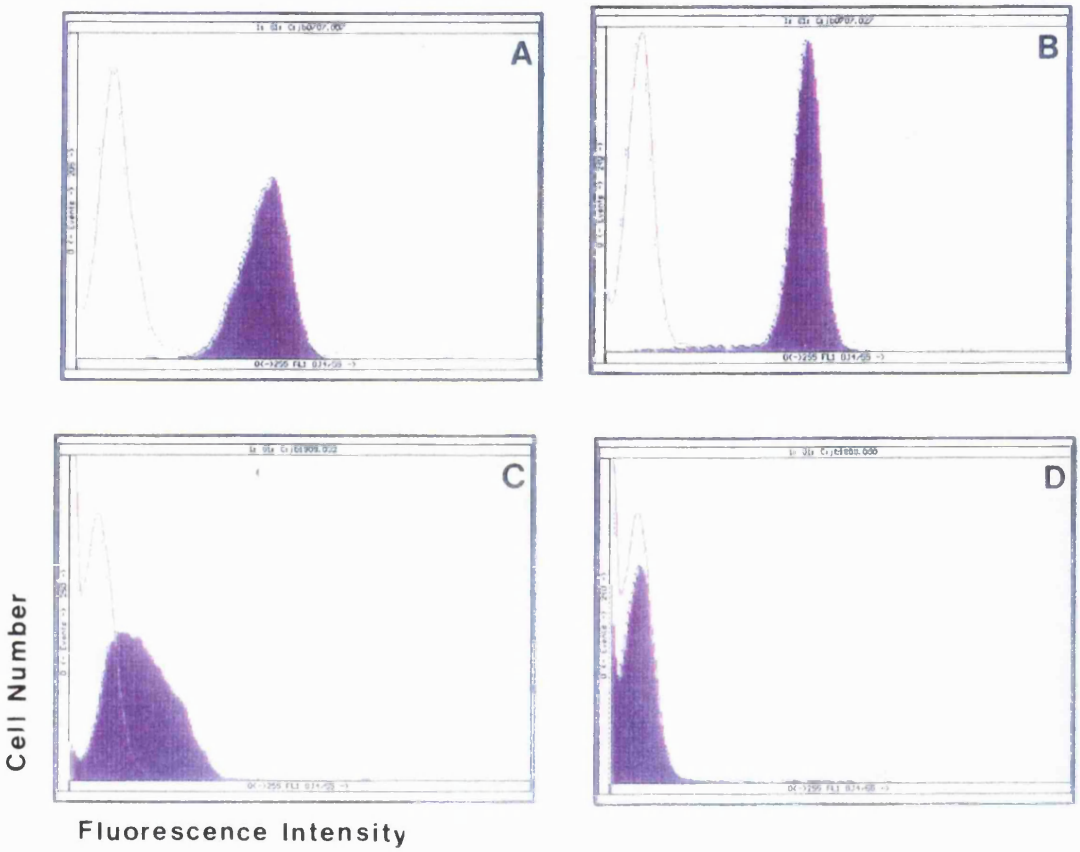

Figure 4.3. Flow cytometric analysis of the rabbit T cell lines (A) RL-5, (B) BJ/873, (C) BJ880 and (D) BJ610 stained with T1/7 (negative control, open histogram), L11/135 (positive control, dotted histogram) and BJ4/55 (solid histogram).

4.2.3. The antigen recognised by mAb R5/23, R9/380 and R9/483 is CD44

mAb R5/23, R9/380 and R9/483, raised against RL-5 cells, gave strong reactivity with the RL-5 and BJ/873 cells by cellular ELISA and immunofluorescence. Immunoblotting of RL-5 cell proteins with mAb R5/23, R9/380 and R9/483 showed that all three mAb labelled a diffuse band with an apparent molecular mass of 90 kDa. R5/23, R9/380 and R9/483 were compared with the anti-CD44 mAb W4/86 and RPN3/24 by immunoblotting RL-5 cell proteins. All five mAb immunostained a diffuse band at 90 kDa (figure 4.4.a and 4.4.b).

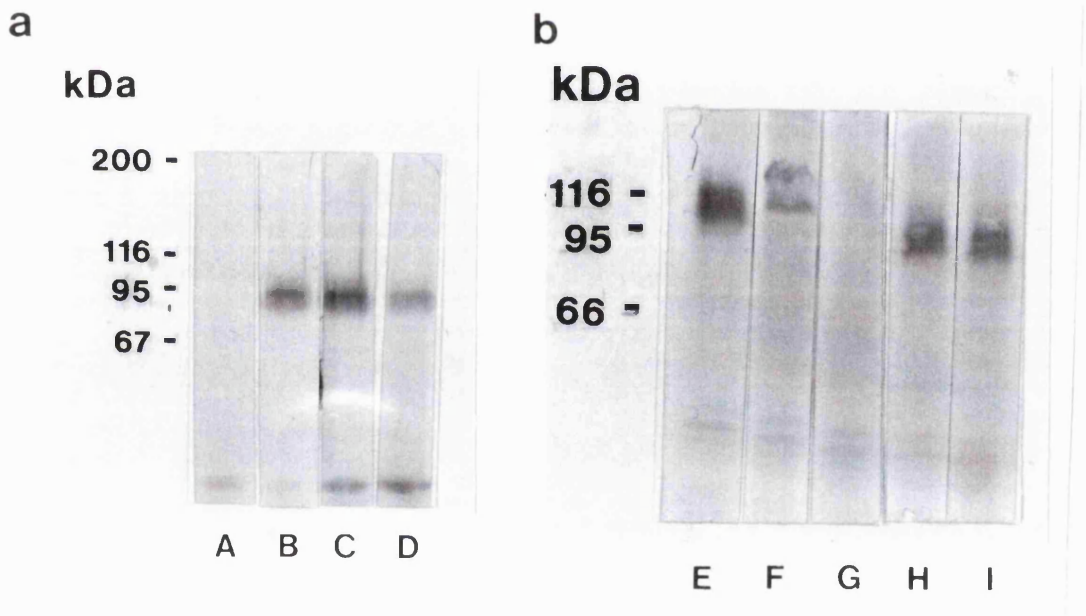

Figure 4.4.a. and 4.4.b. RL-5 cell proteins were separated by SDS-PAGE on (a) 8% and (b) 10% gels under reducing conditions and transferred to nitrocellulose (described 2.4). Proteins were immunostained with (A and G) T1/7 (negative control), (B) R5/23, (C and E) RPN3/24, (D) W4/86, (F) L11/135, (H) R9/380 and (I) R9/483

The antigen recognised by mAb R5/23 was immunoprecipitated from ^{35}S-labelled RL-5 cell lysates and compared with the band precipitated by RPN3/24 (figure 4.5). L11/135 precipitated a major band at 120 kDa. mAb RPN3/24 and R5/23 precipitated a single band at 90 kDa.

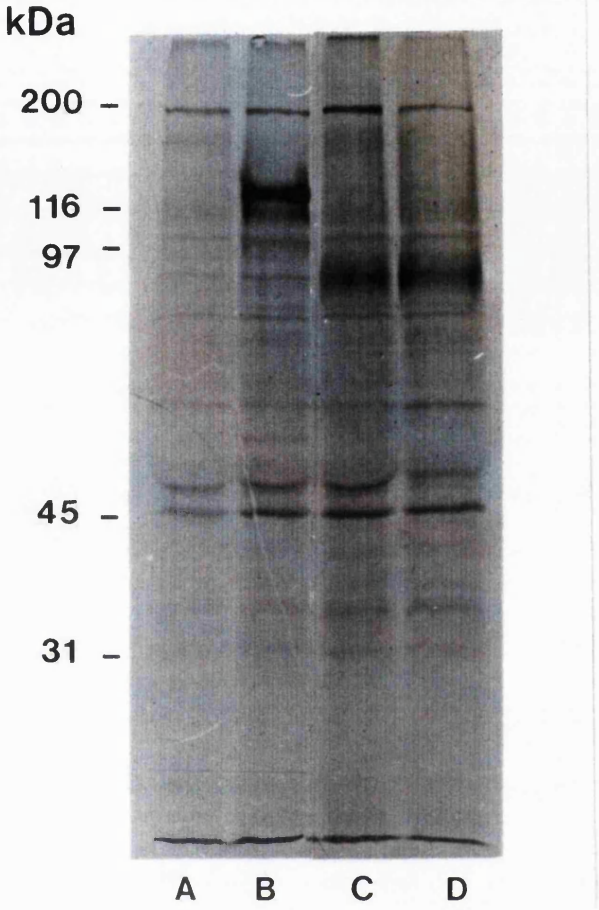

Figure 4.5. *Immunoprecipitation from ^{35}S-labelled RL-5 cell lysates with (lane A) T1/7 (negative control), (lane B) L11/135 (anti-CD43, positive control), (lane C) RPN3/24 (anti-CD44) and (lane D) R5/23. Immunoprecipitates were separated by SDS-PAGE on 8% gels under reducing conditions.*

Previously, rabbit CD44 had been shown to be expressed on peripheral monocytes, lymphocytes and neutrophils (Wilkinson *et al.*, 1992). Rabbit peripheral blood leucocytes were labelled for flow cytometry with mAb R5/23, R9/380 and R9/483. Cells were labelled with T1/7 (negative) and positive control mAb L11/135 (anti-CD43, T lymphocyte and monocyte control), NRBM (anti-μ-chain, B lymphocyte control) and 198 (anti-CD11b, neutrophil control). Cells were gated for analysis according to their light scatter properties. The results of a typical experiment are shown in figure 4.6. Labelling mononuclear cells with either R5/23, R9/380 or R9/483 produced histograms with a bimodal distribution (figures 4.6. A-C). Monocytes and T lymphocytes stained

brightly R5/23 (MFI=203, $MFI_{T1/61}$=29), R9/380 (MFI=207) and R9/483 (MFI=204) and B lymphocytes were stained at intermediate levels ($MFI_{R5/23}$=149, $_{R9/380}$=153, $_{R9/483}$=148). Neutrophils were strongly labelled with these newly developed mAb ($MFI_{T1/61}$=30, $_{R5/23}$=196, $_{R9/380}$=200, $_{R9/483}$=198) (figures 4.6. D-F).

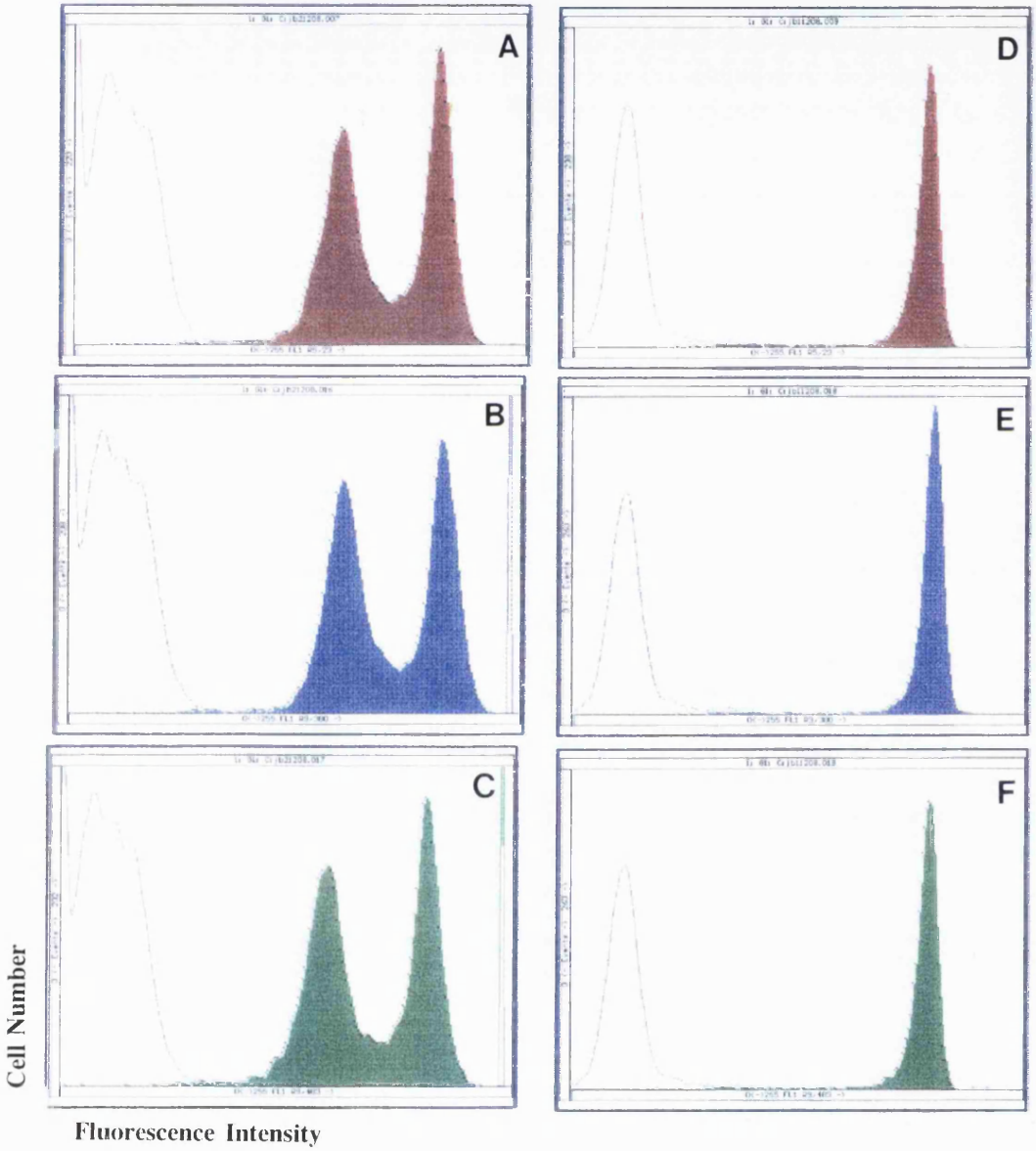

Figure 4.6. *Flow cytometric analysis of (A-C) peripheral mononuclear cells and neutrophils (D-F) stained with (A and D) R5/23, (B and E) R9/380 and (C and F) R9/483. Cells were also stained with the negative control mAb T1/7 (negative control) shown as the open histogram.*

4.2.4. The antigen recognised by mAb R5/151 is CD45

Antibody secreted by the hybridoma cells growing in well 151 of fusion RL-5 showed strong reactivity against the RL-5 cells by cellular ELISA and immunofluorescence and well 151 was selected for cloning. mAb R5/151 was used to precipitate antigen from detergent lysates of ^{35}S-labelled RL-5 cells (figure 4.7). mAb L11/135 and T1/61 (anti-chicken troponin T) (Bird *et al.*, 1985) were used as positive and negative controls respectively. mAb R5/151 precipitated a major band at 200 kDa (lane C). This was similar to the band previously precipitated by mAb 1.24 and L12/201 from RL-5 cell lysates (M.R. Gorden and J.M. Wilkinson, unpublished).

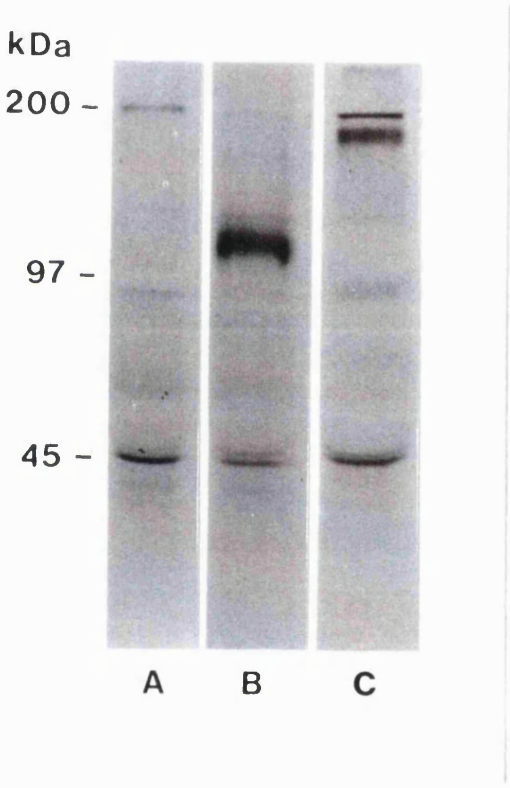

Figure 4.7. Immunoprecipitation from detergent lysates of ^{35}S-labelled RL-5 with mAb (lane A) T1/61 (negative control), (lane B) L11/135 (anti-CD43) and (lane C) R5/151. Immunoprecipitates were separated by SDS-PAGE on 8% gels under reducing conditions and gels were dried under vacuum for autoradiography.

The antigen recognised by mAb R5/151 was compared with the antigen recognised by mAb 1.24 in a series of sequential immunoprecipitation studies from lysates of ^{35}S-labelled RL-5 cells as described in section 2.5.8 (figure 4.8). Both mAb precipitated a band of 200 kDa from these lysates (lanes A and H). mAb R5/151 failed to precipitate a band from lysate sequentially pre-cleared with 1.24 (lane E), although 1.24 precipitated a faint band from lysates sequentially pre-cleared with R5/151 (lane D). That 1.24 was able to precipitate a band from R5/151 pre-cleared lysates is probably a reflection of differences in mAb affinity.

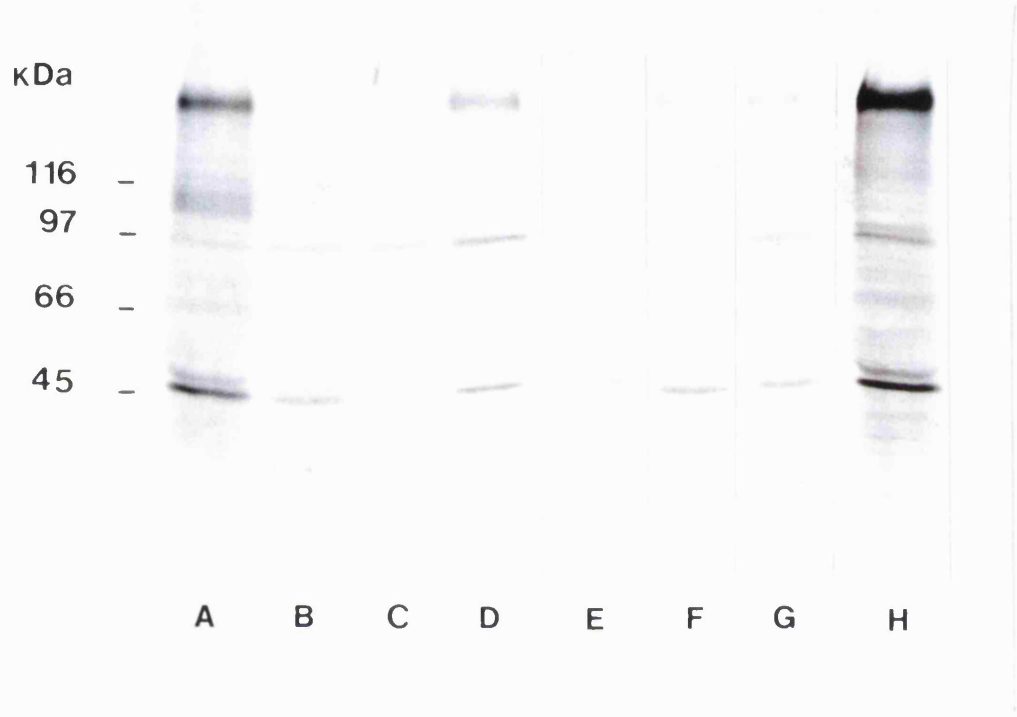

Figure 4.8. Sequential immunoprecipitation from ^{35}S-labelled RL-5 cell lysates with mAb 1.24 and R5/151. Lysates were pre-cleared with 1.24 (lanes A-C) and reprecipitated with R5/151 (lane E) or were pre-cleared with R5/151 (lanes F-H) and reprecipitated with 1.24 (lane D). Precipitated proteins were separated by 8% SDS-PAGE under reducing conditions.

Peripheral leucocytes and RL-5 cells were stained for flow cytometry with R5/151 and L12/201 (figure 4.9). Cells were also stained with mAb T1/7 and L11/135 (anti-CD43) as positive and negative controls. The results of this experiment showed that a population of peripheral lymphocytes expressed intermediate levels of CD45 ($MFI_{L12/201}=122$, $_{R5/151}=155$), while the remaining lymphocyte population and monocytes expressed high levels ($MFI_{L12/201}=148$, $_{R5/151}=187$) (figures 4.9. A and B).

Neutrophils were shown to express intermediate levels ($MFI_{L12/201}$=135, $_{R5/151}$=112) (figure 4.9. C and D). RL-5 cells were stained at high levels with mAb L12/201 ($MFI_{L12/201}$=170) (figure 4.9.E) and at lower levels by R5/151 (MFI=112) (figure 4.9.F).

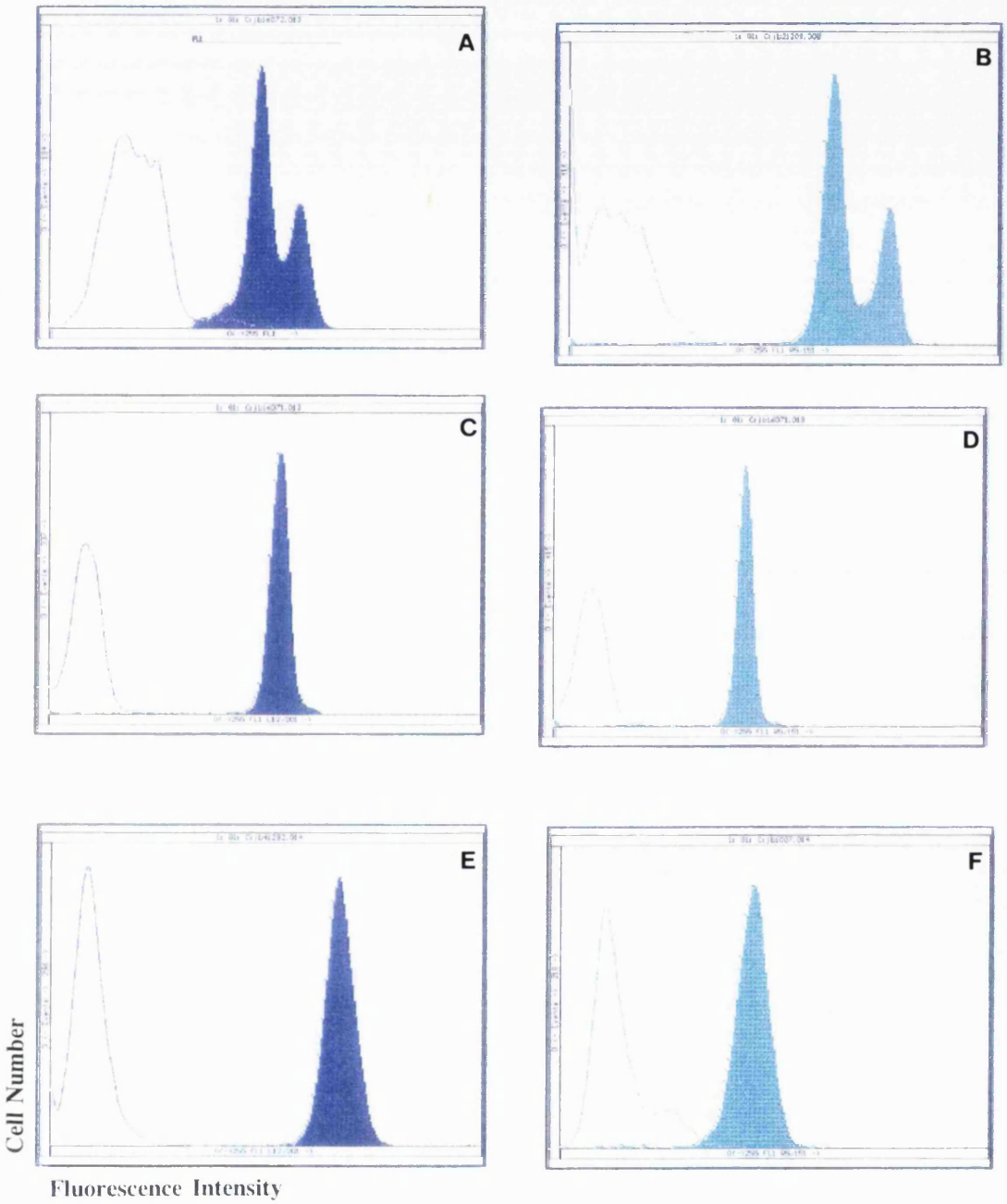

Figure 4.9. *Flow cytometric analysis of (A and B) rabbit mononuclear cells, (C and D) peripheral neutrophils and (E and F) RL-5 cells, stained with (A, C and E) L12/201 (anti-CD45) and (B, D and F) R5/151. Staining with the negative control mAb is shown as open histograms.*

4.2.5. Molecular characterisation of the antibody recognised by mAb R5/443

Initial studies showed that mAb R5/443, raised against RL-5 cells, reacted strongly with RL-5 cells by ELISA and surface immunofluorescence staining. To determine the molecular nature of the antigen recognised by mAb R5/443, it was used in a series of immunprecipitation experiments. mAb L11/135, L13/64 and T1/7 were used as positive and negative controls. From ^{35}S-metabolically labelled RL-5 cell lysates, R5/443 precipitated a major band at 110 kDa and a minor band at 45 kDa under reducing and non-reducing conditions (figure 4.10 lanes C and E).

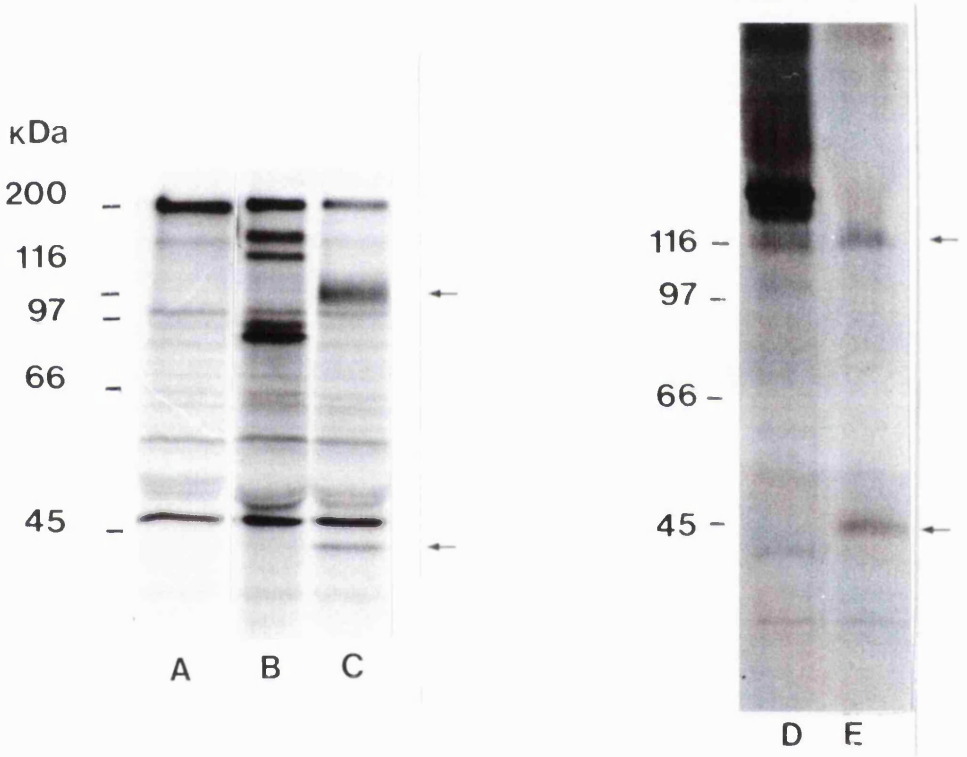

Figure 4.10. Immunoprecipitation of the antigen recognised by mAb R5/443 from ^{35}S-labelled RL-5 cells, (lane A) T1/7 (negative control), (lane B) L13/64 (positive control), (lane C) R5/443, (lane D) L11/135 and (lane E) R5/443. Precipitates were separated on 8% gels under (lanes A-C) reducing and (lanes D and E) non-reducing conditions.

When BJ/873 cells were metabolically ^{35}S-labelled or surface labelled with biotin, R5/443 precipitated a single band with an apparent molecular weight of 120 kDa whether precipitates were run under reducing (figure 4.11 lanes C and F) or non-reducing conditions (figure 4.11 lanes D and G).

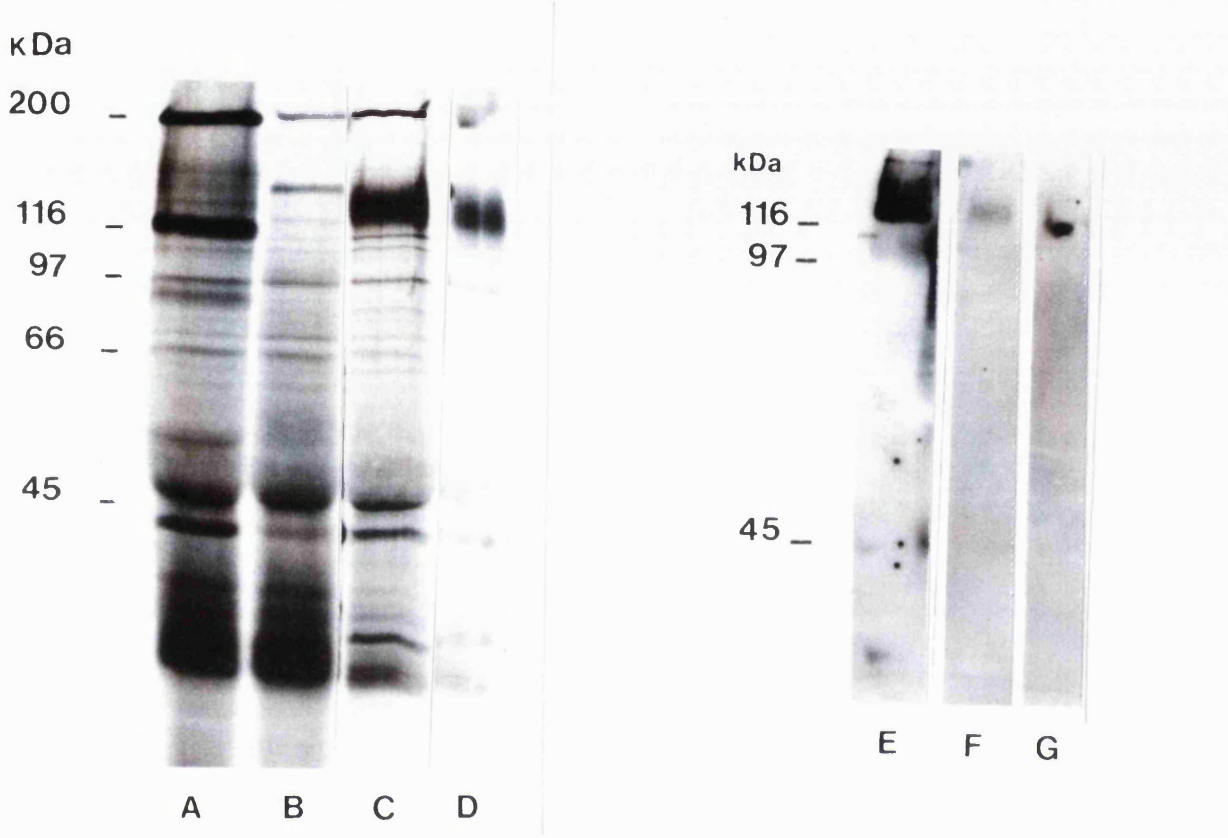

Figure 4.11. Immunoprecipitation from (lanes A-D) ^{35}S-labelled BJ/873 cells lysates and (lanes E-G) biotin labelled BJ/873 cell lysates with (lanes A and E) L11/135, (lane B) T1/7 (negative control), (lanes C, D, F and G) R5/443. Precipitates were separated on a 8% gel under (lanes A, B, C, E, and F) reducing and (lanes D and F) non-reducing conditions.

Rabbit peripheral blood leucocytes were stained for flow cytometry with R5/443. Cells were also stained with control mAbs T1/7 (negative control), L11/135 (CD43), NRBM (anti-μ chain) and NR185 (anti-CD11a, chapter six). The results from a typical experiment are shown in figure 4.12. Mononuclear cells were stained as a single cell population and gates were set for data analysis according to light scatter profiles. B lymphocytes were labelled at low levels ($MFI_{R5/443}=50$, $_{neg}=31$) (figure 4.12.B), monocytes at intermediate levels ($MFI_{R5/443}=81$) (figure 4.12.D) and T lymphocytes

brightly ($MFI_{R5/443}$=131) (figure 4.12.B). Immunolabelling of peripheral neutrophils with R5/443 showed that these cells express very low levels of this antigen ($MFI_{R5/443}$=50, $_{neg}$=29) (figure 4.12.F). R5/443 labelled RL-5 cells at intermediate levels (MFI_{neg}=29, $_{R5/443}$=98) (figure 4.12.H).

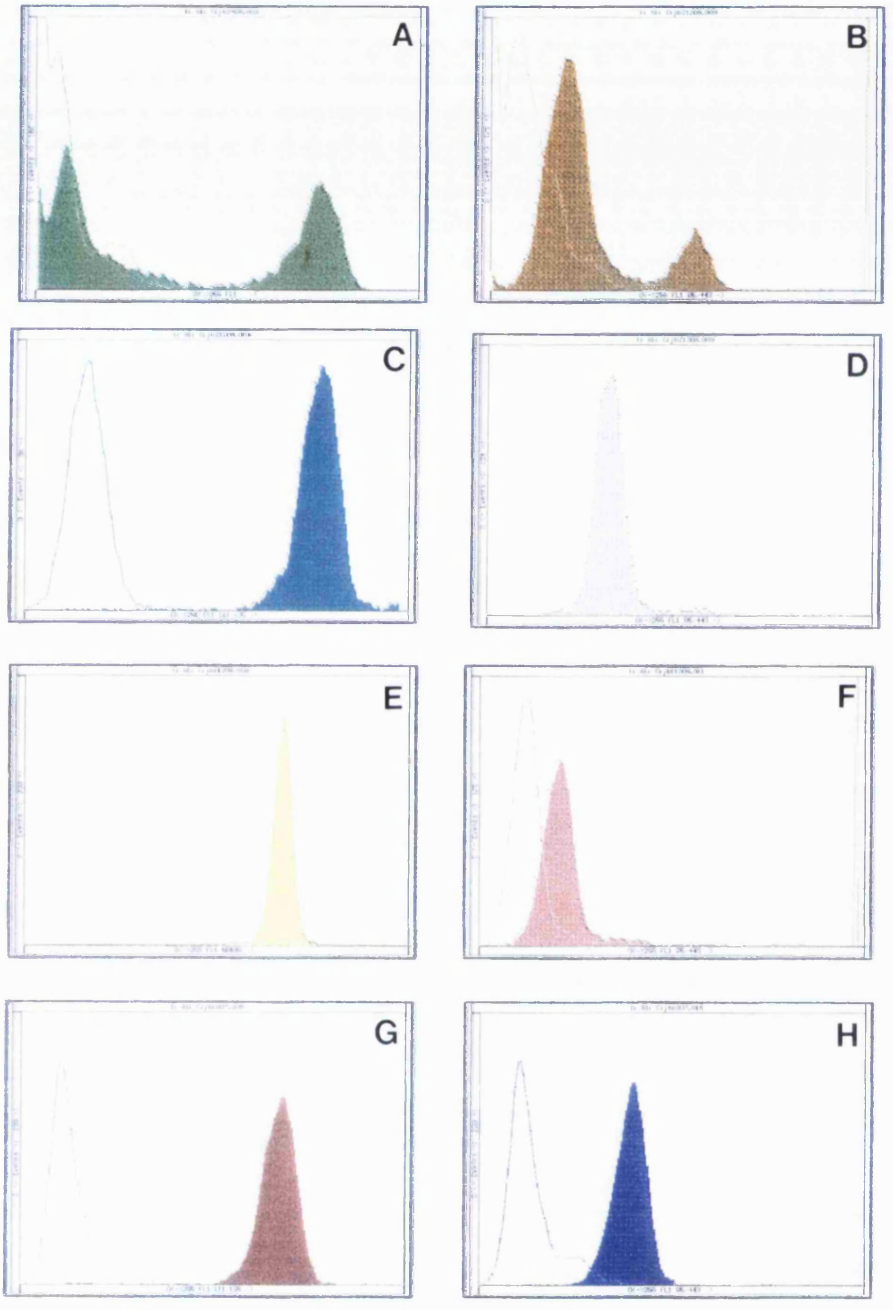

Figure 4.12. *Flow cytometric analysis of rabbit peripheral (A and B) lymphocytes, (C and D) monocytes, (E and F) neutrophils and (G and H) RL-5 cells, stained with (A, C and G) L11/135 (anti-CD43) and (E) NR185 (anti-CD11a), and (B,D, F and H) R5/443. Cells were also stained with T1/7 (negative control) shown as open histogram.*

4.2.6. mAb R9/17 recognises a 23 kDa antigen

mAb R9/17, raised against RL-5 cells, showed strong reactivity by cellular ELISA and surface immunofluorescence with RL-5 and BJ/873 cells. Labelling of peripheral blood leucocytes showed that R9/17 reacted with peripheral rabbit mononuclear cells, but not peripheral neutrophils and on this basis R9/17 was selected for further investigation.

R9/17 was used to immunoprecipitate antigen from ^{35}S-labelled RL-5 and BJ/873 cells. mAb T1/7 and L13/64 (anti-CD18) were used as negative and positive controls respectively. mAb R9/17 precipitated a single band of 23 kDa from these cell lysates (figure 4.13 lanes C and F). The R9/17 antigen had the same electrophoretic mobility under non-reducing conditions.

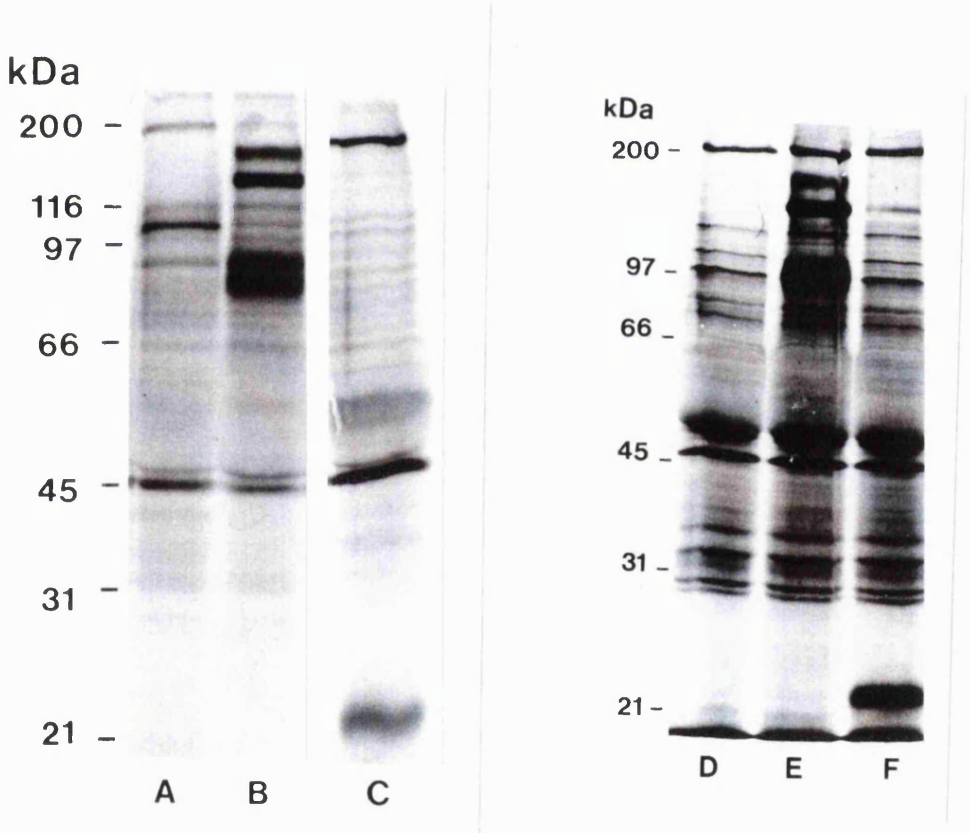

Figure 4.13. Immunoprecipitation from (lanes A-C) ^{35}S-labelled RL-5 cells and (lanes D-F) BJ/873 cells with (lanes A and D) T1/7 (negative control), (lanes B and E) L13/64 (anti-CD18, positive control) and (lanes C and F) R9/17. Precipitates were separated on 8% SDS-PAGE gels under reducing conditions.

The experiment was repeated using surface biotinylated RL-5 cell lysates. mAb L11/135 (anti-CD43) and Ken-4 (anti-CD4) were used as positive controls and precipitates were separated by SDS-PAGE on 15% gels, electroblotted to nitrocellulose membrane and developed by enhanced chemiluminescence (section 2.5.7). L11/135 precipitated a band at 120 kDa and Ken-4 a band at 48 kDa, R9/17 did not precipitate antigen from biotin surface labelled RL-5 cells.

Proteins from RL-5 cells were separated by SDS-PAGE under reducing or non-reducing conditions and electroblotted to nitrocellulose. mAb R9/17 failed to immunolabel proteins using this technique, while positive control mAb R5/23 (anti-CD44) and L11/135 (anti-CD43) labelled bands at 90 kDa and 110 kDa respectively.

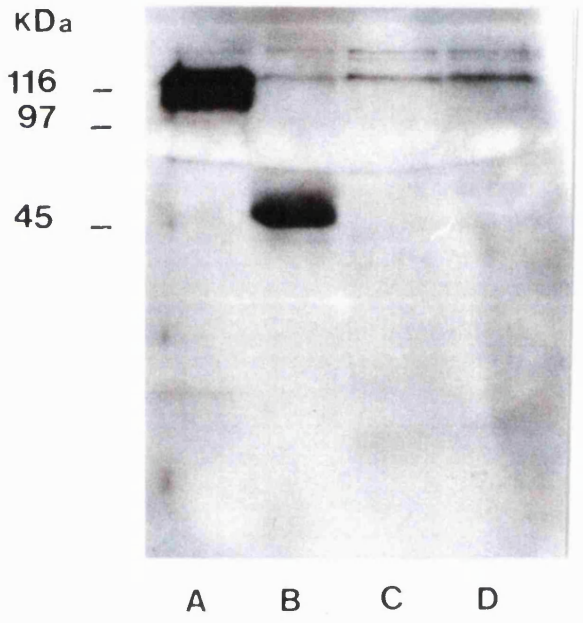

Figure 4.14. *Immunoprecipitation from biotinylated RL-5 cells lysates with (lane A) L11/135 (anti-CD43), (lane B) Ken-4 (anti-CD4) (lane C) R9/17 and (lane D) T1/7 (negative control). Precipitates were separated on 15% gels, protein was transferred to nitrocellulose and bands were detected by ECL.*

mAb R9/17 was used to stain rabbit peripheral blood leucocytes and rabbit T cell lines for flow cytometry and the results of a typical experiment are shown in figure 4.15. Monocytes and lymphocytes were stained as a single population and gates were set for data analysis according to light scatter properties. Cells were stained with T1/7 and

L11/135 (anti-CD43) as negative and positive controls. All lymphocytes express the R9/17 antigen. B lymphocytes and a population of T lymphocytes were labelled at intermediate levels (MFI_{neg}=39, $MFI_{R9/17}$=117) (figure 6.15.A). Monocytes (figure 6.15.B) and the remaining T lymphocyte population were labelled by R9/17 at high levels (MFI_{neg}=39, $MFI_{R9/17}$=155). The histogram produced from labelling isolated neutrophils overlaid the histogram obtained from labelling with the negative control mAb T1/7 (MFI_{neg}=50, $MFI_{R9/17}$=60) (figure 6.15.C). The antigen recognised by mAb R9/17 is expressed at intermediate levels by all four rabbit T cell lines (figure 6.15.D-G).

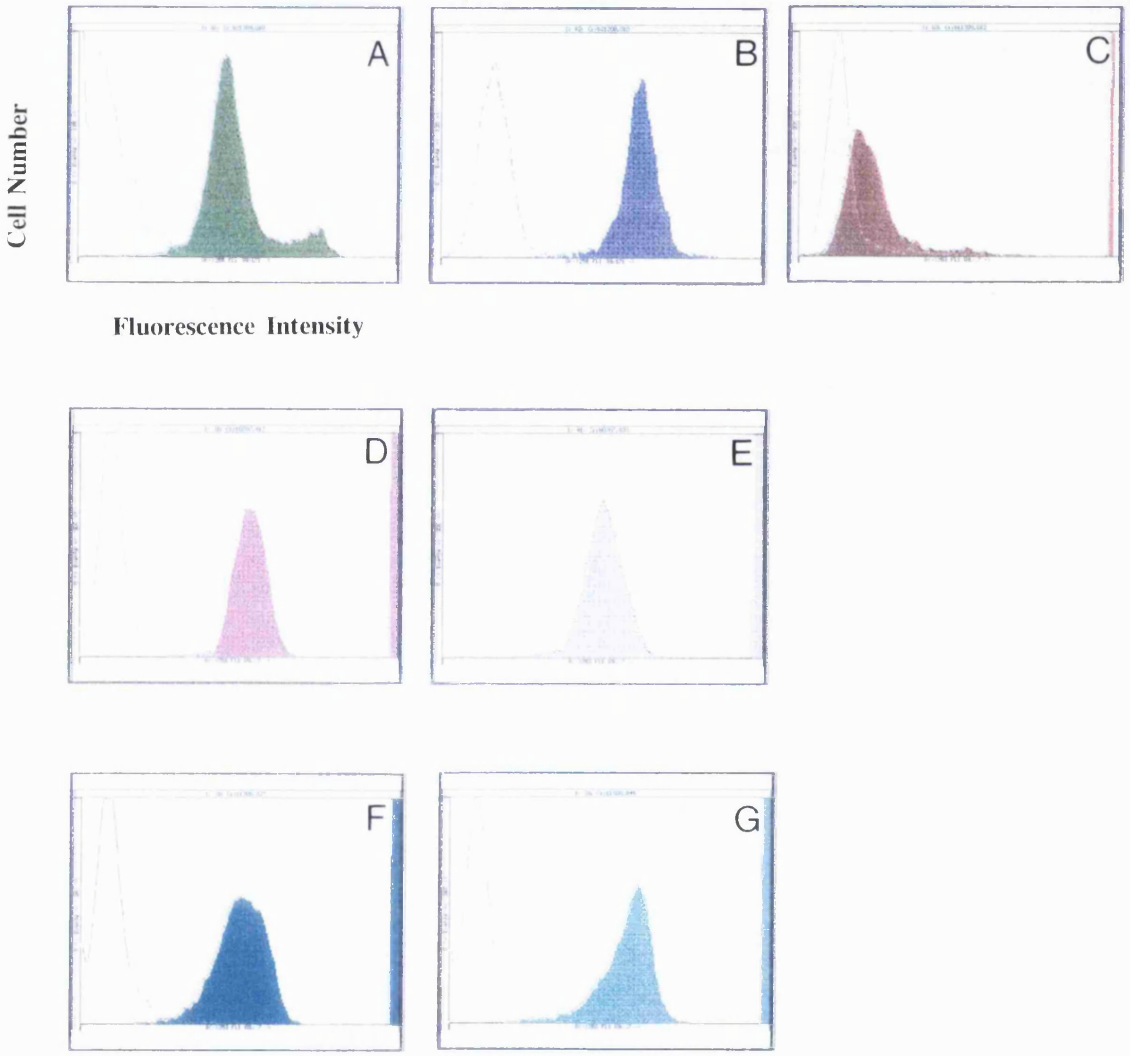

Figure 4.15. Flow cytometric analysis of rabbit peripheral leucocytes and T cell lines stained with R9/17: (A) lymphocytes, (B) mononuclear cells, (C) neutrophils, (D) RL-5 cells, (E) BJ/873 cells, (F) BJ610 cells and (G) BJ610 cells. Staining with T1/7 (negative control) is represented by the open histogram.

Purified mAb R9/17 was coupled to CnBr sepharose as described (2.6) and the immunoadsorbant was used to purify the R9/17 antigen. The antigen recognised by mAb R9/17 has an apparent molecular mass of 23 kDa, while mouse IgG light chain has a molecular mass of 25 kDa. To prevent contamination of the 23 kDa antigen by mouse IgG light chain, the samples were run on a 15% polyacrylamide gel, 200 x 160 x 1.5 mm. The proteins were electroblotted on to Immobilon-P membrane and analysed by peptide mass-fingerprint analysis which is described in section (2.6.5). The tryptic peptides released from the R9/17 antigen were matched, by mass, to those released from the chicken analogue of the CD28 precursor molecule. This suggests that R9/17 may recognise the rabbit analogue of CD28 or a CD28 like protein.

4.2.7. Characterisation of the Antigen Recognised by mAb BJ5/69

Initial screening of mAb BJ5/69, showed that it reacted strongly with BJ/873 cells, but weakly with RL-5 cells and on this basis was selected for further investigation. SDS-extracts of BJ/873 cells were separated by SDS-PAGE, transferred to nitrocellulose and immunostained with BJ5/69 and control mAb using methods described in section 2.4. mAb BJ5/69 did not label proteins separated under reducing or non-reducing conditions. mAb BJ5/69 was used to immunoprecipitate antigen from metabolically or surface labelled BJ/873 cell lysates as described in section 2.5. mAb BJ5/69 failed to immunoprecipitate protein from these cell lysates, while mAb L13/64 (anti-CD18), used as a positive control, precipitated proteins of 90, 145 and 158 kDa.

Rabbit T cell lines were immunolabelled with BJ5/69 and the results from a typical experiment are shown in table 4.3 and figure 4.16. Cells were labelled with mAb T1/7 as a negative control and L11/135 (CD43) as a positive control. The histograms produced from labelling the rabbit $CD8^+$ T cell lines with mAb BJ5/69 show that the antigen is expressed at intermediate levels by BJ880 and BJ/873 cells (figure 4.16. E and F), and at high levels by BJ610 cells (figure 4.16.D). RL-5 cells were shown to be heterogenous in their expression of the BJ5/69 antigen. Approximately 50% of the cells expressed low levels of this antigen, with the remaining cells overlapping the histogram produced from labelling cells with mAb T1/7 (figure 6.16.C).

	Mean Fluorescence Intensity		
Cell Line	**L11/135**	**T1/7**	**BJ5/69**
RL-5	174	30	(50%) 29
			(50%) 96
BJ/873	166	30	107
BJ880	166	27	111
BJ610	186	37	218

Table 4.3. Flow cytometric analysis of rabbit T cell lines stained with BJ5/69, negative control mAb T1/7 and positive control L11/135.

Rabbit peripheral leucocytes were isolated from whole blood (described in section 2.1.4) and labelled with BJ5/69 for flow cytometry. The results from a typical experiment are shown in table 4.4 and figure 4.16. Cells were labelled with T1/7 (negative control) and positive control mAb L11/135 (T lymphocytes and monocyte control), NRBM (B lymphocyte control) and 198 (neutrophil control). The antigen recognised by mAb BJ5/69 was shown to be expressed on all peripheral mononuclear cell populations (figure 4.16.A). Monocytes expressed intermediate levels of the BJ5/69 antigen, while approximately 60% of lymphocytes expressed low levels and 40% higher levels. Peripheral neutrophils expressed low levels of the BJ5/69 (figure 4.16.B).

	Mean Fluorescence Intensity		
Gated cell population	**-ve control**	**+ve control**	**BJ5/69**
Lymphocytes	27	160	60% - 94 40% - 126
Monocytes	14	159	107
Neutrophils	24	138	60

Table 4.4. Mean fluorescence intensity levels obtained from FACS analysis of peripheral leucocytes labelled with BJ5/69. Cells were labelled with T1/7, L11/135 and 198 as controls.

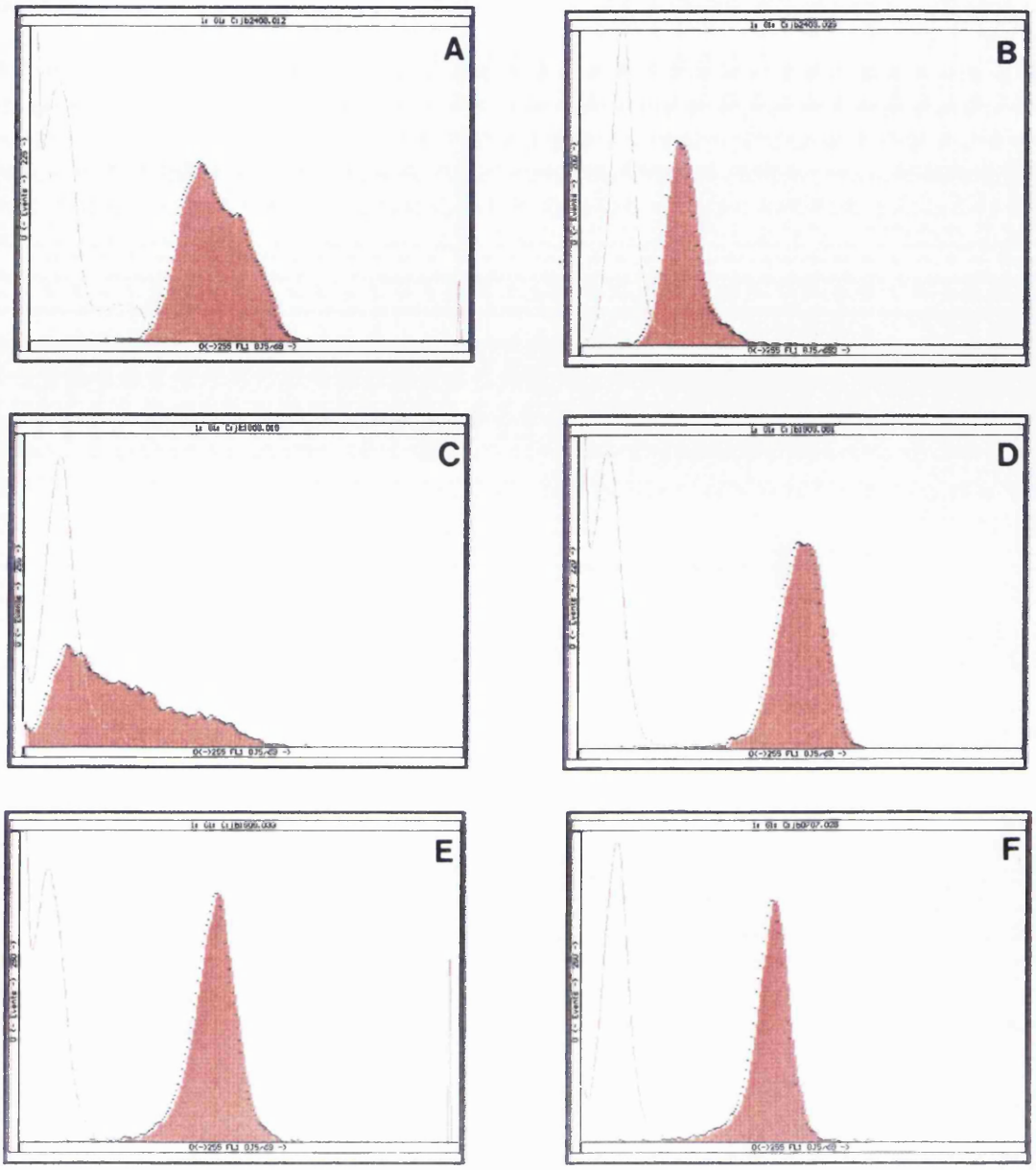

Figure 4.16. *Flow cytometric analysis of (A) rabbit peripheral mononuclear cells (B) neutrophils and T cell lines (C) RL-5, (D) BJ610, (E) BJ/880 and (F) BJ/873 stained with BJ5/69. Staining with the negative control mAb T1/61 is shown as the open histogram.*

4.2.8. Characterisation of the Antigen Recognised by mAb BJ5/74

Cells in well 74 of BJ873 cell fusion 5, reacted strongly with BJ/873 cells but not with RL-5 cells during screening and, on this basis, this well was cloned and tissue culture supernatant was prepared and used in the following studies. mAb BJ5/74 failed to stain proteins from BJ/873 cells by immunoblotting and was unable to immunoprecipitate antigen from detergent lysates of metabolically or surface labelled BJ/873 cells.

The expression of the antigen recognised by mAb BJ5/74 on rabbit T cell lines was determined by flow cytometry and the results of a typical experiment are shown in table 4.5 and figure 4.17. Cells were stained with T1/7 as the negative control and L11/135 as the positive control. The BJ5/74 antigen was shown to be expressed at high levels on BJ/873 cells (figure 4.16.F) and at intermediate levels on BJ/610 (figure 4.16.D) and BJ/880 (figure 4.16.E) cell lines. The broad base of the histograms produced by labelling pure populations of BJ/880 and BJ/610 cells with BJ5/74 suggests heterogenous expression of the antigen by the cells within each population. mAb BJ5/74 does not label the RL-5 cell line (figure 4.17.C).

	Mean Fluorescence intensity		
Cell Line	**L11/135**	**T1/7**	**BJ5/74**
RL-5	174	30	33
BJ/873	166	30	179
BJ/880	166	27	122
BJ610	186	27	131

Table 4.5. Mean fluorescence intensities from staining rabbit T cell lines with BJ5/74 mAb compared with control mAb staining (L11/135 +ve control, T1/7 -ve control).

Rabbit peripheral leucocytes were isolated from whole blood, as described 2.1.4, and stained for flow cytometry with BJ5/74 and T1/7 (negative control) and positive control mAbs L11/135 (anti-CD43) and 198 (anti-CD11b). The results from a typical experiment are shown in table 4.6 and figure 4.16. Mononuclear cells were stained and analysed as a whole population and the histogram obtained showed three levels of expression (figure 4.16.A). Approximately 67% of cells are essentially negative for BJ5/74 expression. This negative peak contained both lymphocytes and monocytes. The remaining lymphocyte population was labelled at intermediate and high levels. Isolated peripheral neutrophils expressed very low levels of the R5/74 antigen (figure 4.16.B).

		Mean Fluorescence Intensity	
Antibody	**Antigen**	**Mononuclear cells**	**Neutrophils**
T1/7	-ve control	22	24
L11/135	CD43	47% - 30 22% - 160 31% - 191	-
198	CD11b	-	135
BJ5/74	n/d	67% - 35 10% - 114 22% - 175	28

Table 4.6. Mean fluorescence levels obtained from FACS analysis of peripheral leucocytes stained with BJ5/74. Cells were also labelled with T1/7 (negative control), L11/135 (mononuclear cell control) and 198 (neutrophil control). n/d - not determined, - experiment not performed

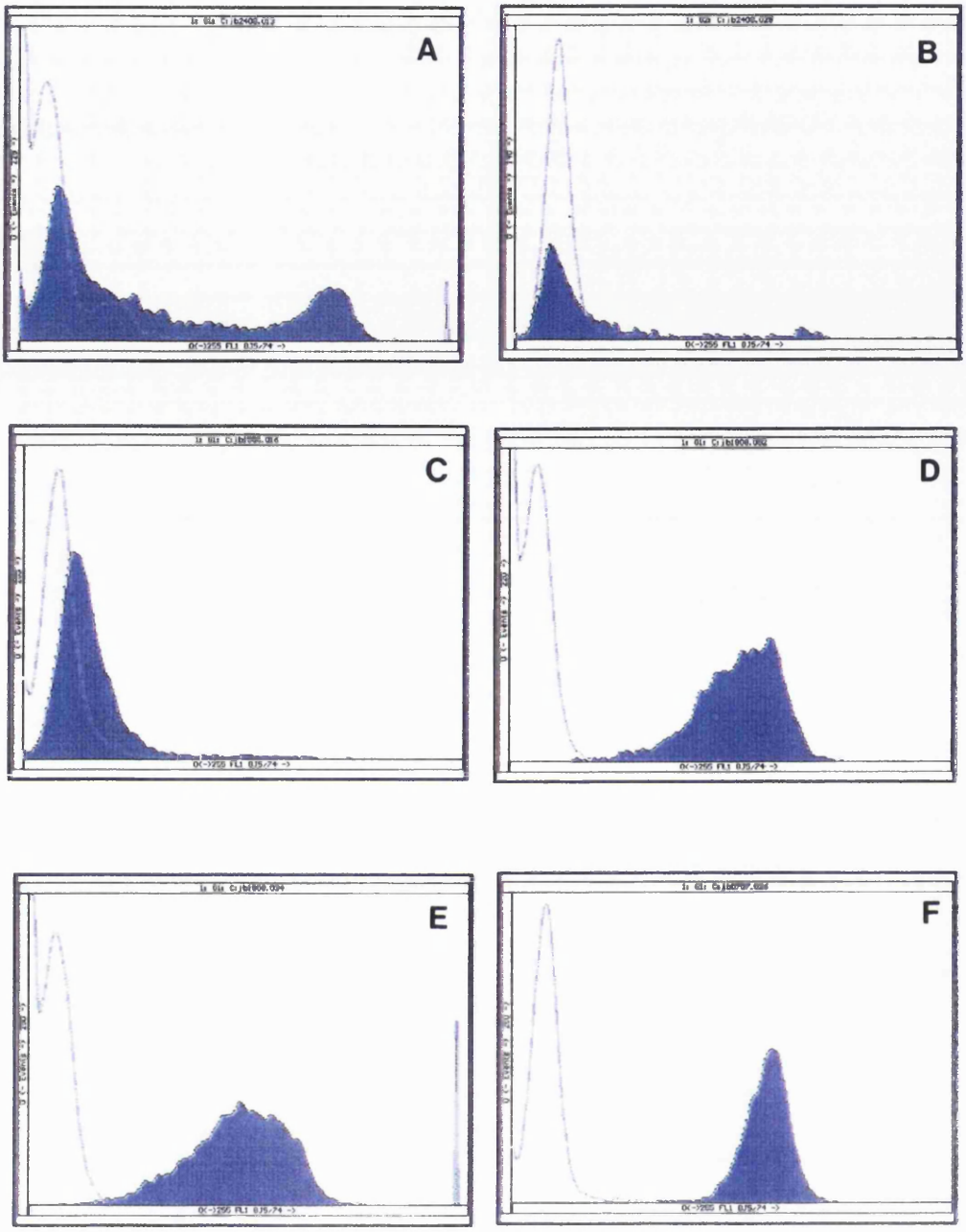

Figure 4.17. *Flow cytometric analysis of (A) rabbit peripheral mononuclear cells, (B) neutrophils, (C) RL-5 cells, (D) BJ610 cells, (E) BJ880 cells and (F) BJ/873 cells stained with BJ5/74. Staining with the negative control mAb, T1/7, is represented by the open histograms*

4.3. Discussion

mAb BJ4/55, raised against the BJ/873 cell line, was shown to immunostain a 120 kDa protein from RL-5 cell extracts. The BJ4/55 antigen has the same mobility by SDS-PAGE as the rabbit CD43 analogue which is recognised by mAb L11/135. Staining of peripheral blood mononuclear cells with BJ4/55 for flow cytometry produced FACS profiles which were almost identical to those produced from staining the cell population with L11/135. Mean fluorescence intensities obtained from staining with mAb BJ4/55 was lower than from staining with L11/135 and this is likely to be a reflection of mAb affinity. These data strongly suggest that mAb BJ4/55 recognises CD43.

The rabbit T cell lines RL-5, BJ/873, BJ610 and BJ880 were all labelled by L11/135, whereas BJ4/55 labelled RL-5, BJ/873 and BJ880, but not BJ610 cells. This suggests that the L11/135 epitope is distinct from the BJ4/55 epitope. In the human, CD43 shows cell-type-associated variation in glycosylation patterns (Cyster *et al.*, 1991). Cell type-specific glycosylation of rabbit CD43 may account for the different staining patterns obtained from staining rabbit T cell lines with L11/135 and BJ4/55. It has been suggested that L11/135 recognises a protein epitope because L11/135 labelled RL-5 cells following neuramidase treatment, although the antigen had an higher apparent molecular mass of 138 kDa (J. Galea-Lauri, unpublished observation). BJ4/55 may recognise a carbohydrate epitope although further studies are required before the nature of the BJ4/55 epitope is determined. Previously Cyster *et al.*, (1991) showed that the accessibility of protein epitopes displayed by rat CD43 are modified by glycosylation patterns. mAb BJ4/55 may prove to be a valuable tool in the study CD43 isoform expression in the rabbit.

mAb R5/23, R9/380 and R9/483 have been shown to recognise the rabbit CD44 analogue. The FACS profiles obtained from immunolabelling rabbit peripheral leucocytes with R5/23, R9/380 and R9/480 were identical to each other, and to those obtained from immunolabelling the same cell populations with the anti-rabbit CD44 mAb W4/86 and RPN3/24 (Wilkinson *et al.*, 1992). The three newly developed mAb immunostained a 90 kDa protein from SDS-extracts of RL-5 cells which was identical

to the protein immunostained by RPN3/57. Further, R5/23 and RPN3/24 immunoprecipitated a band of 90 kDa from ^{35}S-labelled RL-5 cell lysates. These data strongly suggests that R5/23, R9/380 and R9/483 recognise the rabbit CD44 homologue, described by (Wilkinson *et al.*, 1992). Several isoforms of CD44 have been described in the human and mouse though such variants have not been described for the rabbit. The preliminary biochemical data collected during this study suggests that the newly developed mAb recognise the same isoform as W4/86 and RPN3/24 which is probably homologous to the major form expressed on human leucocytes, CD44H.

Staining of peripheral leucocytes with mAb R5/151 produced FACS profiles that were comparable with those obtained from staining cells with L12/201, which recognises rabbit CD45. Both mAb stained a lymphocyte sub-population and neutrophils at intermediate levels and monocytes and the remaining lymphocyte population, presumably T lymphocytes, strongly. Differences in the mean fluorescence intensities obtained from staining cells with R5/151 and L12/201 are probably a reflection of antibody affinity or concentration since R5/151, which is an IgM molecule, was used as tissue culture supernatant rather than purified antibody. mAb R5/151 precipitated a single protein with an apparent molecular weight of 200 kDa from ^{35}S-labelled RL-5 cell lysates and sequential immunoprecipitation studies showed this was identical to the band precipitated by the anti-CD45 mAb, 1.24. These data suggest that the R5/151 antigen is the same as that recognised by mAb L12/201 and 1.24. Whether rabbit leucocytes are like other species and express different isoforms of CD45 has not been defined. However, if CD45 isoforms are expressed in the rabbit, L12/201 and 1.24 are likely to be reactive with multiple isoforms since they are reactive with the majority of circulating leucocytes and cells in lymphoid tissues, (Jackson *et al.*, 1983; Wilkinson *et al.*, 1993; J.M. Wilkinson and J. Galea-Lauri, unpublished observation). Whether R5/151 recognises multiple CD45 isoforms or a restricted epitope is not evident from these studies, this could be investigated by staining rabbit lymphoid tissues with R5/151 or by immunoprecipitation from labelled peripheral leucocytes.

The molecule recognised by mAb R5/443, raised against the RL-5 cell line, has not been identified during these studies. Data generated from studies with R5/443 cannot be compared with those generated from studies with other rabbit mAbs suggesting that R5/443 recognises a novel antigen. The antigen is expressed at low levels on all peripheral leucocytes except a small population of T lymphocytes which express high levels. Whether these are Th or Tc cells has not been determined, further studies in which cells are stained with R5/443 and Ken-4 (anti-CD4) or 12.C7 (anti-CD8) are required to address this question.

From metabolically labelled RL-5 cell lysates, R5/443 precipitated two bands of 110 and 45 kDa. The mobility of these bands by SDS-PAGE was the same regardless of whether reducing or non-reducing conditions were used, which suggests that these two proteins are not disulphide linked. Immunoprecipitation with R5/443 from surface labelled RL-5 cells is required to determine whether both the 110 and 45 kDa molecules are expressed at the cell surface. From BJ/873 cell lysates, R5/443 immunoprecipitated a single band of 120 kDa. The 45 kDa protein co-precipitated with the 110 kDa band from RL-5 cells by R5/443 was not precipitated from BJ/873 cell lysates. The 150 kDa integrin subunit α_4 (CD49d), which associates with β_1 and β_7 subunits, undergoes post-translation cleavage generating two fragments of 70 and 80 kDa. Cleavage of α_4 is variable and incomplete and there is no evidence for disulphide linkage of the two fragments, although a potential protease cleavage site has been identified (Hedqvist, 1994). It is possible that the antigen recognised by R5/443 undergoes similar post-translational modifications. Thus the antigen may be cleaved in RL-5 cells and expressed as two associated chains of 110 and 45 kDa, while in BJ/873 cells the antigen is not modified and is expressed as a 120 kDa molecule. Further studies including immunoprecipitation of the R5/443 antigen from other rabbit lymphoid cells, immunostaining rabbit lymphoid tissues and amino-acid sequence analysis are required before this antigen can be identified.

The antigen recognised by R9/17 has not be determined during this study. The antigen recognised by R9/17 is expressed at high levels on a T lymphocyte sub-population and monocytes, and at intermediate levels on the remaining T lymphocyte population and

B lymphocytes. The rabbit T cell lines expressed intermediate levels of the R9/17 antigen. The antigen was not expressed on neutrophils. R9/17 immunoprecipitated a major band of 23 kDa under reducing and non-reducing conditions from detergent lysates of metabolically labelled RL-5 and BJ/873 cells. mAb R9/17 did not precipitate antigen from surface-biotinylated cell lysates, although immuofluorescence staining data clearly shows that R9/17 recognises a cell surface antigen. It is possible that the R9/17 antigen was not labelled by biotin and this may be as a result of a low lysine content. To confirm the expression of the 23 kDa antigen at the cell surface, R9/17 needs to be precipitated from lysates of, for example, surface-iodinated RL-5 and BJ/873 cells.

The 23 kDa band was analysed by mass-peptide fingerprinting techniques and tryptic peptides from the R9/17 antigen were matched, by mass, to tryptic peptides from the precursor of the chicken CD28 analogue (Young *et al.*, 1994). CD28 is an Ig superfamily member which is expressed on 95% of $CD4^+$ and 50% of $CD8^+$ human peripheral lymphocytes (Yamada *et al.*, 1985). Binding of CD28, expressed on T lymphocytes, to its ligand B7, expressed on B lymphocytes, in conjunction with TCR-MHC/antigen engagement, results in T lymphocyte activation and increased lymphokine secretion which appears to be the result of stabilisation of lymphokine mRNAs (Linsley *et al.*, 1991). The predicted molecular mass of CD28, after signal peptide cleavage, is 23 kDa (Aruffo and Seed, 1987). Immunoprecipitation of CD28 from surface labelled human T lymphocytes followed by analysis by SDS-PAGE under reducing and non-reducing conditions, showed that CD28 is a homodimer composed of two 44 kDa subunits. The increased apparent molecular weight of the cell surface protein by SDS-PAGE is the result of N-linked glycosylation. It is possible that the 23 kDa band precipitated from metabolically labelled cells represents an intracellular, unmodified form of rabbit CD28 or a novel CD28-like protein.

The molecular nature of the antigens recognised by mAb BJ5/69 and BJ5/74 have not been determined since they both failed to immunoprecipitate antigen from detergent lysates of metabolically and surface labelled cells. It is reasonable to assume that the affinity of BJ5/69 and BJ5/74 for antigen is too low for antigen precipitation. It has been suggested that immunoprecipitation requires minimum binding affinities of 10^8

mol^{-1} and mAbs with lower binding affinities are unable to precipitate antigen (Harlow and Lane, 1988). Alternatively, it is possible that the BJ5/69 and BJ5/47 antigens are not available in BJ/873 cell lysates for immunoprecipitation. Detergent lysis of the cells may be too harsh and denature the antigen preventing mAb-antigen interaction, or may be too mild to solubilise the antigen which would be removed with other insoluble material following ultracentrifugation.

The BJ5/69 antigen is expressed on all peripheral leucocyte populations tested to some degree. Neutrophils expressed only low levels of the BJ5/69 antigen and monocytes and 60% of lymphocytes low to intermediate levels. Typically 40% of lymphocytes labelled brightly, though from this data, it is not clear to which lymphocyte sub-populations these cells belong. Two colour analysis of cells labelled with BJ5/69 and NRBM (B lymphocyte marker) or L11/135 (T lymphocyte marker), 12.C7 (CD8) and Ken-4 (CD4) will confirm which lymphocytes express high levels of the BJ5/69 antigen. RL-5 cells did not express, or expressed only low levels of the BJ5/69 antigen, while the $CD8^+$ T cell lines expressed intermediate levels. Further studies are required to identify the antigen recognised by mAb BJ5/69 which may be useful as a rabbit lymphocyte marker.

mAb BJ5/74 stained only a small population of peripheral lymphocytes strongly. Analysis of scatter plots showed that cells which expressed the antigen had low FSC and SSC indicative of lymphocytes. Whether these cells are Tc, Th or B lymphocytes is not clear from the data obtained during this study. The rabbit $CD8^+$ T cell lines express the BJ5/74 antigen, but the antigen is absent from the $CD4^+$ T cell line, RL-5. While this implies that it is T lymphocytes in peripheral blood which express this antigen, future studies using two colour flow cytometric analysis of cells stained with BJ5/74 and mAb NRBM (B cell marker) or 12.C7 (CD8) and Ken-4 (CD4) are required. Interestingly, L11/135 which stains T lymphocytes brightly (Jackson *et al.*, 1983), labelled 30% of cells which compares with the number of cells stained with BJ5/74.

More rigorous investigations are required before definite conclusions regarding the nature of the BJ5/74 antigen can be drawn, although the preliminary data collected

during this study suggest that expression of the antigen by peripheral leucocytes is restricted to T lymphocytes. For this reason mAb BJ5/74 may prove to be particularly useful since few mAb which exclusively label all rabbit T lymphocytes but not other cell populations have been described. The exception is mAb 9AE10 which recognises peripheral T cells, over 90% of thymocytes and T cells in peripheral lymphoid tissues (McNicholas *et al.*, 1981). The antigen recognised was a 25 kDa glycoprotein and it was compared with the mouse Thy-1 antigen. The 9AE10 antigen is expressed by RL-5 cells and therefore differs from the BJ5/74 antigen which is absent from RL-5 cells.

CHAPTER FIVE

mAb R9/96 Recognises the Rabbit Homologue of CD4

5.1. Introduction

CD4 has been identified as a 55 kDa glycoprotein expressed by 60-70% of human peripheral T lymphocytes (Parnes, 1989). Molecular cloning of the CD4 gene shows that it contains 4 tandem Ig-like domains in the extracellular region, a transmembrane domain and a 38 residue cytoplasmic tail (Maddon *et al.*, 1985). Crystal structures are available for the two NH_2-terminal domains (D1 and D2) of human CD4 (Wang *et al.*, 1990: Ryu *et al.*, 1990) and the two C-terminal domains (D3 and D4) of rat CD4 (Brady *et al.*, 1993) and evidence from these, and electron micrographs, suggest that the extracellular region of CD4 is rod-like and extends up to 120Å from the cell surface (Kwong *et al.*, 1990; Lange *et al.*, 1994).

Functionally CD4 acts as a T lymphocyte accessory molecule which is involved in the recognition of peptide antigens bound to MHC class II. Interaction of CD4 and the TCR/CD3 complex on the T lymphocyte with MHC class II/peptide antigen expressed on the antigen presenting cell (APC), combined with co-stimulation from other receptor-ligand pairs, stimulates T helper lymphocyte activation. This triggers secretion of cytokines which play a role in the antibody and cytotoxic T lymphocyte responses (Male *et al.*, 1993). Site directed mutagenesis studies have shown that D1 of human CD4, parts of D2 and the region between the two domains are involved in CD4 binding to non-polymorphic regions of MHC class II (Moebius *et al.*, 1993) and it has been proposed that one function of CD4 is to provide stability to the T lymphocyte-APC interaction (Doyle and Strominger, 1987). However the ability of anti-CD4 mAb to inhibit T lymphocyte activation in response to non-specific triggers such as mitogen suggest that CD4 has a signalling function. The cytoplasmic domain of CD4 is associated with $p56^{lck}$, a 56 kDa intracellular protein which is a member of the *src* family of tyrosine kinases (Veillette *et al.*, 1988). Following MHC class II mediated TCR/CD3 and CD4 cross-linking, the CD4/$p56^{lck}$ complex is able to phosphorylate the CD3ζ chain on tyrosine residues and these signals may further promote T lymphocyte

activation (Veillette *et al.*, 1989). During activation CD4 itself is phosphorylated on serine residues and this may lead to modulation from the cell surface by internalisation. CD4 has been shown to be essential for thymocyte development, but this function of CD4 is not mediated through $p56^{lck}$ signalling events (Killeen and Littman, 1993).

CD4 has been shown to act as the receptor for HIV-1 (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984) and regions within D1 of CD4 are essential for HIV-1 envelope glycoprotein gp120 binding (Houlgatte *et al.*, 1994). The discovery that rabbit cell lines can be infected with HIV-1, reviewed by Varnier and Kindt (1992), combined with the need for a small animal model for studying acquired immunodeficiency syndrome (AIDS), prompted the molecular cloning of rabbit CD4 (Hague *et al.*, 1992). The deduced amino-acid sequence of rabbit CD4 shares a high level of sequence identity with human CD4 (Maddon *et al.*, 1985). mAb Ken-4, raised against rabbit thymocytes, has been described by Kotani *et al.*, (1993) and shown to react with rabbit CD4. Ken-4 precipitates two bands, of 42 and 50 kDa, from surface radio-labelled thymocytes and labels thymocytes and T cell areas in peripheral lymphoid tissues. Other than Ken-4, no mAb which recognise rabbit CD4 have been described (Wilkinson *et al.*, 1984).

mAb R9/96, raised against RL-5 cells, reacted strongly with the RL-5 cells but not with the rabbit $CD8^+$ cell line BJ/873 by cellular ELISA and surface immunofluorescence. The data presented in this chapter show that R9/96 recognises rabbit CD4. mAb R9/96 may be expected to be a useful tool for the study of infection of rabbit $CD4^+$ lymphocytes with HIV-1, and for identifying $CD4^+$ lymphocytes from other infiltrating leucocytes in rabbit models of disease such as rheumatoid arthritis.

5.2. Results

5.2.1. Molecular characterisation of the antigen recognised by mAb R9/96

The antigen recognised by mAb R9/96 was immunoprecipitated from detergent lysates of ^{35}S-labelled RL-5 cells (figure 5.1). R9/96 precipitated a protein with an apparent molecular weight of 48 kDa under reducing conditions (lane C). This band was similar to the band precipitated by the anti-rabbit CD4 mAb, Ken-4.

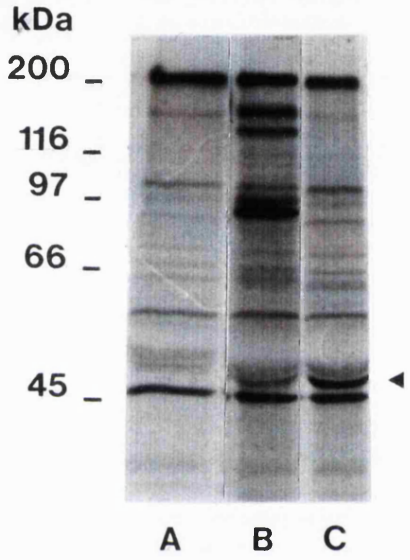

Figure 5.1. Immunoprecipitation from ^{35}S-labelled RL-5 cell lystaes with (A) T1/7 (negative control), (B) L13/64 (positive control) and (C) R9/96. Precipitates were run under reducing conditions on 8% SDS-PAGE gels.

mAb R9/96 and Ken-4 were compared by sequential immunoprecipitation from ^{35}S-labelled RL-5 cell lysates (figure 5.2). mAb R9/96 failed to precipitate antigen from a cell lysate pre-cleared with Ken-4 (lane F). When Ken-4 was used to precipitate antigen from lysate pre-cleared with R9/96 a faint band at 48 kDa was visible (lane G). The results of this experiment strongly suggest that R9/96 recognises the rabbit CD4 homologue. R9/96 and Ken-4 were used to precipitate antigen from ^{35}S-labelled RL-5 cells lysates and precipitates were run under non-reducing conditions. Major bands at 48 kDa and minor bands at 97 kDa were seen (lanes K and L).

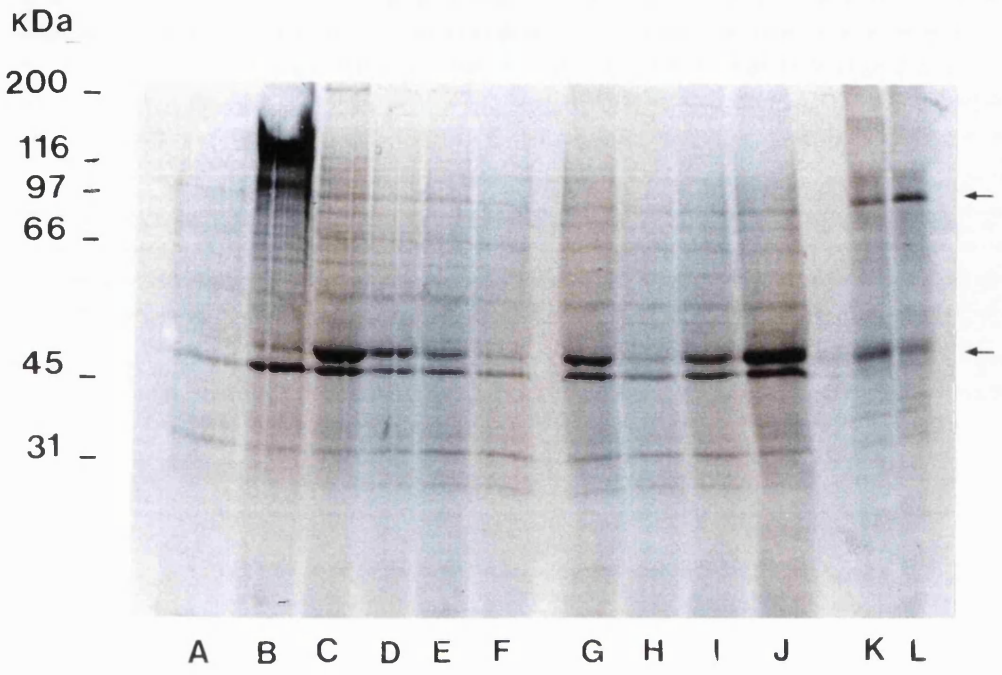

Figure 5.2. Comparison of the antigens recognised by mAb R9/96 and Ken-4 by sequential immunoprecipitation from ^{35}S-labelled RL-5 cell lysates. Lysate was pre-cleared with R9/96 (lanes C-E) and re-precipitated with Ken-4 (lane G) or was pre-cleared with Ken-4 (lanes H-J) and re-precipitated with R9/96 (lane F). Precipitation with negative control mAb T1/61 (lane A) and positive control mAb L11/135 (lane B) is shown. Precipitates were separated under reducing conditions by 8% SDS-PAGE. Precipitation with mAb (lane K)Ken-4 and (lane L) R9/96 separated under non-reducing conditions.

mAb R9/96 and Ken-4 were used to immunoprecipitate CD4 from detergent lysates of biotin surface-labelled RL-5 cells. Samples were separated on 8% gels and proteins were transferred to nitrocellulose and detected by enhanced chemiluminescence (figure 5.3). Only a single band at 48 kDa was obtained when R9/96 and Ken-4 precipitates were separated under reducing conditions (lanes C and D), while a major band at 48 kDa and a minor band at 97 kDa were seen when precipitates were separated under non-reducing conditions (lanes E and F).

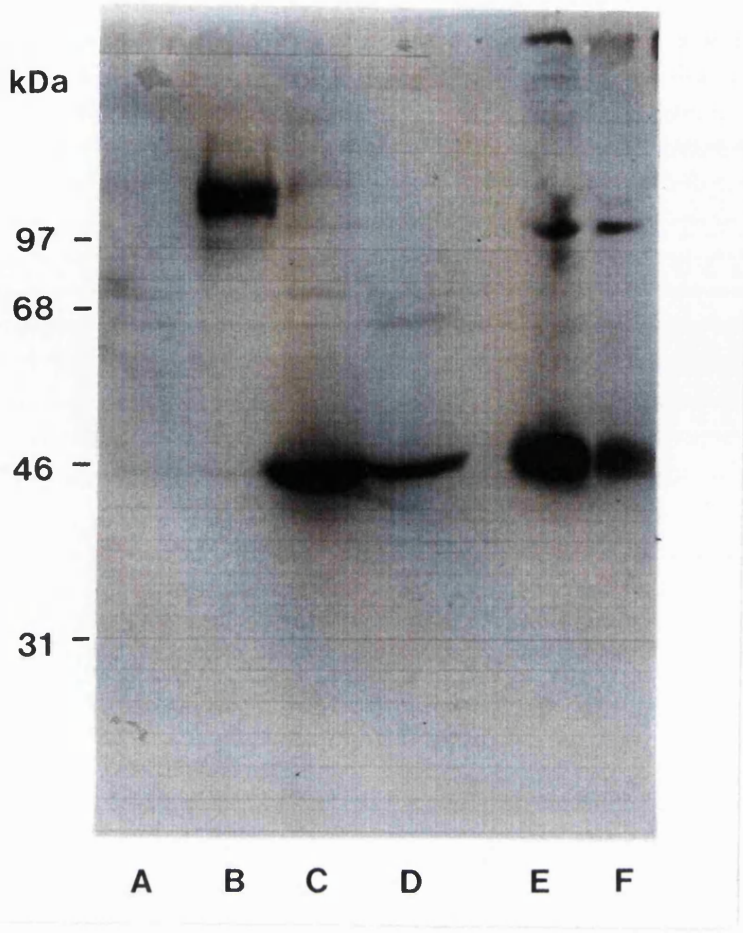

Figure 5.3. *Immunoprecipitation of CD4 from biotinylated RL-5 cell lysates with Ken-4 and R9/96, (lane A) T1/7 (negative control), (lane B) L11/135 (positive control), (lane C) Ken-4 (reduced), (D) R9/96 (reduced), (lane E) Ken-4 (non-reduced) and (lane F) R9/96 (non-reduced).*

5.2.2 mAb R9/96 inhibits Ken-4 binding to RL-5 cells

Sequential immunoprecipitation experiments show that mAb R9/96 is reactive with the rabbit CD4 homologue. To confirm this observation, mAb R9/96 and Ken-4 were tested in competitive binding assays designed to determine whether binding of R9/96 inhibited the binding of the Ken-4 to RL-5 cells (as described in section 2.3.5). RL-5 cells were incubated with increasing amounts of R9/96 unconjugated mAb, washed and incubated with fluorescinated Ken-4 (figure 5.4). During this experiment mAb R9/96, and the control anti-CD45 mAb, were used as TCSN while Ken-4 was used as a purified IgG. This was because R9/96 is a murine IgM molecule and therefore difficult

to purify. The results of a typical experiment show that pre-incubation of RL-5 cells with saturating amounts of mAb R9/96 TCSN inhibited the binding of mAb Ken-4 by 54% (figure 5.5), while an isotype matched anti-CD45 mAb had no effect on the level of Ken-4-FITC binding. This supports the proposal that R9/96 recognises rabbit CD4.

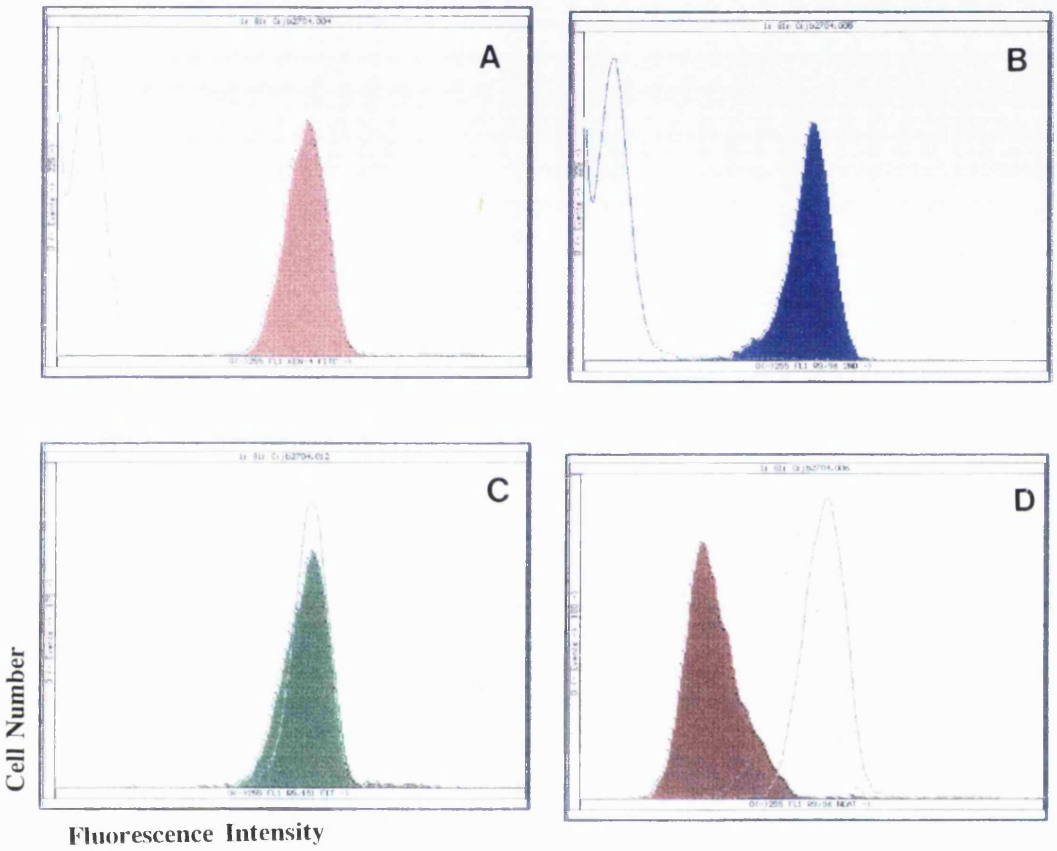

Figure 5.4. *Inhibition of direct immunofluorescence of Ken-4-FITC by R9/96. RL-5 cells were stained with (A) Ken-4-FITC, (B) R9/96 followed by rabbit anti-mouse Ig FITC-conjugate, (C) pre-incubation with R5/151 (anti-CD45, control mAb) followed by Ken-4-FITC (solid histogram) compared with Ken-4-FITC alone (open histogram) and (D) pre-incubation with neat R9/96 TCSN (solid histogram) or 1:80 dilution R9/96 TCSN (dotted histogram) followed by incubation with Ken-4-FITC, compared with incubation with Ken-4-FITC alone (open histogram). Staining with the negative control mAb T1/7 is shown as open histogram (A and B).*

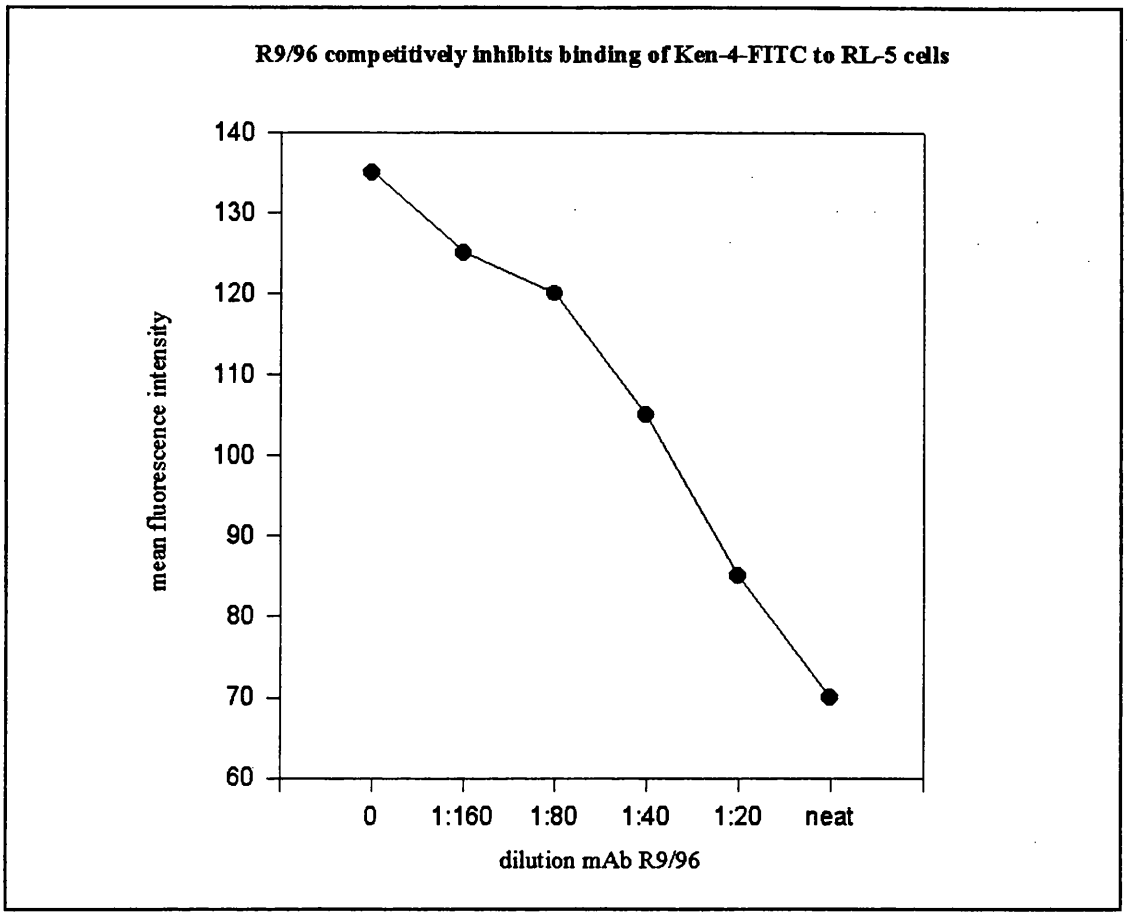

Figure 5.5. Inhibition of direct immunofluorescence of Ken-4-FITC binding to RL-5 cells by mAb R9/96. Cells were pre-incubated with increasing concentrations of R9/96, before incubation with Ken-4-FITC .

5.2.3 Expression of CD4 by rabbit leucocytes

Rabbit peripheral leucocytes were stained for flow cytometry with R9/96 and Ken-4. The experiment was repeated three times and the results from a typical experiment are shown in figure 5.6. mAb T1/7 gave background staining levels (MFI = 50) and L13/64 labelled all mononuclear cells as expected (MFI = 213). mAb R9/96 stained 24% of the mononuclear cell population at intermediate levels (MFI = 145) (figure 5.6.A). R9/96 positive cells had low SSC and FSC, indicative of lymphocytes. To confirm that only a sub-population of T lymphocytes were stained by R9/96, mononuclear cells were labelled with R9/96 and L11/135 for two colour flow

cytometric analysis according to the methods described in section 2.3.4 (figure 5.6.C). L11/135 stains T lymphocytes and monocytes brightly, but does not stain B lymphocytes. Cells which labelled brightly with R9/96 also stained brightly with L11/135 and these presumably are the Th population. The L11/135 negative population which are B lymphocytes were not stained by R9/96. Monocytes and the remaining T cell population were labelled brightly with L11/135 but not R9/96. mAb Ken-4 stained 37% of cells brightly (MFI = 172) (figure 5.6.B). The majority of the cells stained were lymphocytes, though it appeared that some monocytes (high FSC and SSC) were positive. In the human and rat, but not the mouse, CD4 is expressed by monocytes and macrophages and so labelling of monocytes is not unusual. This difference in the staining of monocytes by R9/96 and Ken-4 may be a reflection of mAb affinity. Immunoprecipitation and mAb inhibition of data suggested that, of the two mAb, Ken-4 had the higher affinity for CD4 and would therefore label cells expressing low levels of CD4. Neutrophils were not stained with R9/96 or Ken-4 (figure 5.6. D and F). The RL-5 rabbit T cell line stained at intermediate levels with R9/96 (MFI = 115) (figure 5.6.E) and Ken-4 (MFI = 138), while histograms produced from staining BJ/880 and BJ/873 cells with R9/96 or Ken-4 overlapped negative control histograms (figure 5.6 G and H).

Immunofluorescence staining of rabbit leucocytes with mAb R9/96

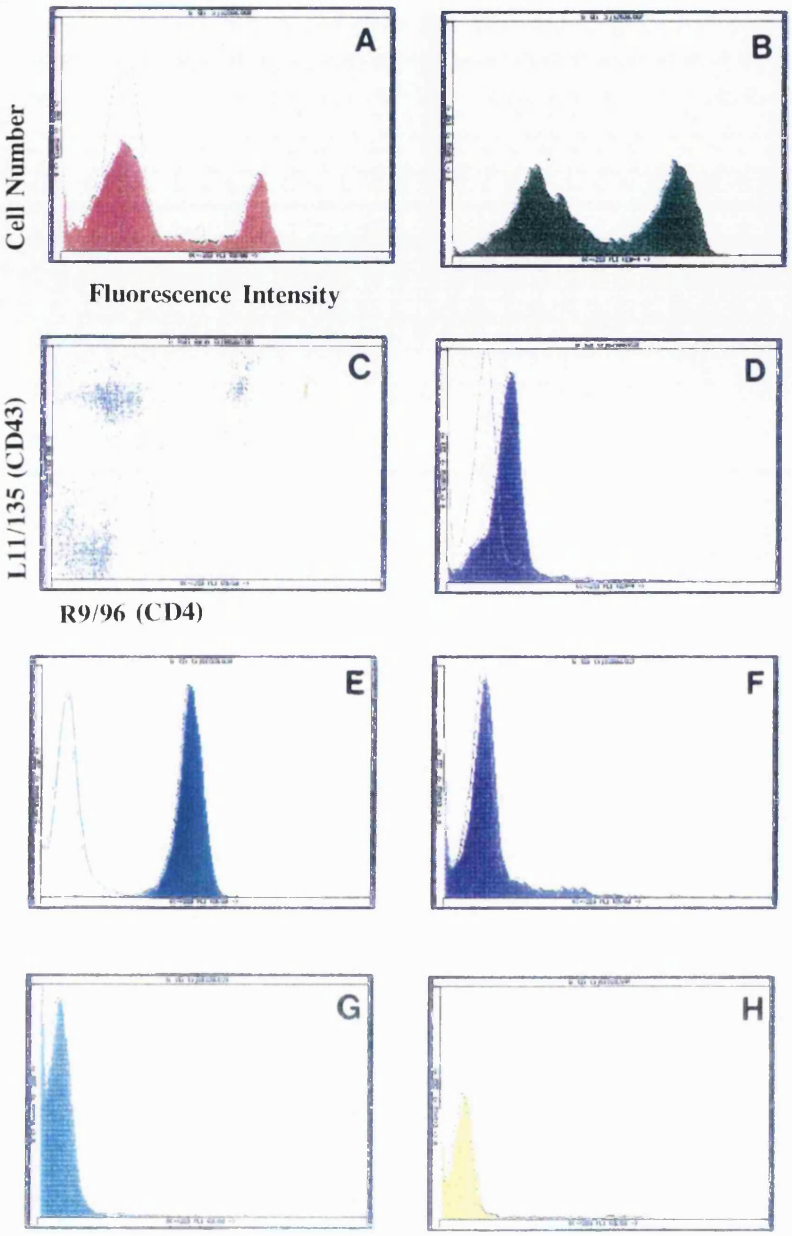

Figure 5.6. *Peripheral monunuclear cells (A-C) were stained with (A) R9/96, (B) Ken-4 and (C) R9/96 and L11/135. Neutrophils were stained with (D) R9/96 or (F) Ken-4. The rabbit T cell lines (E) RL-5, (G) BJ/880 and (H) BJ/873 were stained with R9/96 and control staining is shown as the open histogram.*

5.3. Discussion

The data presented here show that mAb R9/96 is reactive with the rabbit CD4 analogue. Like the anti-rabbit CD4 mAb Ken-4 (Kotani *et al.*, 1993), R9/96 is reactive with a sub-population of peripheral lymphocytes and precipitates a major band of 48 kDa from lysates of the rabbit T cell line RL-5. Previously, when reverse transcriptase polymerase chain reaction was used to test for CD4 transcription, a strong signal was gained from RL-5 cells, but expression of CD4 at the cell surface had not been confirmed (Hague *et al.*, 1992). These results clearly show that the RL-5 cell line is derived from a cell of CD4/helper cell lineage.

Ken-4 was reported to precipitate a major band of 50 kDa and a minor band of 42 kDa under reducing and non-reducing conditions from rabbit thymocytes (Kotani *et al.*, 1993). Precipitation from metabolically ^{35}S-labelled and biotin surface-labelled RL-5 cell lysates with R9/96 and Ken-4 showed that under reducing conditions only a single band of 48 kDa was precipitated. When Ken-4 and R9/96 were used to precipitate CD4 from RL-5 cell lysates and samples were run under non-reducing conditions a faint band at 97 kDa was also visible. This suggests that a small percentage of CD4 molecules expressed by virally transformed RL-5 cells exist as homodimers. Many members of the Ig superfamily are expressed as homodimers, including CD8 and CD28, and the suggestion that CD4 can form a homodimer has been made previously (Davis *et al.*, 1990). Models have been proposed in which CD4 forms a dimer allowing interaction with MHC class II (Langedijk *et al.*, 1993; Frey *et al.*, 1993). Recently, Sakihama *et al.*, (1995), proposed a model in which oligomerisation of CD4 is required for stable binding to MHC class II. They suggest that one or two CD4 molecules form the initial contact with the MHC class II dimer and other interactions become involved creating a complex which allows T cell receptor cross-linking. Whether CD4 is expressed as a dimer on rabbit peripheral cells has not been determined in this study, although another group did not find any evidence of a possible dimer band in lysates of rabbit thymocytes (Kotani *et al.*, 1993).

CHAPTER SIX

Characterisation of Anti-Rabbit CD11/CD18 Integrin mAb

6.1 Introduction

The β_2 integrins are $\alpha\beta$ heterodimers in which the β subunit, CD18, associates non-covalently with one of three α subunits, CD11a, CD11b and CD11c, to give LFA-1, Mac-1 and p150,95 respectively. Recently a fourth α-chain, α_d, has been identified in the human and a homologous molecule has been described in the dog (Danilenko *et al.*, 1992; Danilenko *et al.*, 1995). The structure and function of these molecules have been previously discussed in this work and have been extensively reviewed (Larson and Springer, 1990; Kishimoto and Rothlein, 1994; Imhof and Dunon, 1995).

The rabbit has proved to be a useful model in which to examine the interactions of CD11/CD18 molecules with ligands both *in vitro* and *in vivo*. The anti-human CD18 mAb 60.3 (Beatty *et al.*, 1983), 7E4 (Nortamo *et al.*, 1988) and IB4 (Wright *et al.*, 1983) cross react with rabbit CD18 and have been used *in vitro* to inhibit the adhesion of phorbol ester stimulated rabbit neutrophils to cultured human umbilical vein endothelial cells (HUVEC) (Lindbom *et al.*, 1990). mAb IB4 has also been shown to inhibit neutrophil attachment to rabbit mesentery venular endothelium (Von Andrian *et al.*, 1991). mAb R15.7 (Entman *et al.*, 1990) which reacts with canine, human and rabbit CD18 (Argenbright *et al.*, 1991), was used to examine the effects of treatment of antigen-induced arthritis in the rabbit with anti-CD18 mAb (Jasin *et al.*, 1992). Results showed that administering mAb R15.7 inhibited the acute phase of the disease and diminished the severity of the chronic phase in this rheumatoid arthritis (RA) model. mAb R15.7 and 60.3 block basal and fMLP-stimulated binding of rabbit neutrophils to gelatin coated plastic (Galea-Lauri *et al.*, 1993).

mAb L13/64, raised against isolated glycoproteins from the rabbit T cell line RL-5 (Wilkinson *et al.*, 1984) and RCN1/21, raised against rabbit peripheral neutrophils, have been shown to recognise the rabbit CD18 molecule homologue (Galea-Lauri *et al.*, 1993). L13/64 and RCN1/21 precipitated a complex of four proteins from ^{35}S-labelled RL-5 cells, which, under reducing conditions, have apparent M_r of 158, 145, 95 and 90 kDa. These were similar to the 150, 130 and 95 kDa bands precipitated from human peripheral mononuclear cells by the anti-CD18 mAb, 60.3 (Beatty *et al.*, 1983). mAb 60.3 was shown to precipitate three bands of 158, 145 and 95 kDa from ^{35}S-labelled RL-5 cells (Galea-Lauri *et al.*, 1993) and these were compared with the bands precipitated by L13/64 and RCN1/21 in a series of sequential immunoprecipitation experiments. These studies showed that the 95 kDa bands is the rabbit equivalent of CD18, hence the 150 and 145 kDa bands may be assumed to be rabbit CD11 proteins. The 90 kDa band is still precipitated by L13/64 following clearance of lysates with 60.3 and it is presumed to be an intracellular, partially glycosylated precursor form of rabbit CD18, designated CD18'. Attempts were made to obtain N-terminal sequence data for the 158, 145 and 95 kDa bands. No data could be obtained for the 95 kDa band and this is consistent with the finding that CD18 has an N-terminal glutamine residue (Kishimoto *et al.*, 1987) which may be expected to be cyclised and block sequencing. Sequence data obtained from the 158 and 145 kDa bands suggests that these are the rabbit CD11 molecules. Firm conclusions could not be drawn as to which of the CD11 molecules they represent.

The anti-rabbit CD11b mAb, 198, raised against rabbit peripheral blood leucocyte (Smet *et al.*, 1986), was shown to precipitate two polypeptide chains of 165 and 95 kDa from surface labelled rabbit granulocytes. mAb 198 inhibits complement receptor-mediated functions such as phagocytosis and binding of rabbit neutrophils to gelatin coated plastic (Galea-Lauri *et al.*, 1993). mAb 198 and L13/64 precipitate two proteins from surface labelled peritoneal neutrophils with apparent M_r of 155 and 95 kDa under reducing conditions. N-terminal sequence data were obtained from the 155 kDa band which showed it to be homologous to CD11b and its murine equivalent (Galea-Lauri *et al.*, 1993). mAb 198 is not reactive with the RL-5 T cell line and thus it is probable that the 158 and 145 kDa bands precipitated from RL-5 cell lysates by L13/64 and RCN1/21 represent CD11a and CD11c proteins.

In the rabbit all leucocytes express CD11a as determined by staining with the cross reactive anti-human mAb R7.1 (Galea-Lauri *et al.*, 1993). Monocytes express high levels and T cells intermediate levels, while B cells and neutrophils only express low levels of this molecule. Spleen cells were also found to express CD11a weakly, while 55% of thymocytes have low expression and 45% intermediate expression. Expression of CD11b is restricted to macrophages, neutrophils and 4-8% of peripheral lymphocytes. Immunohistochemical techniques were used to determine expression of CD11b in rabbit tissues (Wilkinson *et al.*, 1993). Macrophages in the spleen and the medulla of the thymus express CD11b, as do Kupffer cells in the liver and synovial infiltrating macrophages in the inflamed synovium of rabbits with adjuvant induced RA. CD18 is expressed on B lymphocytes at a low level, T lymphocytes at intermediate levels and on monocytes at high levels. Different populations of spleen cells express low, intermediate and high levels of CD18. Peritoneal and blood neutrophils stain brightly with anti-CD18 mAb. Thymocytes appear to have either low or intermediate CD18 expression. Staining of frozen-sections of spleen showed that L13/64 stained macrophages in the red pulp and lymphocytes and macrophages in the white pulp areas. Staining of thymus sections showed that CD18 expression was restricted to lymphocytes. Kupffer cells of the liver are CD18 positive. Thus it appears that expression of the rabbit equivalents of the CD11/CD18 proteins is similar to that described in other species (Larson and Springer, 1990; Danilenko *et al.*, 1992; Gupta *et al.*, 1993).

In this study, attempts were made to generate a panel of mAb reactive with rabbit T lymphocyte antigens by immunising mice with the rabbit T cell lines RL-5 and BJ/873. Data presented in this chapter show that mAb BJ3/120 and BJ3/41 recognise rabbit CD18, mAb NR185 and BJ3/343 recognise rabbit CD11a and mAb BJ3/22, BJ6/112 and BJ7/208 recognise rabbit CD11c. These mAb have been used to examine the expression of these antigens on rabbit lymphoid cells. Partial sequence data have been obtained for CD18 and CD11c purified from the rabbit T cell lines and show that the rabbit proteins are homologous to their human counterparts.

6.2. Results

6.2.1. mAb BJ3/120 and BJ3/41 recognise rabbit CD18

Tissue culture supernatant from fusion wells BJ3/120 and BJ3/41 reacted strongly with RL-5 and BJ/873 cells by ELISA and immunofluorescence and were assessed for their ability to inhibit PMA-induced RL-5 cell homotypic aggregation (section 2.7). mAb BJ3/120 completely inhibited PMA-induced aggregation of RL-5 cells, while BJ3/41 increased the RL-5 cell aggregation to a level above that seen with PMA alone (chapter seven). On this basis, these mAb were selected for cloning and further investigation.

mAb BJ3/120 and BJ3/41 were used to immunoprecipitate proteins from lysates of ^{35}S-labelled RL-5 cells (figure 6.1). Results from this experiment showed that other than non-specific bands at about 200 and 45 kDa, both mAb precipitated three bands with apparent M_r of 158, 145, and 95 kDa. mAb BJ3/120 also precipitated a fourth band at 140 kDa (lane C), while BJ3/41 precipitated an extra band at 90 kDa (lane B). The experiment was repeated using lysates from ^{35}S-labelled BJ/873. mAb BJ3/120 precipitated the same four bands (lane F), although mAb BJ3/41 precipitated the 158, 145 and 95 kDa bands, but not the 90 kDa (lane E).

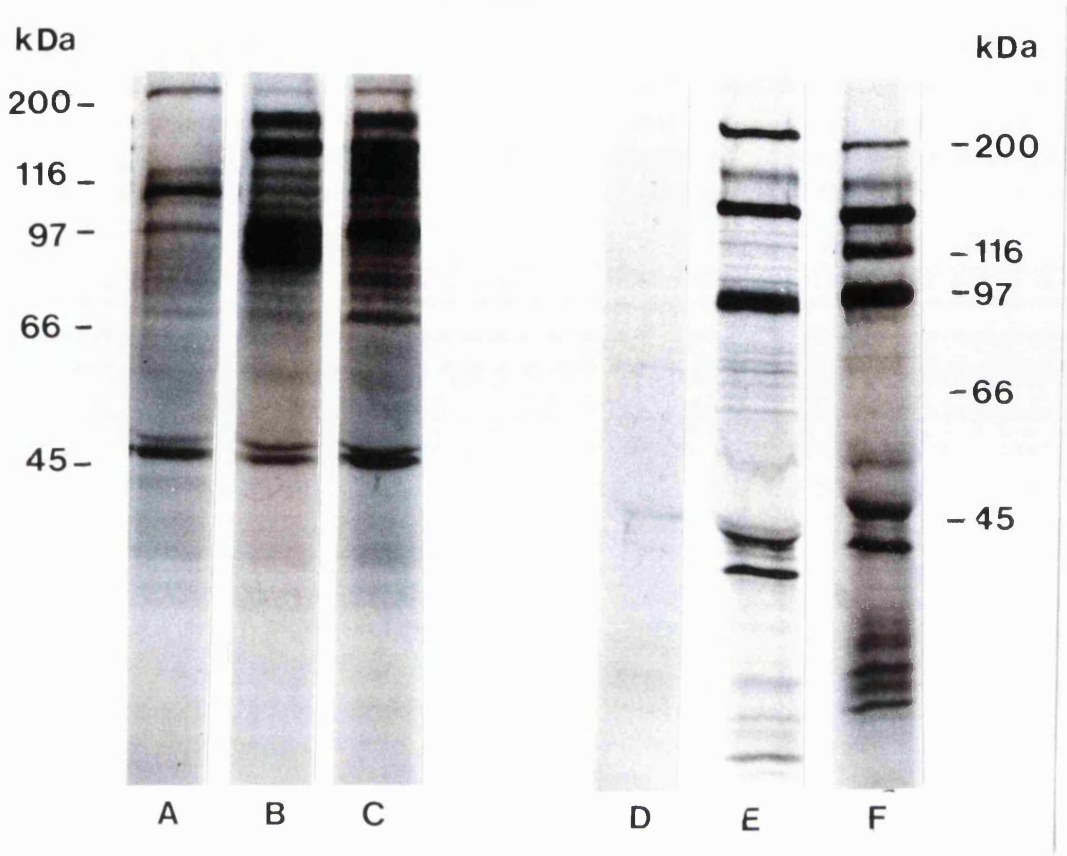

Figure 6.1. Immunoprecipitation from (lanes A-C) ^{35}S-labelled RL-5 cells or (lanes D-F) ^{35}S-labelled BJ/873 cells with (lanes A and D) T1/7 (negative control), (lanes B and E) BJ3/41 and (lanes C and F) BJ3/120. Precipitates were separated by SDS-PAGE on 8% gels under reducing conditions.

The anti-rabbit CD18 mAb L13/64 precipitates two major bands at 145 and 95 kDa and a minor band at 158 kDa from ^{35}S-labelled BJ/873 cell lysates (figure 6.2. lanes H and Q). The relationship between these bands and those precipitated by BJ3/41 and BJ3/120 was assessed in sequential immunoprecipitation experiments. Neither BJ3/41 (lane D) or BJ3/120 (lane M) precipitated bands from lysates pre-cleared with L13/64. Conversely, L13/64 failed to precipitate bands from lysates pre-cleared with either BJ3/41 (lane E) or BJ3/120 (lanes N). These data show that BJ3/41 and BJ3/120 recognise the rabbit CD18 molecule.

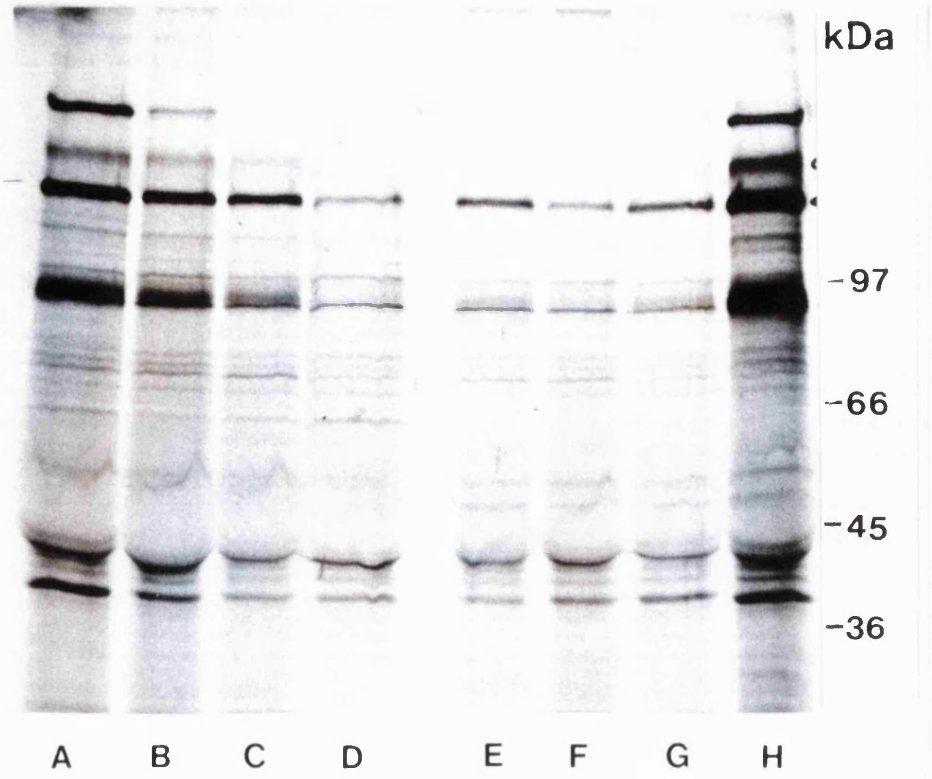

Figure 6.2.a. Detergent lysates of ^{35}S-labelled BJ/873 cells were sequentially cleared with (lanes A-C) BJ3/41 or (lanes F-H) L13/64. Lysate cleared with BJ3/41 was re-precipitated with (lane E) L13/64 and lysate cleared with L13/64 was re-precipitated with (lane D) BJ3/41.

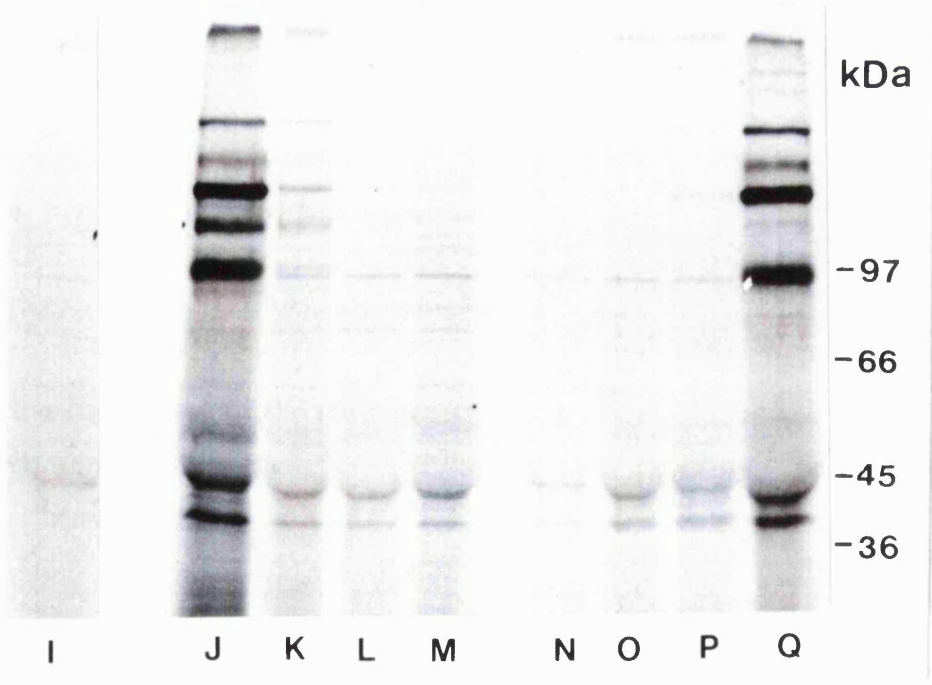

Figure 6.2.b. Detergent lysates of ^{35}S-labelled BJ/873 cells were also sequentially cleared with (lanes J-L) BJ3/120 or with (lanes O-Q) L13/64. Lysates cleared with BJ3/120 were re-precipitated with (lane N) L13/64 and lysates cleared with L13/64 were re-precipitated with (lane M) BJ3/120. Lane (I) precipitated with T1/7 (negative control). Precipitates were separated by SDS-PAGE on 8% gels under reducing conditions.

To determine whether the complex of proteins precipitated by mAb L13/64, BJ3/41 and BJ3/120 are expressed at the surface of RL-5 and BJ/873 cells, antigens were precipitated from detergent lysates of surface-biotinylated cells (figure 6.3). The results showed that all three mAb precipitated the three bands of 158, 145 and 95 kDa from both cell lysates, although there was no evidence of the 90 or 140 kDa bands precipitated from ^{35}S-labelled cell lysates.

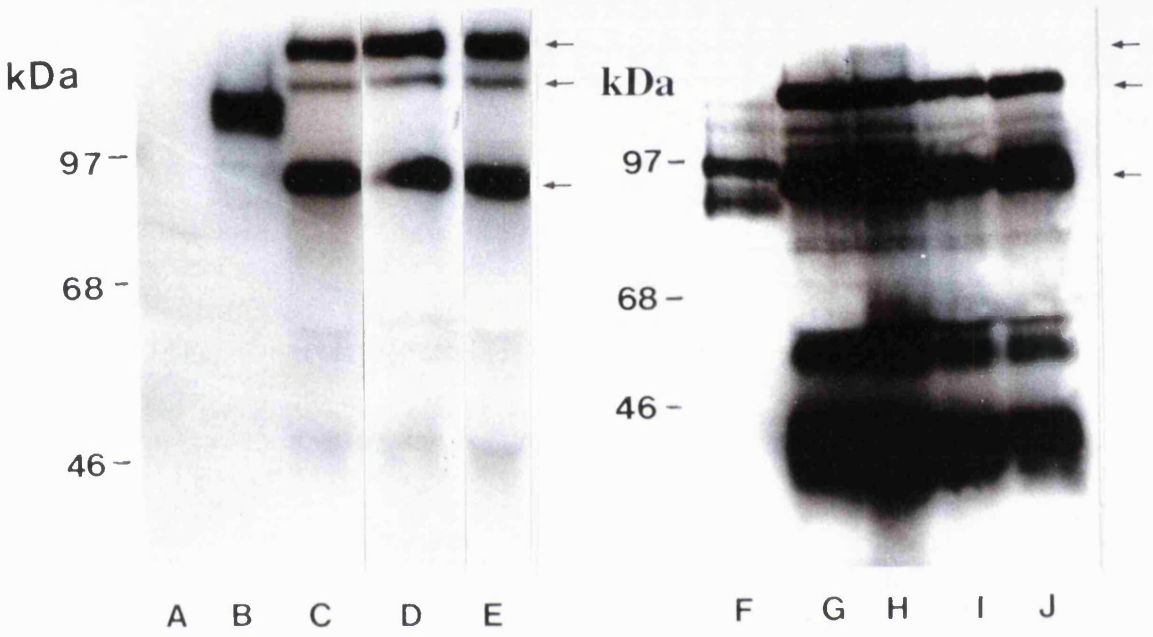

Figure 6.3. Immunoprecipitation from detergent lysates of surface biotin-labelled RL-5 (lane A-E) or BJ/873 (lane F-J) cells with (lane A) T1/7 (negative control), (lanes B and F) L11/135 (positive control), (lanes C and H) L13/64, (lanes D and I) BJ3/120, (lanes E and J) BJ3/41 and (lane G) BJ3/22 (anti-CD11c). Precipitates were separated under non-reducing conditions by SDS-PAGE on 8% gels.

6.2.2. Characterisation of the antigens recognised by mAb BJ3/120 and BJ3/41 by immunoblotting

The anti-rabbit CD18 mAb, L13/64, stains a single band of approximately 95 kDa from RL-5 cell SDS-extracts run under non-reducing conditions, but it does not stain the reduced form of this protein. SDS-extracts of BJ/873 and RL-5 cells were separated by SDS-PAGE under reducing and non-reducing conditions, the proteins were transferred to nitrocellulose and immunostained with mAb BJ3/120 and BJ3/41 (figure 6.4a and 6.4b). mAb BJ3/120 failed to stain proteins from either cell line (6.4a. lane

E and 6.4b. lane E). mAb BJ3/41 stained a band at approximately 95 kDa which was present in both reduced and non-reduced SDS-cell extracts from both cell lines (6.4a. lanes D and I, 6.4b. lanes D and I). The protein had the same apparent molecular weight as that recognised by L13/64, providing further evidence that BJ3/41 recognises rabbit CD18.

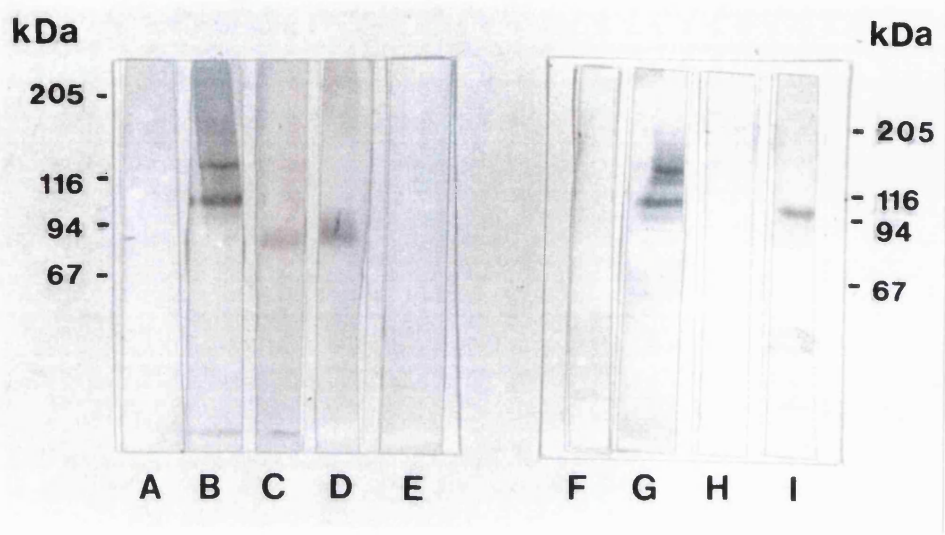

Figure 6.4.a. Immunoblotting of BJ/873 cell proteins separated under (lane A-E) non-reducing and (lane F-I) reducing conditions by 8% SDS-PAGE with (lanes A and F) 198 (anti-CD11b, negative control), (lanes B and G) L11/135 (anti-CD43, positive control), (lanes C and H) L13/64, (lanes D and I) BJ3/41 and (lane E) BJ3/120.

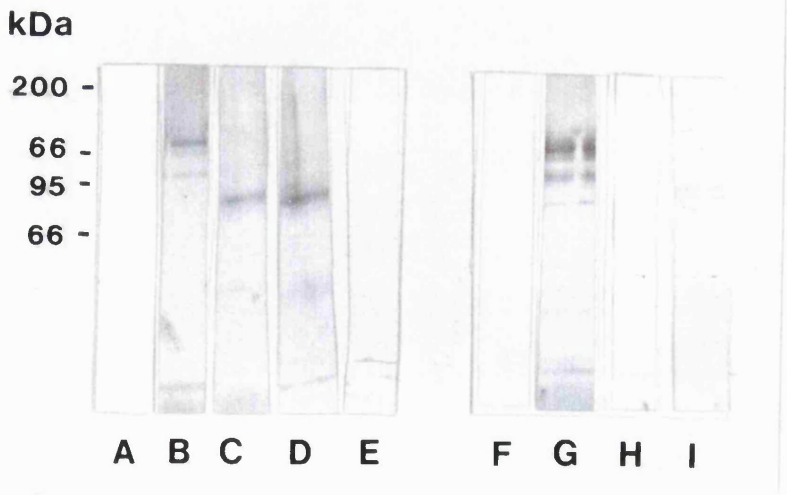

Figure 6.4.b. Immunoblotting of RL-5 cell proteins separated under (lanes A and E) non-reducing and (lanes F and I) reducing conditions by SDS-PAGE 8% gels with (lanes A and F) 198 (anti-CD11b, negative control), (lanes B and G) L11/135 (anti-CD43, positive control), (lanes C and H) L13/64, (lanes D and I) BJ3/41 and (lane E) BJ3/120.

6.2.3. mAb NR185 and BJ2/343 recognise rabbit CD11a

mAb secreted by the hybridoma cell lines NR185 and BJ2/343 reacted strongly with RL-5 cells by ELISA and immunofluorescence. NR185 inhibited PMA-induced homotypic aggregation of RL-5 cells, while BJ2/343 increased the level of RL-5 cell aggregation above that of cells stimulated with PMA alone (chapter seven). Both mAb were selected for further study.

The ability of mAb NR185 and BJ2/343 to immunostain proteins from RL-5 and BJ/873 cells SDS-extracts was investigated. Neither mAb immunostained proteins, although the positive control mAb, L11/135, labelled a band at 120 kDa.

mAb NR185 and BJ2/343 were used to immunoprecipitate antigens from a detergent lysate of ^{35}S-labelled RL-5 cells. The results of these experiments showed that both mAb immunoprecipitated two major bands of 158 and 95 kDa under reducing conditions (figure 6.5, lanes A and G). These corresponded to two of the bands immunoprecipitated by mAb L13/64 (lanes C and F). The relationship between the bands precipitated by mAb NR185 and L13/64 was determined using sequential immunoprecipitation methods. L13/64 was able to precipitate only the 145, 95 and 90 kDa bands from a lysate which had been pre-cleared with NR185 (lane B), while NR185 failed to precipitate bands from lysate pre-cleared with L13/64 (lane D). This shows that NR185 and BJ2/343 recognise the 158 kDa antigen associated with CD18. Since the RL-5 and BJ/873 cells do not express CD11b, the 158 kDa band is likely to be rabbit CD11a.

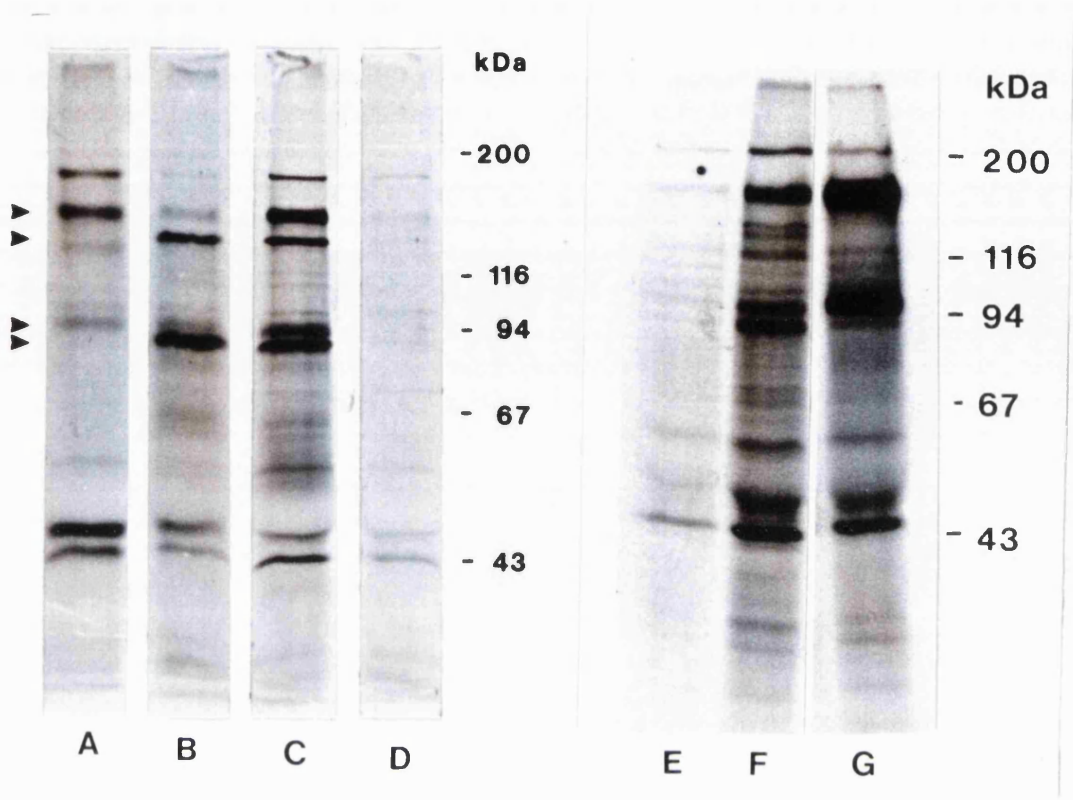

Figure 6.5. Immunoprecipitation from detergent lysates of ^{35}S-labelled RL-5 cells with (lane A) NR185, (lanes C and F) L13/64, (lane E) T1/7 (negative control), (lane G) BJ2/343, (lane B) re-precipitation with L13/64 of lysate pre-cleared with NR185 and (lane D) re-precipitation with NR185 of lysate pre-cleared with L13/64. Precipitates were separated by SDS-PAGE on 8% gels under reducing conditions.

To further characterise the antigens recognised by NR185 and BJ2/343, immunoprecipitation from detergent lysates of biotin surface-labelled RL-5 and BJ/873 cells were carried out (figure 6.6). Both mAb precipitated the bands at 158 and 95 kDa (lanes D and E) from RL-5 cell lysates. However, the bands from BJ/873 cell lysates (lanes I and J) were reduced in intensity compared with those from RL-5 cell lysates. From BJ/873 cell lysates, the positive control mAb, L13/64, precipitated the two major bands at 145 and 95 kDa (lane H), while the 158 kDa band was seen at a reduced intensity. This data suggests that BJ/873 cells express reduced levels of the 158 kDa molecule compared with RL-5 cells, but similar levels of CD18.

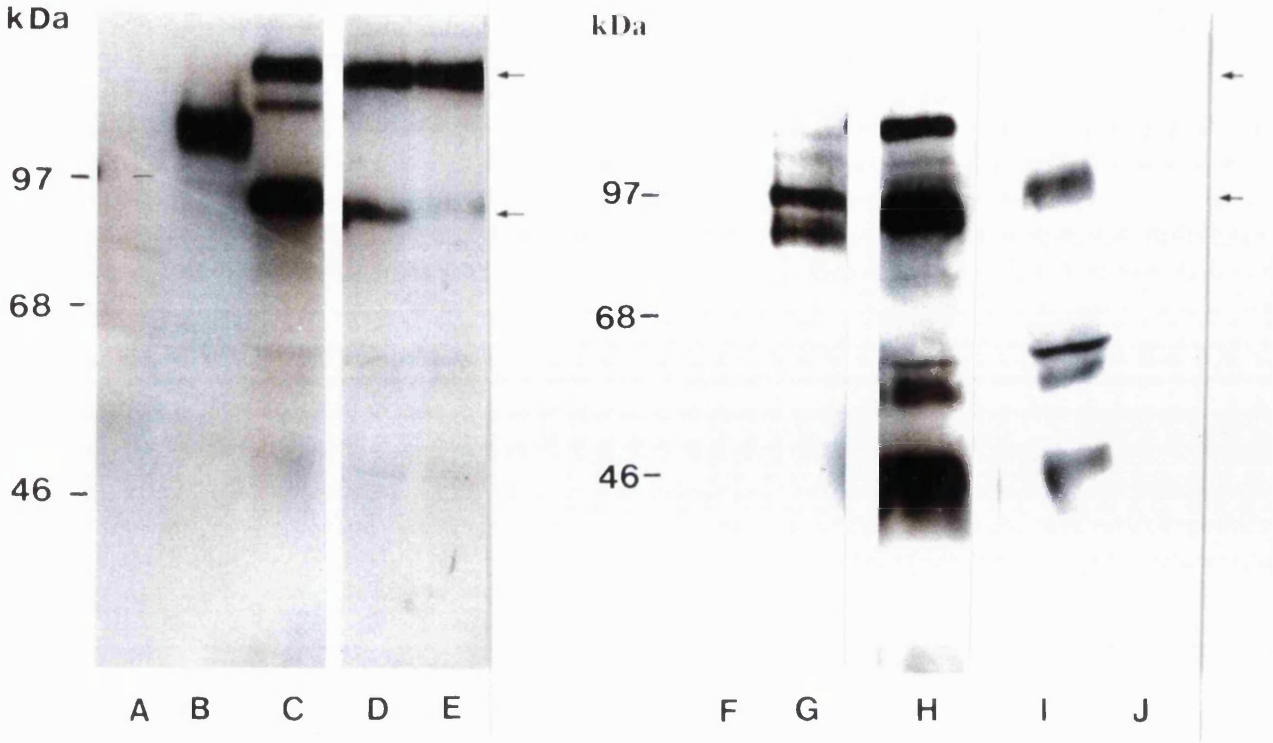

Figure 6.6. Immunoprecipitation from detergent lysates of biotin-surface labelled (lanes A-E) RL-5 and (lanes F-J) BJ/873 cells using mAb (A and F) T1/7 (negative control), (lanes B and G) L11/135 (anti-CD43, positive control), (lanes C and H) L13/64, (lanes D and I) NR185 and (lanes E and J) BJ2/343. Precipitates were run under reducing conditions on 8% SDS-PAGE gels. Proteins were electroblotted to nitrocellulose and detected by ECL.

6.2.4. mAb BJ3/22, BJ6/112 and BJ7/208 recognise rabbit CD11c

mAb BJ3/22, BJ6/112 and BJ7/208 reacted strongly by ELISA and immunofluorescence staining with the BJ/873 cells, but only weakly with the RL-5 cells, and on this basis were selected for further study.

The ability of mAb BJ3/22, BJ6/112 and BJ7/208 to immunostain proteins from BJ/873 and RL-5 cell SDS-extracts was examined. All three mAb failed to label proteins, while control mAb L13/64 and L11/135 stained bands at 95 and 120 kDa respectively. mAb BJ3/22, BJ6/112 and BJ7/208 were used to precipitate antigens from lysates of metabolically ^{35}S-labelled BJ/873 cells. All three mAb precipitated two bands at 145 and 95 kDa (figure 6.7, lanes C, F and G) and these were similar to two of the bands immunoprecipitated by the anti-rabbit CD18 mAb L13/64.

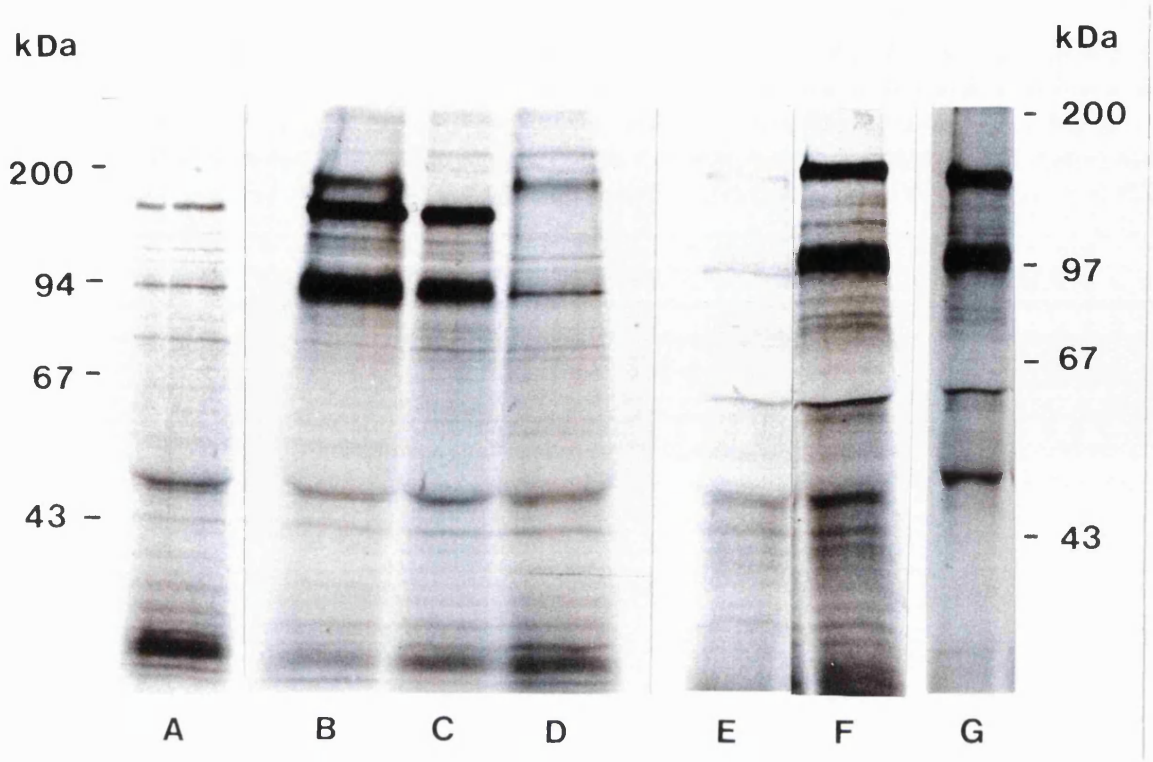

Figure 6.7. Immunoprecipitation from lysates of ^{35}S-labelled BJ/873 cells with (lanes A and E) T1/7 (negative control), (lane B) L13/64, (lane C) BJ3/22, (lane D) NR185 (anti-CD11a), (lane F) BJ6/112 and (lane G) BJ7/208. Precipitates were separated by SDS-PAGE under reducing conditions on 8% gels.

The relationship between mAb BJ3/22 and L13/64 was investigated by sequential immunoprecipitation from ^{35}S-labelled BJ/873 cells (figure 6.8). mAb L13/64 precipitated two major bands at 145 and 95 kDa and a minor band at 158 kDa (lane C), while BJ3/22 precipitated the bands at 158 and 95 kDa (lane J). Pre-clearing lysate with BJ3/22 completely removed the 145 and 95 kDa bands precipitated by L13/64, although the 158 kDa band was still present (lane F). mAb BJ3/22 failed to precipitate the 95 kDa band from lysate pre-cleared with L13/64, but was still able to precipitate the 145 kDa band although it was reduced in intensity (lane G). These data strongly suggest that mAb BJ3/22 recognises the 145 kDa band associated with rabbit CD18 which presumably is rabbit CD11c.

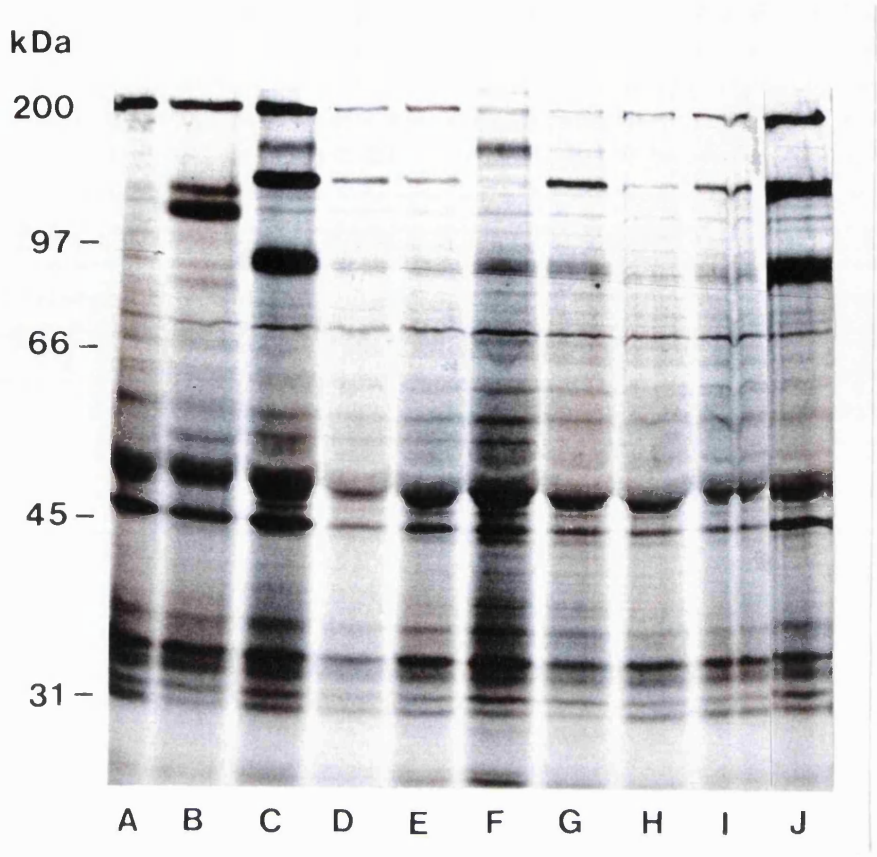

Figure 6.8. *Sequential immunoprecipitation from ^{35}S-labelled BJ/873 cell lysates with mAb BJ3/22 and L13/64. Lysate was pre-cleared with L13/64 (lanes C-E) and re-precipitated with BJ3/22 (lane G) or was pre-cleared with (lanes H-J) BJ3/22 and re-precipitated with L13/64 (lane F). Lysate was also precipitated with (lane A) T1/7 (negative control) and L11/135 (positive control). Precipitates were separated by SDS-PAGE on 8% gels.*

To confirm that mAb BJ6/112 and BJ7/208 recognised the same antigens as mAb BJ3/22 further sequential immunoprecipitation studies were performed and the results are shown in figures 6.9 and 6.10. mAb BJ3/22 completely removed the two bands at 158 and 95 kDa precipitated from ^{35}S-labelled BJ/873 cells lysates by mAb BJ6/112 (figure 6.9, lane D) and BJ7/208 (figure 6.10, lanes D).

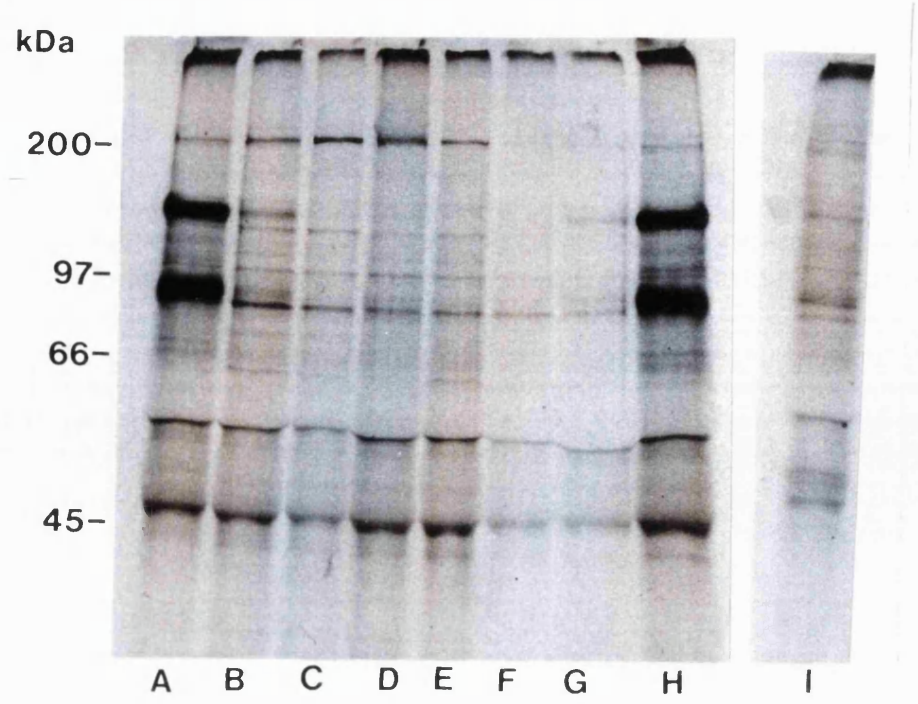

Figure 6.9. Sequential immunoprecipitation from ^{35}S-labelled BJ/873 cell lysate with mAb BJ3/22 and BJ6/112. Lysates were pre-cleared with (lanes A-C) BJ6/112 and re-precipitated with (lane E) BJ3/22 or were pre-cleared with (F-H) with BJ3/22 and re-precipitated with (lane D) BJ6/112. Lysate was also precipitated with (lane I) T1/7 (negative control). Precipitates were separated by SDS-PAGE on 8% gels.

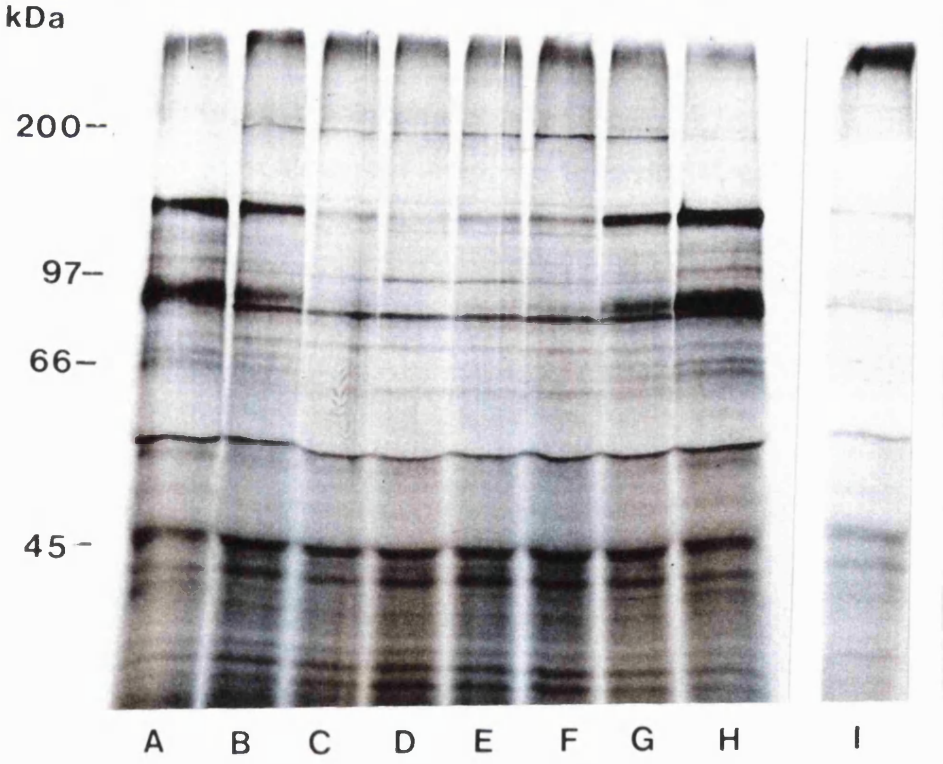

Figure 6.10. Sequential immunoprecipitation from ^{35}S-labelled BJ/873 cell lysates with mAb BJ3/22 and BJ7/208. Lysate was pre-cleared with (lanes A-C) BJ7/208 and re-precipitated with (lane E) BJ3/22 or were pre-cleared with (lanes F-H) BJ3/22 and re-precipitated with (lane D) BJ7/208. Lysate was also cleared with (lane I) T1/7 (negative control). Precipitates were separated by SDS-PAGE on 8% gels.

The bands precipitated by mAb BJ3/22 were compared with those precipitated by mAb NR185 in sequential immunoprecipitation studies from biotin surface-labelled BJ/873 cells (figure 6.11). mAb NR185 precipitated two bands at 158 and 95 kDa (lane A), while BJ3/22 precipitated two bands of 145 and 95 kDa (lane H). mAb BJ3/22 precipitated the same two bands from lysate which had been pre-cleared with NR185 (lane E). Similarly, mAb NR185 was able to precipitated the 158 and 95 kDa bands from lysate pre-cleared with BJ3/22 (lane D). This experiment confirms that mAb NR185 and BJ3/22 recognise the individual α-chains associated with rabbit CD18 which are presumed to be CD11a and CD11c respectively.

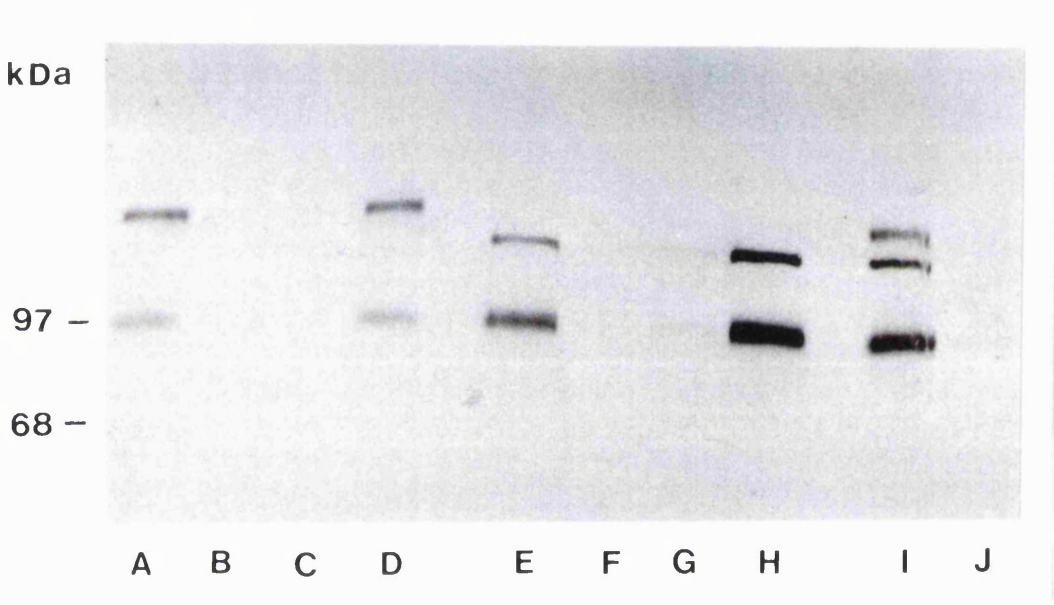

Figure 6.11. Immunoprecipitation from biotin surface labelled BJ/873 cells with BJ3/22 and NR185. Lysate was pre-cleared with (lanes A-C) NR185 and re-precipitated with (lane E) BJ3/22 or was pre-cleared with (lanes F-H) BJ3/22 and re-precipitated with (lane D) NR185. Lysate was also precipitated with (lane I) L13/64 (positive control) and (lane J) T1/7 (negative control). Precipitates were separated by SDS-PAGE on 8% gels.

6.2.5. Purification of rabbit CD11a/CD18 with mAb NR185 for peptide-mass fingerprint analysis

Immunoprecipitation data suggests that mAb NR185 recognises rabbit CD11a/CD18. To confirm this observation, NR185 was coupled to cyanogen bromide activated Sepharose beads and the immunoadsorbant was used to immunoaffinity purify the 158 and 95 kDa complex from RL-5 cells as described in section 2.6. The proteins were separated by SDS-PAGE on 7% gels under the appropriate conditions for sequencing, transferred to Immobilon-P membrane and stained with Sulphorhodamine B. The 158 and 95 kDa bands were cut out, the proteins digested with trypsin and the eluted peptides analysed by peptide-mass fingerprint techniques (2.6.5). Of the peptides released from the 95 kDa band, 6 matched peptides from murine CD18 (ranked 1) and 4 matched peptides from human CD18 (ranked 22). This supports the suggestion that NR185 recognises CD11a/CD18. The data is shown in figure 6.12. Peptides released from the 158 band did not give a specific match, by mass, to those obtained from the human CD11a, CD11b or CD11c which are contained in the database.

Tryptic peptides from rabbit CD18 matched by mass to peptides from mouse and human CD18

Rabbit peptide 1 - mass weight of 1878 matched to:
Mouse peptide ^{380}V T Y D S F C S N G A S S I G K S R^{397}

Rabbit peptide 2 - mass weight of 1693 matched to:
Mouse peptide ^{296}S N E F D Y P S V G Q L A H^{310}
Human peptide ^{296}S N E F D Y P S V G Q L A H^{310}

Rabbit peptide 3 - mass weight of 1484 matched to:
Mouse peptide ^{367}V F L D H S T L P D T L K^{379}
Human peptide ^{367}V F L D H N A L P D T L K^{397}

Rabbit peptide 4 - mass weight of 1401 matched to:
Mouse peptide ^{49}L N F T G P G E P D S L R^{61}

Rabbit peptide 5 - mass peptide of 1382 matched to:
Mouse peptide ^{175}T V L P F V N T H P E K^{186}

Rabbit peptide 6 - mass weight of 1363 matched to:
Mouse peptide ^{746}S Q W N N D N P L F K^{756}
Human peptide ^{745}S Q W N N D N P L F K^{756}

Rabbit peptide 7 - mass weight of 1090 matched to:
Human peptide ^{156}A L N E I T E S G R^{165}

Figure 6.12. Amino-acid sequences of tryptic peptides from human and mouse CD18 which matched by mass weight to peptides from rabbit CD18. Human and mouse fragments were taken from the MOWSE database which holds the peptide mass fingerprints of 72 000 proteins.

6.2.6. Purification of rabbit CD11c/CD18 with mAb BJ3/22 for amino-acid sequencing

mAb BJ3/22 was coupled to cyanogen bromide activated Sepharose as described in section 2.6.1 and the immunoadsorbant was used to isolate the 145 and 95 kDa protein complex from BJ/873 cell lysates. Proteins were separated by SDS-PAGE, transferred to nitrocellulose and the 145 kDa band cut out and subjected to solid-phase N-terminal amino-acid sequencing (2.6.6). Data for the first 18 residues were obtained and of these, 10 are identical to the human CD11c sequence with conservative substitutions at 2 other positions. Figure 6.13 compares the rabbit and human CD11c sequences. The sequences of human CD11b, the rabbit CD11b homologue, and human and dog α_d are also given. The 145 kDa band was also subjected to internal peptide sequence analysis (2.6.7). The isolated band was digested with trypsin, peptides were eluted, purified by HPLC and sequenced. The results of this experiment (figure 6.14) clearly show that, in these regions, there is a high level of homology between the human and rabbit protein sequences. Combined, the N-terminal and internal peptide sequencing data provide confirmation that the 145 kDa band recognised by mAb BJ3/22, BJ6/112 and BJ7/208 is rabbit CD11c.

Antibody	Antigens	Size kDa
BJ3/120	CD18	95
BJ3/41	CD18	95
BJ2/343	CD11a	158
NR185	CD11a	158
BJ3/22	CD11c	145
BJ6/112	CD11c	145
BJ7/208	CD11c	145

Table 6.1. A summary of the anti-CD11/CD18 mAb produced and characterised during this project

N-terminal amino-acid sequences of rabbit and human CD11 proteins

											10								20
BJ/873 145 kDa band	**F**	**N**	**L**	**D**	**T**	**E**	T	**L**	G	T	**F**	X	M	E	(G)	X	(G)	**F**	X	**X**
Human CD11c	**F**	**N**	**L**	**D**	**T**	**E**	E	**L**	T	A	**F**	R	V	D	S	A	**G**	**F**	G	D
Rabbit CD11b	**F**	**N**	**L**	E	**T**	**E**	**N**	T	**M**	T	**F**	(S)	X	**N**	E	X	**X**	(**F**)	X	(**Q**)
Human CD11b	**F**	**N**	**L**	D	**T**	**E**	**N**	A	**M**	T	**F**	Q	E	**N**	A	R	**G**	**F**	G	**Q**
Human α_d	**F**	**N**	**L**	**D**	V	**E**	E	P	T	I	**F**	Q	E	D	A	G	**G**	**F**	G	Q
Human CD11a	Y	**N**	**L**	**D**	V	R	G	A	R	S	**F**	S	P	P	R	A	**G**	R	H	F

Figure 6.13. The N-terminal sequence of the 145 kDa band immunoaffinity purified from BJ/873 cells with mAb BJ3/22 compared with that of human CD11c (Corbi et al., 1993). The sequences of rabbit CD11b (Galea-Lauri, et al., 1993) and human CD11a (Larson et al., 1989), CD11b (Corbi et al., 1988) and α_d (Danilenko et al., 1995) are shown for comparison. Residues shown in bold type are conserved between pairs of sequences. Residues in brackets could not be identified unequivocally.

Amino-acid sequence of rabbit and human CD11c peptides

PEPTIDE 1	Rabbit 145 kDa	**I**	**L**	**I**	**V**	**I**	**T**	**D**	**G**	**O**	**K**									
		253									262									
	Human CD11c	**I**	**L**	**I**	**V**	**I**	**T**	**D**	**G**	K	**K**									
		236									245									
	Human CD11b	**I**	**L**	V	V	**I**	**T**	**D**	**G**	E	**K**									
	Mouse CD11b	**I**	**L**	V	V	**I**	**T**	**D**	**G**	E	**K**									
PEPTIDE 2	Rabbit 145 kDa	**I**	**A**	**G**	**A**	**Q**	**L**	**S**	**P**	T	**L**	**Q**	**Y**	**F**	**G**	**O**	**A**	**L**	**S**	**G**	**G**	**Q**
		572																				592
	Human CD11c	**I**	**A**	**G**	S	**Q**	**L**	**S**	S	R	**L**	**Q**	**Y**	**F**	**G**	Q	**A**	**L**	**S**	**G**	**G**	**Q**
		550																				570
	Human CD11b	**I**	**A**	**G**	S	K	**L**	**S**	**P**	R	**L**	**Q**	**Y**	**F**	**G**	Q	S	**L**	**S**	**G**	**G**	**Q**
	Mouse CD11b	**I**	I	**G**	**A**	H	F	**S**	**P**	**G**	**L**	**Q**	**Y**	**F**	**G**	Q	S	**L**	**S**	**G**	**G**	K
PEPTIDE 3	Rabbit 145 kDa	**L**	**L**	**L**	**T**	**A**	**A**	**V**	**S**	**M**	**E**	**V**	**F**	**A**								
		894												907								
	Human CD11c	**L**	**L**	**L**	**T**	**A**	N	**V**	**S**	S	**E**	N	N	**T**								
		879												901								
	Human CD11b	**L**	**L**	**L**	K	N	N	**V**	T	**S**	**E**	**N**	**N**	**M**								
		880												992								
	Mouse CD11b	**L**	**L**	**L**	K	**A**	I	**V**	A	**S**	**E**	**N**	**N**	**M**								

Figure 6.14. Amino-acid sequence of peptides obtained from the 145 kDa band immunoprecipitated from BJ/873 cell lysates by mAb BJ3/22. The sequences aligned to human CD11c which is shown for comparison (Corbi et al., 1987), as is human and mouse CD11b (Corbi et al., 1988; Pytela, 1988). Residues shown in bold type are conserved between species.

6.2.7. Cellular Distribution of the Rabbit CD11/CD18 Integrins

The panel of mAb recognising rabbit β_2 integrin molecules were used to stain rabbit peripheral leucocytes, rabbit lymphoid cell populations and RL-5 and BJ/873 cells using indirect immunofluorescence labelling methods described in section 2.3.3. Cell populations were stained with L13/64 (anti-CD18), NR185 (anti-CD11a), 198 (anti-CD11b) and BJ3/22 (anti-CD11c). Unstained cells were used to set data collection parameters. Experiments were repeated a minimum of three times. L11/135 (anti-CD43) and T1/7 (anti-chicken troponin T) were used as positive and negative controls respectively.

Expression of CD18: CD18 was expressed on all of the leucocyte populations examined. The histogram obtained from staining peripheral mononuclear cells with L13/64 (figure 6.15.C; table 6.2) had three distinct peaks indicative of low, intermediate and high expression. B lymphocytes expressed low levels of CD18, T lymphocytes expressed intermediated levels and monocytes high levels, which is in line with the previous findings of (Galea-Lauri *et al.*, 1993). Neutrophils appeared to express high levels of CD18 (figure 6.15.I; table 6.2) which confirmed previous findings of (Galea-Lauri *et al.*, 1993). Labelling thymocytes produced a histogram with a bimodal distribution (figure 6.16.C; table 6.3). Approximately 60% of thymocytes expressed low levels of CD18, while the remaining population expressed intermediate levels. Spleen cells labelled at two levels (figure 6.17.I; table 6.3). Approximately 20% of cells expressed low to intermediate levels of CD18, while 80% expressed high levels. Labelling of lymph node cells with L13/64 (figure 6.17.C; table 6.3) showed that a small population of cells do not stain above the level produced by staining with the negative control mAb, 20% of cells expressed intermediate levels of CD18 and 70% expressed high levels. Labelling of bone marrow cells (6.16.I; table 6.3) produced a histogram with a broad base and three overlapping peaks, 30% of the cells stained weakly with mAb L13/64, 70% at intermediate levels and a small population of cells stained brightly.

Expression of CD11a: The results obtained from labelling rabbit peripheral mononuclear cells with NR185 showed that B lymphocytes expressed intermediate levels of CD11a, T lymphocytes expressed high levels, while monocytes expressed very high levels (figure 6.15.D; table 6.2). These data were similar to those obtained from staining peripheral rabbit leucocytes with the anti-human CD11a mAb R7.1 (Galea-Lauri *et al.*, 1993), which further supports the characterisation of NR185 as an anti-CD11a mAb. All thymocytes stained with NR185 with 60% of cells staining at intermediate levels and 40% at high levels (figure 6.16.D; table 6.3). A small population of spleen cells were shown to express low levels of CD11a, while the majority of cells expressed high levels (6.17.J; table 6.3). Lymph node cells had three levels of CD11a expression. Approximately 10% of cells expressed low levels of CD11a, 20% expressed intermediate levels while the rest expressed high levels (figure 6.17.D; table 6.3). Labelling of bone marrow cells with anti-CD11a mAb shows that 40% of cells stained at low levels and 55% at intermediate levels, with a small population staining brightly (figure 6.16.J; table 6.3)

Expression of CD11b: Labelling of peripheral lymphocytes with CD11b mAb, 198, showed that the majority of cells expressed low levels of surface CD11b (figure 6.15.E; table 6.2). Previously it has been reported that only between 4-8% of lymphocytes express this marker (Galea-Lauri *et al.*, 1993). Neutrophils (figure 6.15.K; table 6.2) and monocytes (figure 6.15.E; table 6.2) stained brightly with mAb 198. The majority of thymocytes expressed low levels of CD11b, but there was a distinct sub-population (20%) that expressed intermediate levels (figure 6.16.E; table 6.3). Similarly in the spleen, approximately 20% of cells expressed high levels of CD11b, whereas the remaining cells expressed low to intermediate levels (figure 6.17.K; table 6.3). The majority of lymph node cells did not express CD11b, although a small population of cells that expressed intermediate levels was detected (figure 6.17.E; table 6.3). Labelling of bone marrow cells showed that a small population of cells, approximately 10% of the total population, expressed high levels of CD11b. However, the remaining cells do not express CD11b, or only expressed it at low levels (figure 6.16.K; table 6.3).

Expression of CD11c: Expression of CD11c by rabbit lymphoid cells is highly restricted. The anti-rabbit CD11c mAb, BJ3/22, labelled 20% of peripheral mononuclear cells (figure 6.15.F; table 6.2). Analysis showed that monocytes and a small population of T lymphocytes expressed CD11c at low to intermediate levels. B lymphocytes did not express CD11c. Neutrophils were not stained with mAb BJ3/22 (figure 6.15.L; table 6.2). In the thymus only 10% cells expressed CD11c (figure 6.16.F; table 6.2). Expression of CD11c is greatest in the spleen with 40% of cells staining at intermediate to high levels (figure 6.16.L; table 6.3). Labelling of lymph node cells produced a histogram with a single peak, 90% of which overlapped that produced by cells labelled with the negative control mAb. The rest of the population expressed low levels of CD11c (figure 6.17.F; table 6.3). Labelling of bone marrow cells with mAb BJ3/22 showed that 80% of cells were negative for CD11c, while the remaining 20% of cells expressed only low levels (figure 6.16.L; table 6.3).

Expression of CD11/CD18 proteins on the rabbit T cell lines BJ/873 and RL-5: Both cell lines were shown to express intermediate levels of CD18 (figures 6.18 A and E; table 6.4). RL-5 cells expressed intermediate levels of CD11a (figure 6.18 F; table 6.3), but did not express CD11b (figure 6.18 G; table 6.4). The expression of CD11c on RL-5 cells ranged from low to intermediate levels (figure 6.18 H; table 6.4). BJ/873 cells expressed both CD11a and CD11c (figures 6.18 B and D; table 6.4) but only very low levels of CD11b (figure 6.18 C and D; table 6.4).

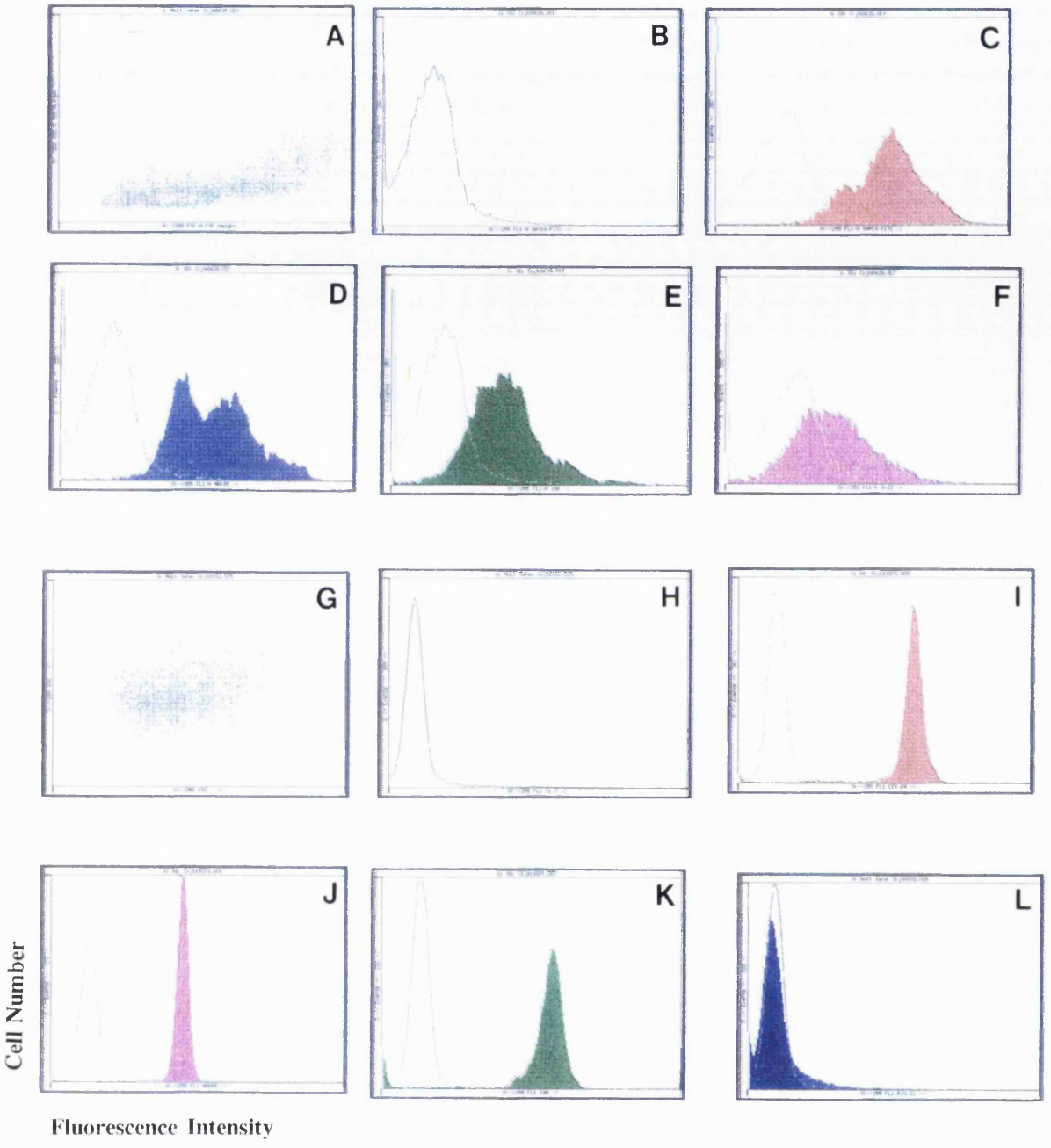

Figure 6.15. *Flow cytometric analysis of rabbit (A-F) peripheral leucocytes and (G-L) neutrophils stained with anti-CD11/CD18 mAb. (A and G) Scatter plots, (B and H) negative control, (C and I) L13/64 (anti-CD18), (D and J) NR185 (anti-CD11a), (E and K) 198 (anti-CD11b) and (F and L) BJ3/22 (anti-CD11c).*

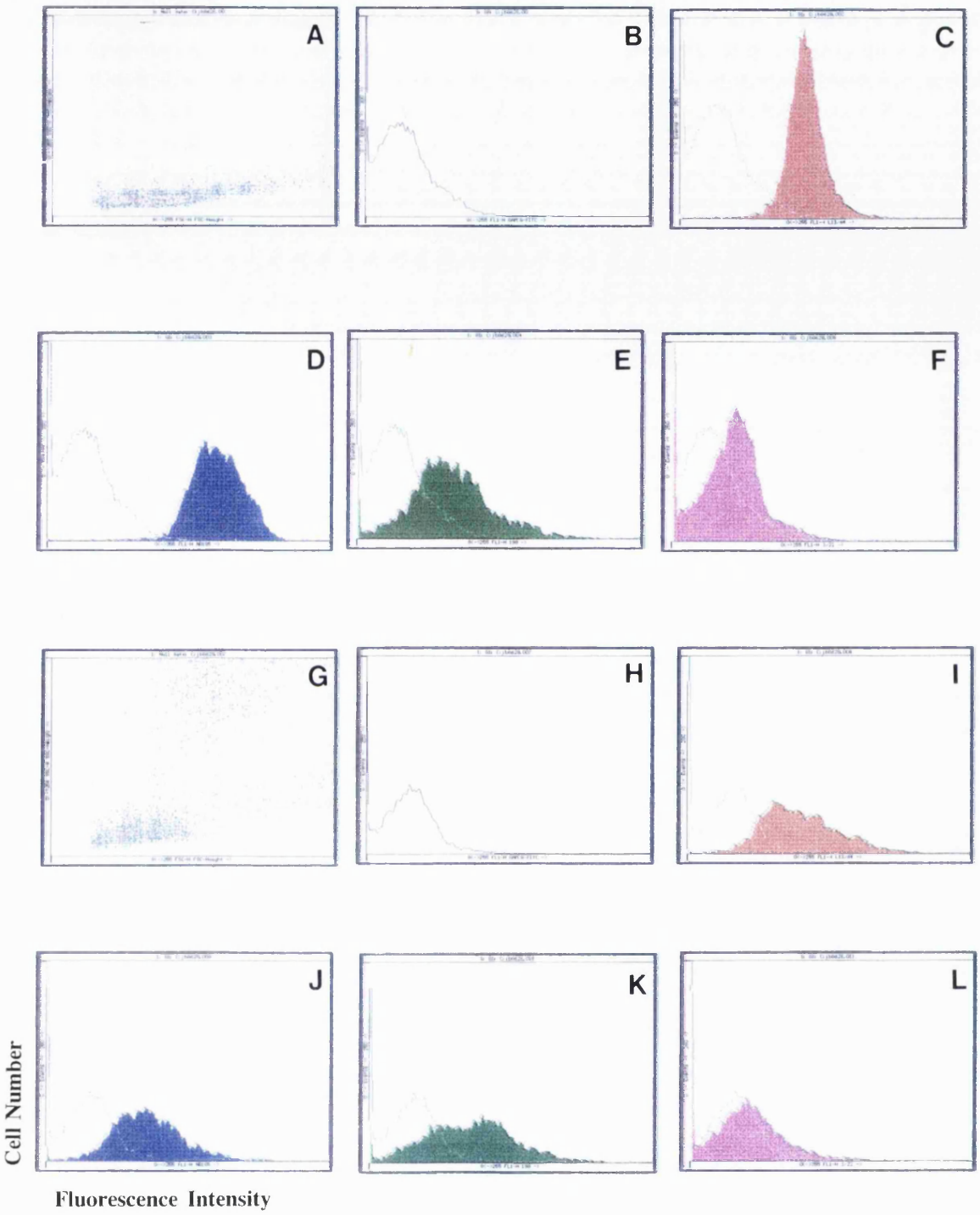

Figure 6.16. *Flow cytometric analysis of rabbit (A-F) thymocytes and (G-L) bone marrow cells stained with anti-CD11/CD18 mAbs. (A and G) scatter plots, (B and H) negative control mAb, (C and I) L13/64 (anti-CD18) (D and J) NR185 (anti-CD11a), (E and K) 198 (anti-CD11b) and (F and L) BJ3/22 (anti-CD11c).*

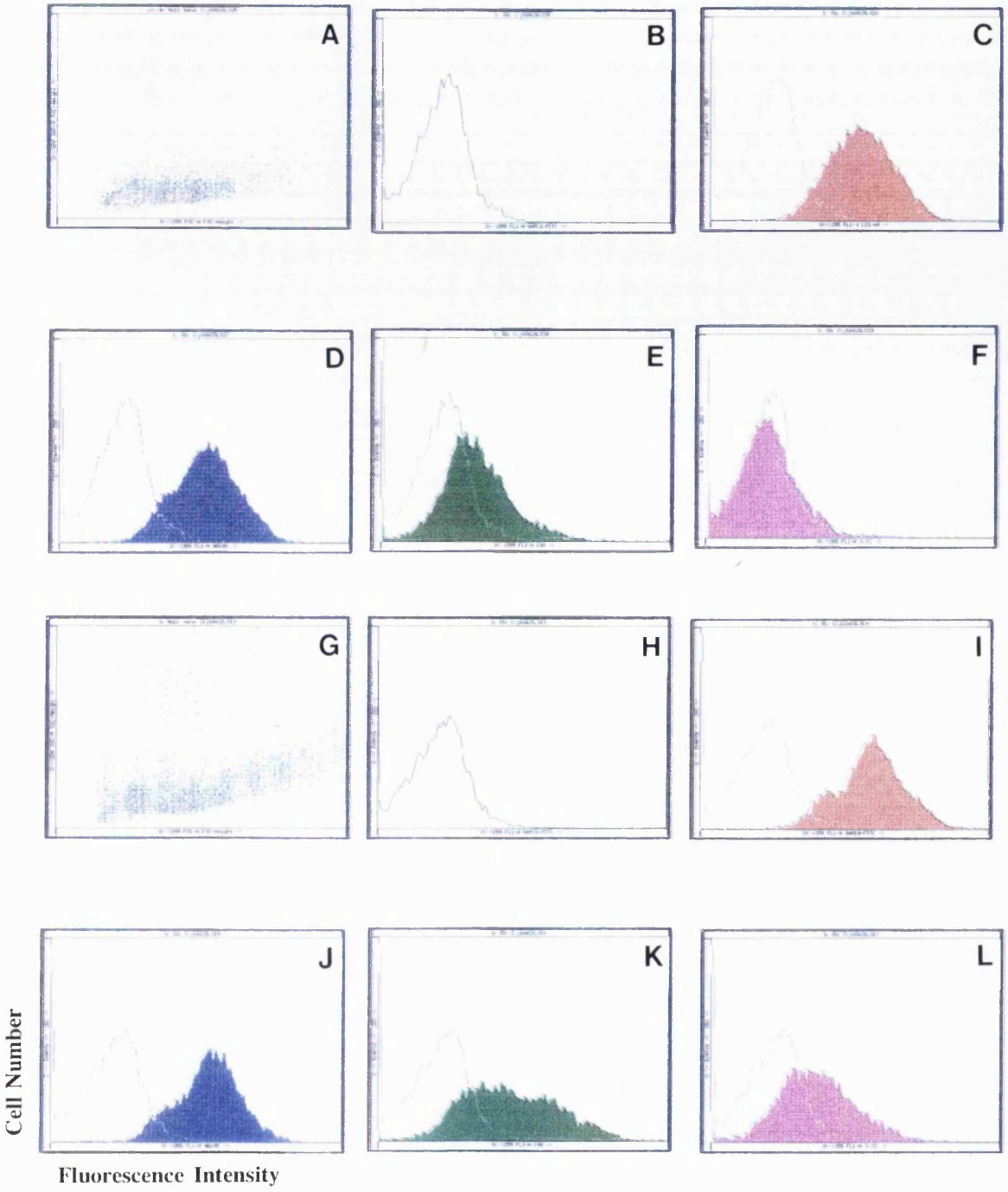

Figure 6.17. *Flow cytometric analysis of rabbit (A-F) lymph node and (G-L) spleen cells stained with anti-CD11/CD18 mAbs. (A and G) scatter plots, (B and H) negative control mAb, (C and I) L13/64 (anti-CD18), (D and J) NR185 (anti-CD11a), (E and K) 198 (anti-CD11b) and (F and L) BJ3/22 (anti-CD11c).*

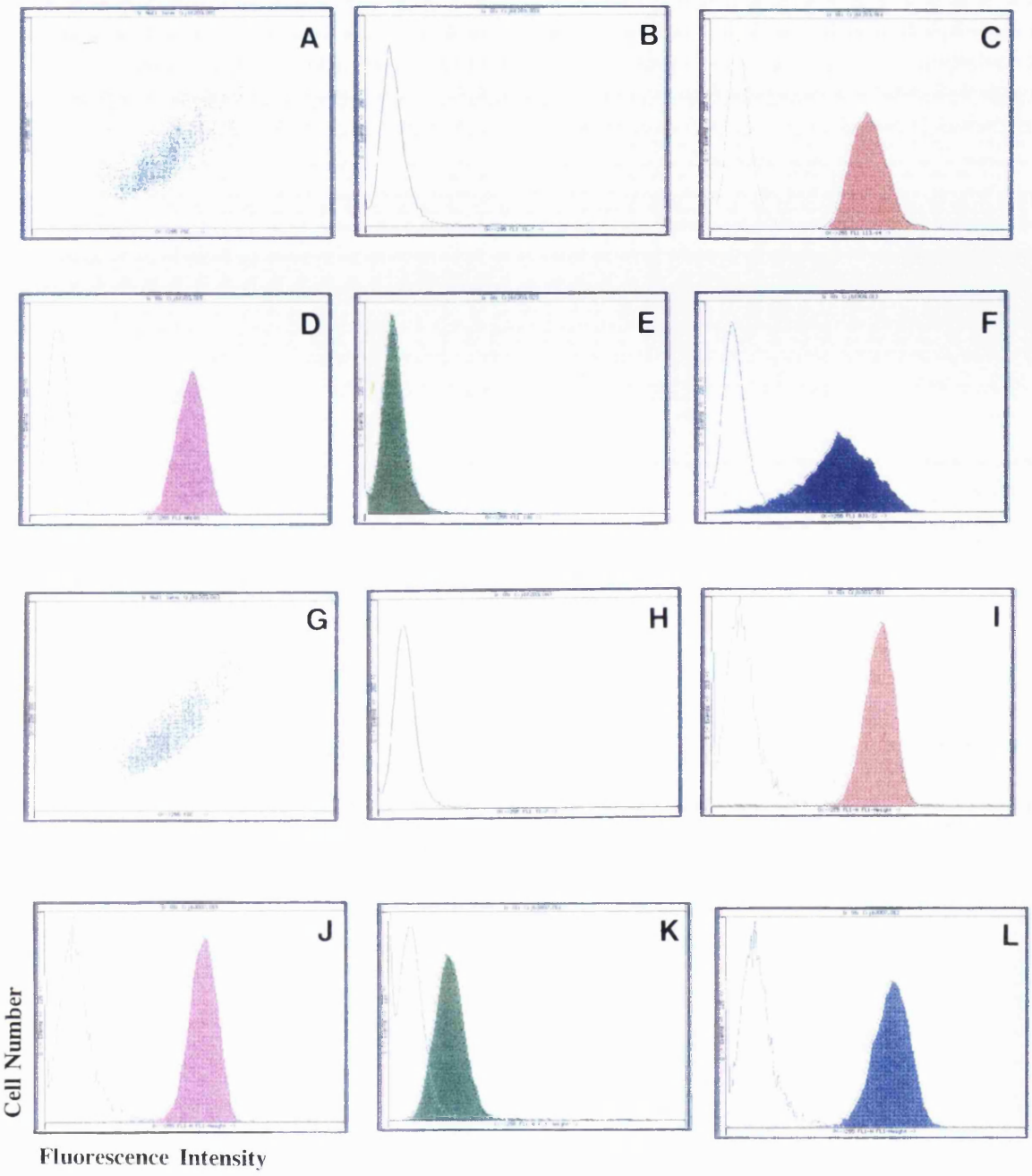

Figure 6.18. *Flow cytometric analysis of (A- F) RL-5 cells and (G-L) BK/873 cells with anti-CD11/CD18 mAbs. (A and G) scatter plots, (B and H) negative control mAb, (C and L) L13/64 (anti-CD18), (D and J) NR185 (anti-CD11a), (E and K) 198 (anti-CD11b) and (F and L) BJ3/22 (anti-CD11c).*

Mean Fluorescence Intensity of rabbit peripheral leucocytes labelled with anti-CD11/CD18 mAbs

mAb	Antigen	Lymphocytes	Monocytes	Neutrophils
FITC-conjugated	-ve	40	40	20
L13/64	CD18	B (55%) 119 T (45%) 144	164	151
NR185	CD11a	B (50%) 103 T (50%) 139	157	112
198	CD11b	(40%) 65 (60%)104	(15%) 70 (85%) 123	(10%) 86 (90%) 167
BJ3/22	CD11c	(80%) 51 T (20%) 108	(22%) 113	22

Table 6.2. *Mean fluorescence intensity was determined by flow cytometry after labelling rabbit peripheral leucocytes with anti-CD11/CD18 mAb. Lymphocyte and monocyte data was collected simultaneously and cells were gated for analysis according to FCS and SSC, mAb were detected with rabbit anti-mouse f(ab')$_2$ -FITC. Neutrophils were collected from the blood of a different animal and stained with the same batch of primary mAb, which were detected with rabbit anti-mouse f(ab')$_2$ - FITC. B = B lymphocyte, T = T lymphocyte*

Mean Fluorescence Intensity of Cells Isolated from Rabbit Lymphoid Tissue Stained with anti-CD11/18 mAb

mAb	Antigen	Thymus	Spleen	Lymph Node	Bone Marrow
FITC	-ve	35	52	52	40
L13/64	CD18	(9%) 80	(4%) 83	(7%) 79	(31%) 64
		(51%) 103	(14%) 107	(23%) 104	(69%) 105
		(40%) 125	(82%) 157	(70%) 144	(10%) 161
NR185	CD11a	(61%) 138	(11%) 88	(11%) 78	(38%) 60
		(39%) 174	(89%) 151	(22%)103	(54%) 98
				(67%) 141	(8%) 148
198	CD11b	(63%) 60	(30%) 73	(61%) 65	(40%) 51
		(19%) 102	(53%) 135	(30%) 103	(51%) 106
		(18%) 142	(17%) 196	(9%) 147	(9%) 162
3/22	CD11c	(92%) 47	(62%) 71	(90%) 49	(80%) 43
		(8%) 110	(30%) 113	(10%)106	(20%) 99
			(8%) 153		

Table 6.3. Mean fluorescence intensity was determined by flow cytometry after labelling rabbit lymphoid cells with anti-CD11/CD18 mAbs. mAbs were detected with goat-anti mouse IgG-FITC. Organs were collected from the same animal.

mAb	Specificity	BJ/873 cells	RL-5 cells
T1/7	negative	24	23
L13/64	CD18	141	134
NR185	CD11a	120	135
198	CD11b	54	23
BJ3/22	CD11c	132	112

Table 6.4. Mean fluorescence intensity of rabbit T cell lines stained with anti-CD11/CD18 mAb.

6.3. Discussion

Biochemical studies undertaken during this investigation demonstrate that mAb BJ3/120 and BJ3/41 recognise rabbit CD18 and that the rabbit CD18 protein is homologous to the human and mouse proteins. mAb BJ2/343 and NR185 were shown to recognise rabbit CD11a. Two mAb which recognise the rabbit CD11c homologue are described. Data show that in the rabbit, CD11c expression is restricted to peripheral monocytes, a subset of T cells and subsets of cells in the spleen, bone marrow, thymus and lymph node, rabbit neutrophils do not express CD11c. Sequence data for rabbit CD11c show that it is homologous to the human protein.

The anti-rabbit CD18 mAb, L13/64, precipitates three major bands at 158, 145 and 95 kDa from lysates of rabbit T cell lines surface-labelled with biotin. These are the same as the bands precipitated by the newly generated mAb BJ3/120 and BJ3/41. Sequential immunoprecipitation studies confirmed that L13/64, BJ3/120 and BJ3/41 recognised the same protein. This observation was supported by immunoblotting experiments which showed that mAb BJ3/41 and L13/64 stained proteins of the same molecular mass. Immunoprecipitation from ^{35}S-metabolically labelled cell lysates demonstrated that, like mAb L13/64, mAb BJ3/41 precipitates a band at 90 kDa, in addition to the 158, 145 and 95 kDa bands. It can be assumed that the 90 kDa band is an intracellular protein

and probably represents the rabbit CD18 precursor molecule (CD18') as previously suggested by Galea-Lauri *et al.*, (1993). The 90 kDa band is not precipitated from BJ/873 cells presumably because they have only a small pool of intracellular CD18'.

mAb BJ3/120 did not precipitate the 90 kDa band from ^{35}S-labelled cell lysates and apparently only reacts with an epitope displayed on the mature form of rabbit CD18. However, mAb BJ3/120 co-precipitated an extra band of 140 kDa with the 95 kDa rabbit CD18 band, in addition to the 158 and 145 bands. Like CD18, the CD11 proteins are synthesised as distinct precursor molecules which can form heterodimers and undergo post-translational modification before expression at the cell surface (Sanchez-Madrid *et al.*, 1983). The 140 kDa band is not expressed at the cell surface and presumably represents an intracellular precursor form of the 158 or 145 kDa bands. Since mAb BJ3/120 only recognises the mature form of rabbit CD18 these studies suggest that, in the rabbit, mature CD18 may associate with unmodified CD11 proteins.

mAb NR185 and BJ2/343 precipitated two bands with apparent Mr of 158 and 95 kDa from rabbit T cell lysates. Sequential immunoprecipitation studies compared NR185 and BJ2/343 with L13/64. The results of these experiments showed that the 95 kDa band is CD18 and that the epitope recognised by BJ2/343 and BJ3/120 is present on the 158 kDa band. mAb NR185 was used to immunoaffinity purify CD18 and the 158 kDa band from RL-5 cells. The purified proteins were analysed by mass-peptide fingerprinting techniques (Pappin *et al.*, 1993) and these showed that rabbit, mouse and human CD18 proteins are quite strongly conserved. However, peptides from the 158 kDa band could not be matched to those obtained from human and mouse CD11b, or human CD11a and CD11c which are contained in the database. Since expression of CD18 does not occur without expression of a CD11 protein, the 158 kDa band must represent a rabbit CD11 protein. Previously, N-terminal sequencing of the 158 kDa band showed that it shared some homology to human CD11 proteins (Galea-Lauri *et al.*, 1992). CD18 in other species non-covalently links to three α-chains of approximately Mr 180, 165 and 150 kDa which represent CD11a, CD11b and CD11c respectively. In the rabbit, CD18 associated with three α-chains of 158, 155 and 145 kDa. The 155 kDa band associated with CD18 in neutrophils is CD11b. Thus, by

comparison, the 158 kDa band recognised by BJ2/343 and NR185 is probably rabbit CD11a (Blackford *et al.*, 1993).

mAb BJ3/22, BJ6/112 and BJ7/208 were shown to precipitate two proteins of 145 and 95 kDa from lysates of biotin-surface labelled or ^{35}S-metabolically labelled BJ/873 cells. Sequential immunoprecipitation experiments with L13/64 confirmed that the 95 kDa band was CD18. The 145 kDa band was isolated from BJ/873 cells using a BJ3/22-immunoaffinty column. Internal peptide sequence and N-terminal sequence information was obtained and showed that the 145 kDa band was homologous to human CD11c (Blackford *et al.*, 1996). Comparison of the N-terminal sequences of rabbit CD11c and CD11b with those of human CD11a, CD11b and CD11c, shows that in the rabbit, as in the human, rabbit CD11b and CD11c share a high level of homology.

In the dog, CD18 has been shown to associate with CD11a (180 kDa), CD11b (165 kDa) and CD11c (150 kDa) giving LFA-1, Mac-1 and p150,95 (Danilenko *et al.*, 1992). A fourth CD11 chain, designated α_d, has been recently identified in the dog (Danilenko *et al.*, 1995). Using an anti-canine α_d probe, a novel cDNA clone encoding human α_d-chain has been identified and the protein sequence deduced. The canine α_d is homologous to human α_d at its N-terminus and cytoplasmic tail. No evidence of a novel CD11/CD18 protein has been found following immunoprecipitation with anti-CD18 mAb from lysates of rabbit T cell lines. However, in the dog, expression of α_d is restricted to macrophages and a T lymphocyte sub-population. It is therefore possible that rabbits express an α_d-chain on cells other than those examined here.

Staining rabbit peripheral blood leucocytes with mAb against CD18, CD11a and CD11b was in line with previous reports (Galea-Lauri *et al.*, 1993) with a few exceptions. The percentage of cells from the spleen and thymus positive for CD18 and CD11a increased, as did numbers of peripheral lymphocytes labelling with anti-CD11b mAb. These differences may be a reflection of improved cell isolation methods, which have resulted in increased T lymphocyte recovery.

Reports of CD11b expression by rabbit thymocytes and spleen cells differ. Staining tissue sections of thymus and spleen identified CD11b positive macrophages (Wilkinson

et al., 1993), which were not detected when isolated thymocytes and spleen cells were stained with the same anti-CD11b mAb and analysed by flow cytometry (Galea-Lauri *et al.*, 1993). In this study, a small population of thymocytes and spleen cells stained brightly with anti-CD11b mAb and these cells may represent resident macrophages.

In the human, CD11c is expressed on monocytes, macrophages, granulocytes and some lymphocytes (Lanier *et al.*, 1985; Schwarting *et al.*, 1985; Springer *et al.*, 1986; Miller *et al.*, 1986). It is also highly expressed on dendritic cells (Freudenthal *et al.*, 1990). Rabbit monocytes and a population of lymphocytes express CD11c. However, no expression of CD11c on rabbit neutrophils has been detected, which is similar to the situation in sheep (Metlay *et al.*, 1990) and mouse (Gupta *et al.*, 1993), but in contrast to human (Schwarting *et al.*, 1985), dog (Danilenko *et al.*, 1992) and cattle (Splitter *et al.*, 1991). In lymphoid tissues, only a minor population of cells expressed CD11c and these cells may represent the resident dendritic cell population. The exception to this was in the spleen where 30% of cells stained at intermediate levels and 8% stained brightly with anti-CD11c mAb. This is in line with other species, tissue staining of sections of dog (Danilenko *et al.*, 1992) and mouse (Gupta *et al.*, 1993) spleen showed that many cells in the white pulp areas and the marginal zone stained with anti-CD11c mAb.

In conclusion, mAb have been produced which recognise distinct epitopes of rabbit CD18. mAb NR185 and BJ2/343 have been shown to react with CD11a, and BJ3/22, BJ6/112 and BJ7/208 to react with CD11c (Blackford *et al.*, 1996). These are the first mAb described which specifically recognise rabbit CD11a and CD11c. Previously, anti-human cross reactive mAb have been used in rabbit models to define the importance of the CD11/CD18 molecules in leucocyte adhesion. However, due to a lack of mAb which react with the rabbit CD11 proteins, studies to define the roles of CD11a, CD11b or CD11c in leucocyte adhesion have been limited. These mAb may prove to be useful reagents in studying the roles of these proteins *in vivo* using a rabbit models of inflammation.

CHAPTER SEVEN

Identification of the Adhesion Molecules involved in Rabbit T Lymphocyte Homotypic Aggregation induced by Phorbol Esters and Stimulatory Monoclonal Antibodies

7.1. Introduction

Rothlein and Springer (1986), reported that incubation of human leucocytes with phorbol esters induced homotypic cell aggregation which was dependent on temperature, divalent cations and an intact cytoskeleton. Aggregation was completely inhibited by anti-CD11a/CD18 mAb and appeared to involve a different ligand, since CD11a/CD18-positive cells were shown to bind to CD11a/CD18-deficient cells. To identify ligands of CD11a/CD18, or other cell surface molecules involved in cell adhesion, antibodies raised against CD11a/CD18-deficient cells were screened for inhibition of phorbol ester-induced aggregation of cells from the Epstein-Barr virus-transformed line, JY. A single mAb, RR1/1, was obtained and was shown to recognise a 90 kDa molecule designated intercellular adhesion molecule-1 (ICAM-1) (Rothlein *et al.*, 1986). mAb RR1/1 inhibited aggregation of JY cells to the same extent as anti-CD11a/CD18 mAb and it was proposed that ICAM-1 was a ligand for CD11a/CD18. Direct evidence for this interaction of CD11a/CD18 with ICAM-1 was demonstrated by Makgoba *et al.*, (1988), who showed that CD11/CD18$^+$ cells bound to purified ICAM-1 and that adhesion could be blocked by anti-ICAM-1 and anti-CD11a/CD18 mAb.

The failure of mAb RR1/1 to block all CD11a/CD18-dependent adhesion suggested that other ligands of CD11a/CD18 existed (Rothlein *et al.*, 1986; Dustin and Springer, 1988). Staunton *et al.*, (1989) identified a second ligand for CD11a/CD18 using a procedure for DNA cloning of functionally active adhesion molecules. The molecule, designated intercellular adhesion molecule-2 (ICAM-2), shared sequence homology with ICAM-1, and antibodies raised against it inhibited several CD11a/CD18-dependent, ICAM-1-independent cell interactions (De Fougerolles *et al.*, 1991). A third ligand for CD11a/CD18 was identified from studies using the human T cell lymphoma cell line SKW3. This cell line homotypically aggregated in the presence of PMA. Aggregation

could not however, be blocked by anti-ICAM-1 and ICAM-2 mAb. mAb raised against SKW3 cells were screened in combination with anti-ICAM-1 and ICAM-2 mAb for the ability to inhibit adhesion of the cell line to purified CD11a/CD18. One mAb was selected that recognised a novel cell surface molecule which was designated intercellular adhesion molecule-3 (ICAM-3) (De Fougerolles and Springer, 1992). Molecular cloning of ICAM-3 showed that it shared homology with both ICAM-1 and ICAM-2 (Fawcett *et al.*, 1992; Vazeux *et al.*, 1992).

Phorbol ester induced CD11a/CD18-ICAM-1 dependent homotypic aggregation of leucocytes is not associated with increased expression of the molecules at the cell surface (Rothlein and Springer, 1986). It was assumed therefore, that aggregation results from an increase in the affinity of these molecules for each other. This observation was confirmed by experiments in which unstimulated and phorbol ester-stimulated cells were allowed to bind to purified CD11a/CD18 and ICAM-1 immobilised on plastic. Binding to CD11a/CD18 was the same, regardless of whether cells were stimulated or unstimulated, suggesting that ICAM-1 is constitutively avid for CD11a/CD18. However, while stimulated cells bound to purified ICAM-1, unstimulated cells did not. Thus, phorbol esters increase the affinity of CD11a/CD18 for ICAM-1 (Dustin and Springer, 1989).

The precise mechanisms by which CD11a/CD18 increases its affinity for its ligands remain obscure but seem to involve activation of protein kinase C (PKC), which phosphorylates serine and threonine residues in target proteins. Phorbol esters are analogues of diacylglycerol and activate PKC and increase the affinity of CD11a/CD18 for its ligands. Additionally, cross-linking a number of cell surface molecules including the TCR (Dustin and Springer, 1989; Pardi *et al.*, 1992), CD2, CD3 (Van Kooyk *et al.*, 1989), and CD14 (Lauener *et al.*, 1990) has been shown to transiently increase CD11a/CD18-dependent adhesion. This adhesion could be reversed by PKC inhibitors. Early studies indicated that phorbol esters resulted in phosphorylation of the cytoplasmic domain of CD18, the CD11 chains were constitutively phosphorylated, and that this was associated with cell adhesion (Chatila and Geha, 1988; Buyon *et al.*, 1990; Valmu *et al.*, 1991). Subsequently, Hibbs *et al.*, (1991a) showed that serine 756 became phosphorylated following stimulation with phorbol esters. Phosphorylation of this

residue however, did not correlate with changes in CD11a/CD18 affinity. Accordingly, CD11a/CD18 expressed on COS cells was reported to be constitutively active and phorbol ester did not stimulate additional ICAM-1 binding (Larson *et al.*, 1990), although phorbol ester was able to induce a substantial increase in CD18 phosphorylation in COS cells (Hibbs *et al.*, 1991b). These findings suggest that phosphorylation of the CD18 cytoplasmic domain does not regulate CD11a/CD18 affinity. It is clear however, that the cytoplasmic domain of CD18 is important in the regulation of the affinity of CD11a/CD18 for ICAM-1 since deletion or mutation of the CD18 cytoplasmic tail was found to inhibit CD11a/CD18-ICAM-1 interactions (Hibbs *et al.*, 1991a; Hibbs *et al.*, 1991b).

It has been suggested that changes in cytoskeletal organisation, may result in increased adhesion through the CD11a/CD18 integrins since CD11a/CD18 dependent adhesion was disrupted by cytochalasin-B (Rothlein and Springer, 1986). CD11a/CD18 was found to co-localise with talin in phorbol ester treated peripheral blood leucocytes (Burn *et al.*, 1988) and binding of anti-CD11a/CD18 mAb to a human T cell clone resulted in cytoskeletal reorganisation (Kelleher *et al.*, 1990). TCR cross-linking stimulated CD11a/CD18 dependent adhesion (Dustin and Springer, 1989), this was associated with rearrangement of the actin cytoskeleton and linking of the cytoplasmic domains of CD11a/CD18 with cytokeletal proteins including α-actinin and vinculin (Pardi *et al.*, 1992). Peter and O'Toole (1995), recently constructed cDNA encoding a chimeric integrin molecule with CD11a/CD18 intracellular domain and $\alpha_{IIb}\beta_3$ transmembrane and extracellular domains. The chimeras were mutated into a high affinity ligand binding state and expressed on COS cells. Mutations to the cytoplasmic tail of CD18 abolished adhesion of transfected COS cells to immobilised ligand. Immunofluorescence microscopy demonstrated a correlation between impaired adhesion and a decrease in the ability to form focal adhesions and to organise the cytoskeleton into stress fibers. These findings support the suggestion that interaction of the cytoplasmic domains of CD11a/CD18 and the cytoskeleton are involved in the regulation of adhesiveness of CD11a/CD18.

Divalent cations also seem to be involved in the regulation of integrin affinity. Different laboratories have found different cation requirements for CD11/CD18-ligand interactions and the regulatory role played by cations in CD11/CD18 adhesion is unclear. The CD11a/CD18-ICAM-1 interaction was reported to be Mg^{2+}-dependent, although at sub-optimal Mg^{2+} levels, Ca^{2+} was able to increase CD11a/CD18-ICAM-1 interactions (Marlin and Springer, 1987). Other studies showed that Mn^{2+} induced CD11a/CD18-ICAM-1 binding, but that Ca^{2+} exerted inhibitory effects on CD11a/CD18 function suggesting that Ca^{2+} may have a negative regulatory role in CD11a/CD18 activity (Dransfield and Hogg, 1989). CD11b/CD18 and CD11c/CD18 adhesion seems to be either Mg^{2+} or Ca^{2+} dependent (Malhotra *et al.*, 1986), but is maximal in the presence of both. Binding of Mn^{2+} to CD11b/CD18 is able to promote adhesion (Altieri, 1991). The results from recent studies suggest that extracellular Ca^{2+} may play an important role in regulating CD11a/CD18 activity. The anti-CD11a mAb NKI-L16, was first reported for its ability to stimulate CD11a/CD18-dependent aggregation of lymphoid cells (Keizer *et al.*, 1988). Subsequently, it was discovered that NKI-L16 binds to a unique Ca^{2+} dependent epitope on CD11a, thus for NKI-L16 binding, Ca^{2+} must be associated with CD11a/CD18. NKI-L16 does not recognise CD11a/CD18 on resting peripheral mononuclear cells which aggregate slowly following activation of CD11a/CD18 and have a dispersed CD11a/CD18 surface distribution. High expression of the NKI-L16 epitope, is associated with activated cells, which rapidly aggregate following stimulation of CD11a/CD28 and have a "patch-like" CD11a/CD18 surface distribution. It has been proposed that Ca^{2+} is involved in the regulation of CD11a/CD18 avidity by clustering CD11a/CD18 molecules at the cell surface (Van Kooyk *et al.*, 1994).

Regardless of the mechanisms which alter the affinity of the CD11/CD18 integrins, the change from a low affinity to a high affinity receptor involves a change in the conformation of the integrin molecule. This is demonstrated by mAb 7E3, which binds to high affinity CD11b/CD18, but not low affinity CD11b/CD18 (Altieri and Edgington, 1988), and mAb 24 which recognises a Mg^{2+}-dependent epitope expressed on CD11 sub-units when in their high affinity ligand binding state (Dransfield and Hogg, 1989).

O'Toole *et al.*, (1990), described a mAb, mAb 62, which recognises the β chain of $\alpha_{IIb}\beta_3$. On binding, this mAb caused an increase in $\alpha_{IIb}\beta_3$-ligand binding and a distinct change in the conformation of the $\alpha_{IIb}\beta_3$ molecules as assessed by the appearance of a novel epitope which was recognised by mAb PAC-1. Several mAb have been described which are able to induce CD11/CD18 integrin dependent adhesion. Binding of the anti-CD11a mAb, MEM-83, induced T lymphocyte adhesion to immobilised ICAM-1 (Landis *et al.*, 1993) and mAb KIM127 and KIM185, which bind to unique epitopes on human CD18, induced CD11a/CD18-dependent JY cell homotypic aggregation and CD11b/CD18-dependent adhesion of neutrophils to protein coated plastic (Robinson *et al.*, 1992; Andrew *et al.*, 1993). Cross-linking of cell surface molecules with antibody can lead to activation of signal transduction pathways which, in turn, can activate cell adhesion molecules. As discussed, cross-linking of the TCR promotes CD11a/CD18-ICAM-1 interactions with intracellular signals being transmitted from the TCR to CD11a/CD18 (Dustin and Springer, 1989). Alternatively, antibody molecules passively binding surface molecules either by their antigen-binding sites alone, or together with their Fc domains in the case of Fc receptor-bearing cells, can cross-link cells. Adhesion induced by mAb MEM-83, KIM127 or KIM185 was not associated with activation of intracellular signals or passive cross-linking of cells. Nor was it associated with increased expression of CD11/CD18 integrins at the cell surface. It is believed that these mAb behave in an analogous manner to mAb 62 and, on binding to CD18, cause a conformational change in the integrin molecule, detected by the expression of the mAb 24 epitope, that results in increased binding to its ligand.

The processes by which intracellular cellular signalling events alter the affinity of the CD11/CD18 integrins at the cell surface, and how these relate to the *in vivo* situation, remains obscure. Studying various models of leucocyte adhesion is likely to improve our understanding of the processes involved. During this study, the ability of rabbit lymphocytes to undergo homotypic aggregation following stimulation with PMA has been investigated. RL-5 cells were found to aggregate and mAb inhibition studies show that this aggregation is CD11a/CD18 dependent, ICAM-1 independent. Anti-rabbit CD11/CD18 integrin mAb have been screened for their ability to stimulate lymphocyte aggregation. Several mAb were found to promote aggregation of rabbit T cell lines. Comparisons have been made between PMA-induced and mAb-induced aggregation.

7.2. Results

7.2.1. Incubation of RL-5 cells with phorbol ester stimulates cell aggregation

To determine whether the rabbit T cell lines RL-5 and BJ/873 undergo homotypic aggregation in the presence of PMA, cells were incubated with PMA as described in section 2.7.1. After one hour, RL-5 cells had rounded and formed into small irregular clusters. By two hours RL-5 cells had formed into large irregular clusters (figure 7.2.A). Incubation of BJ/873 cells in media containing PMA for periods of up to 48 hours did not stimulate aggregation.

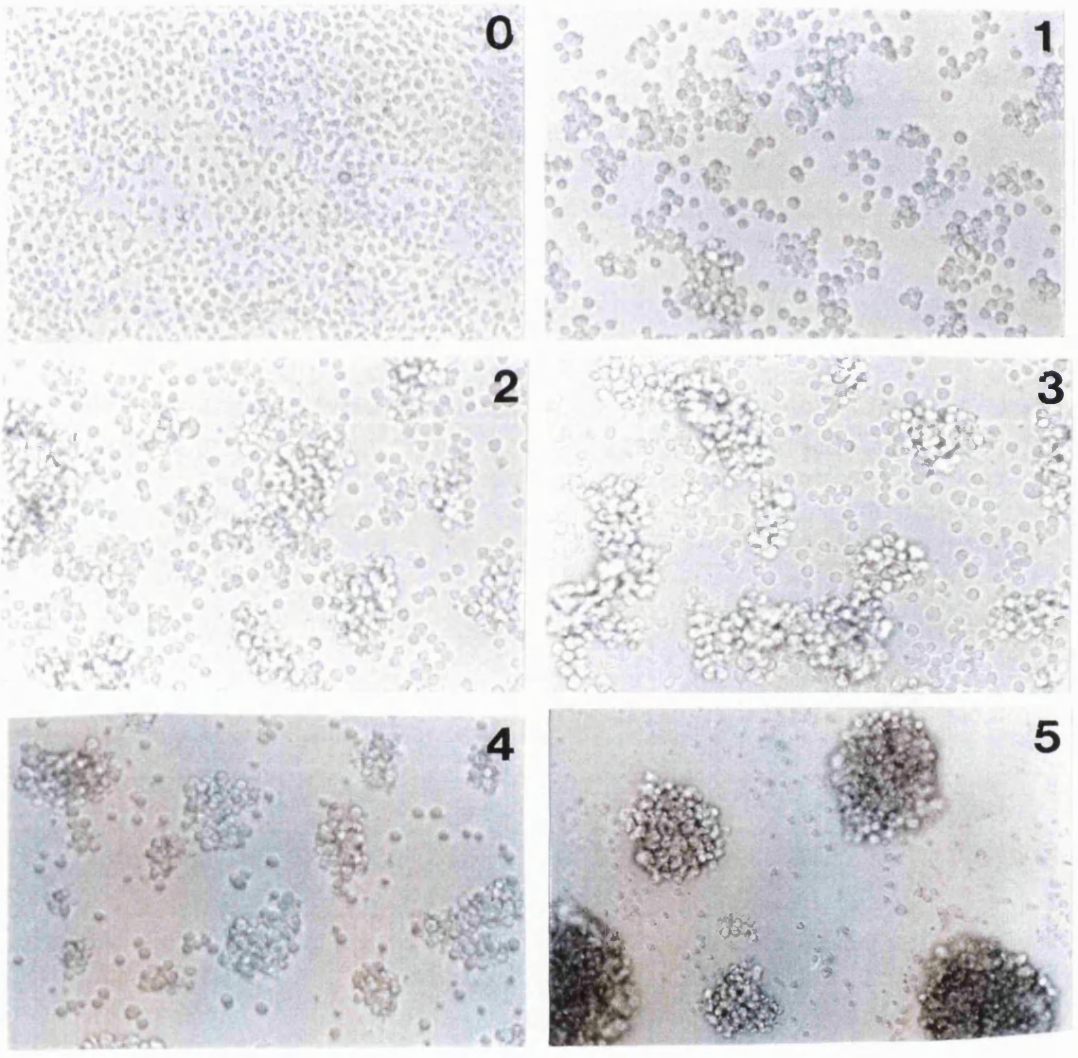

Figure 7.1. Scoring of cell aggregation. Aggregation was scored blindly 2 hours after stimulation of cells on a scale of 0-5 as described by (Rothlein and Springer, 1986): score 0 -no cell aggregation, score 1 -0-20% cell aggregation, score 2 -20-40% cell aggregation, score 3 -40-60% cell aggregation, score 4 -60-80% cell aggregation and score 5 (80-100% cell aggregation).

To identify the adhesion molecules involved in PMA-stimulated RL-5 cell aggregation. Cells were incubated in mAb which recognise various surface molecules prior to stimulation with PMA (section 2.7.3). Aggregation was assessed after 2 hours and the results are summarised in table 7.1 and shown in figure 7.2. The anti-rabbit CD43 mAb L11/135 had no effect on PMA-stimulated aggregation. The cross-reactive anti-human CD18 mAb R15.7 and 6.5E, and the anti-rabbit CD18 mAb L13/64, inhibited aggregation. The anti-human CD11a mAb R7.1 partially inhibited aggregation, whereas the anti-rabbit CD11a mAb, NR185, produced during this study, completely inhibited RL-5 cell aggregation. Aggregation was not inhibited with the anti-rabbit CD18 mAb L13/74 and L13/103, or by the anti-human ICAM-1 mAb, RR1/1. Titration experiments with mAb L13/64 and NR185 were carried out. Both L13/64 and NR185 completely inhibited aggregation at concentrations above 0.8μg/ml.

Antibody	Specificity	Inhibition
R15.7	(H) CD18	+
6.5E	(H) CD18	+
L13/64	(R) CD18	+
L13/74	(R) CD18	-
L13/103	(R) CD18	-
BJ3/120	(R) CD18	+
R7.1	(H) CD11a	+
NR185	(R) CD11a	+
RR1/1	(H) ICAM-1	-
L11/135	(R) CD43	-

Table 7.1. Inhibition of PMA-induced RL-5 cell aggregation with anti-CD11a and CD18 mAb. Cells were pre-incubated for 30 minutes with 25μg/ml mAb prior to stimulation with PMA. (R) anti-rabbit mAb, (H) anti-human mAb, + inhibition, - no inhibition.

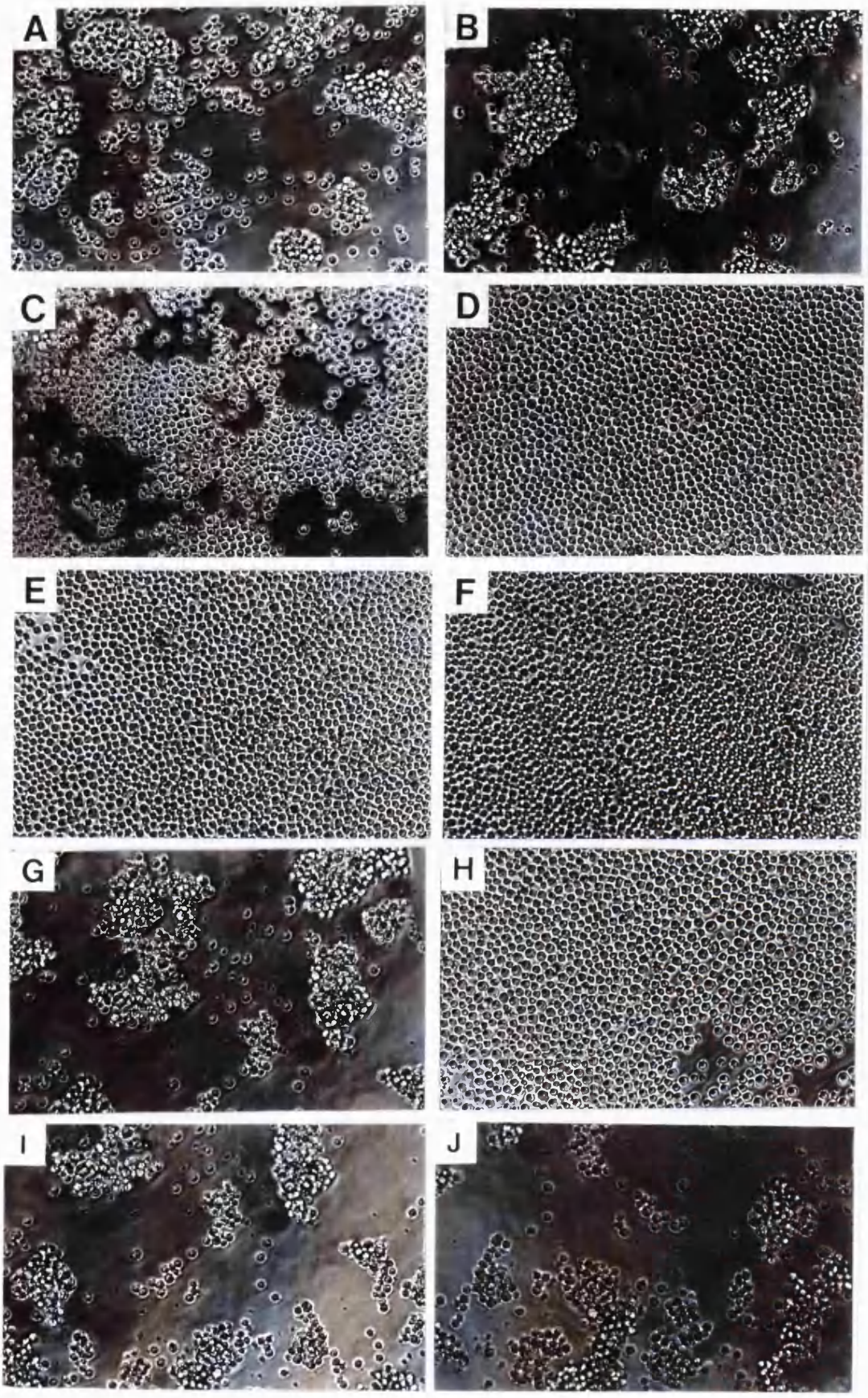

Figure 7.2. Aggregation assay with PMA stimulated RL.5 cells after 2 hours in the presence of (A) 50ng/ml PMA alone, or in the presence of 25μg/ml of mAb (B) L11/135, (C) R7.1, (D) R15.7, (E) 6.5E. (F) L13/64 (G) RR1/1, (H) NR185 (I) L13/74 and (J) L13/103.

Activation of PKC by PMA has been shown to stimulate the aggregation of human B, T and monocytic cell lines, and peripheral blood mononuclear cells (Rothlein and Springer, 1986). This aggregation is associated with an increase in the affinity of CD11a/CD18 for its ligands ICAM-1 (Rothlein *et al.*, 1986), ICAM-2 (De Fougerolles *et al.*, 1991) and ICAM-3 (De Fougerolles and Springer, 1992; Fawcett *et al.* 1992; Vazeux *et al.*, 1992). This suggests that CD11a/CD18 can become activated by intracellular signals. To confirm that PMA-stimulated RL-5 cell aggregation is dependent on PKC activation, RL-5 cells were pre-treated with Staurosporin, a known protein kinase inhibitor, or sodium azide, to deplete intracellular energy stores, according to the methods described in section 2.7.3. Cells retained their membrane integrity after treatment with Staurosporin or sodium azide, as judged by Trypan blue exclusion. Following treatment with Staurosporin or sodium azide, RL-5 cells did not aggregate when stimulated with PMA (table 7.2).

Condition	RL-5 cells	PMA/RL-5 cells
No treatment	0/1	3/4
Staurosporin	1	1
Sodium azide	0/1	1
At 4°C	0	0
At 4°C→37°C	0/1	3/4
EDTA	0	0

Table 7.2. Physiological requirements of PMA-stimulated RL-5 cell aggregation. Cells were treated as described, aggregation was assessed after 2 hours and scored on a scale of 0-5 as described (figure 7.1).

PMA-stimulated aggregation of human lymphocytes requires physiological temperature and the presence of divalent cations, particularly Mg^{2+} and Ca^{2+} (Rothlein and Springer, 1986; Dustin and Springer, 1989). RL-5 cells were stimulated with PMA and incubated at 4°C and 37°C. Cells incubated at 4°C did not aggregate, whereas maximal aggregation was observed at 37°C. Warming of cells to 37°C after incubation at 4°C allowed aggregation to proceed at a normal rate. The requirement for cations in PMA-stimulated aggregation of RL-5 cells was investigated using the methods described in

section 2.7.3. PMA did not stimulate RL-5 cell aggregation in the absence of divalent cations (table 7.2).

These data confirm that the physiological requirements of PMA-stimulation of RL-5 cells are identical to those of human lymphocytes and that aggregation can be inhibited by treatment with EDTA, sodium azide or Staurosporin, or by incubation at 4°C. Since aggregation appears to be ICAM-1-independent, RL-5 cells must express other ligands of CD11a/CD18, presumably ICAM-2 or ICAM-3.

7.2.2. mAb BJ2/343 and BJ3/41 promote CD11a/CD18-dependent homotypic aggregation

The ability of mAb BJ2/343 and BJ3/41, which recognise rabbit CD11a and CD18 respectively (chapter six), to inhibit PMA-stimulated homotypic aggregation of RL-5 cells was assessed. Cells were pre-incubated with control mAb, BJ2/343 or BJ4/41, before aggregation was induced on the addition of PMA (section 2.7.3). The level of cell aggregation in wells pre-incubated with mAb BJ3/41 and BJ2/343 was greater than in wells treated with PMA alone. This phenomenon was investigated further.

RL-5 cells were incubated with medium alone, medium containing 50ng/ml PMA, or medium containing 25μg/ml mAb BJ2/343 or BJ3/41. Cells were also incubated with mAb L11/135 (anti-CD43) as a control. Following incubation with PMA, RL-5 cells formed into irregular aggregates with free cells in the background (figure 7.3. B, table 7.3). Incubation with mAb L11/135 had no effect on the RL-5 cells. Cells incubated with mAb BJ2/343 or BJ3/41 aggregated (figure 7.3. C and D). Aggregation was rapid with small aggregates visible after 30 minutes and by 2 hours, 90% of the cells had aggregated into tight, spherical clusters.

BJ/873 cells do not aggregate in the presence of PMA. However, incubation with mAb BJ2/343 or BJ3/41 promoted BJ/873 cell aggregation, although aggregates were smaller and there was a higher percentage of free cells compared with mAb-stimulated RL-5 cell aggregation (figure 7.3. H and I). Longer incubation times of up to 24 hours, or higher mAb concentrations, did not increase the level of BJ/873 cell aggregation.

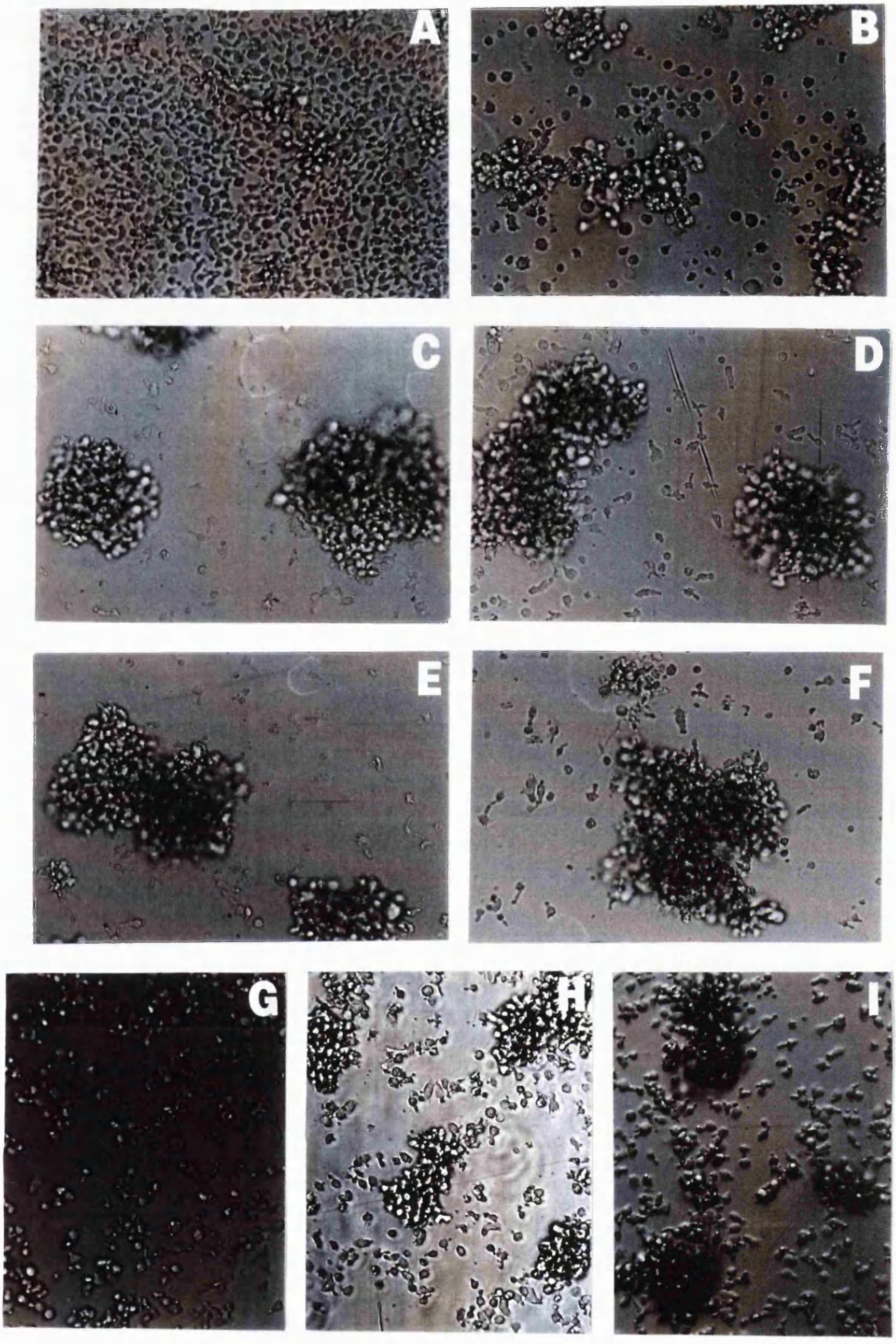

Figure 7.3. Comparison of PMA-induced and mAb-induced aggregation of rabbit T cells. RL-5 cells were incubated with (A) medium, (B) 50ng/ml PMA, (C) 25μg/ml mAb BJ3/343 or (D) 25μg/ml mAb BJ3/41, (E) 25μg/ml BJ/343 Fab fragments and (F) 25μg/ml BJ3/41 Fab fragments. BJ/873 cells were incubated with (G) 50ng/ml PMA, (H) 25μg/ml mAb BJ2/343 or (I) 25μg/ml BJ3/41.

To confirm that cell aggregation induced by BJ2/343 and BJ3/41 was not the result of cross-linking by divalent antibody, cells were incubated with $F(ab')_2$ and Fab fragments of mAb BJ2/343 or BJ3/41, prepared by Cymbus Bioscience, Southampton, U.K. Incubation of the cell lines with 25μg/ml of $F(ab')_2$ or Fab fragments of BJ2/343 or BJ3/41 induced aggregation (figure 7.3 E and F, table 7.3). $F(ab')_2$ and Fab fragments were as effective as whole Ig and titration experiments showed that Ig, $F(ab')_2$ and Fab fragments were able to induce maximal aggregation at concentrations above 0.4ng/ml.

	Aggregation	
Stimulus	**RL-5 cells**	**BJ/873 cells**
No stimulus	0	0/1
PMA	3/4	0
L11/135	0	0
BJ3/41 Ig	5	3
BJ3/41 $F(ab')_2$	5	3
BJ3/41 Fab	5	2
BJ2/343 Ig	5	2
BJ2/343 $F(ab')_2$	5	2
BJ2/343 Fab	5	2

Table 7.3. Aggregation was induced by whole Ig, $F(ab')_2$ and Fab fragments. Cells were incubated with 25μg/ml of Ig, $F(ab')_2$ and Fab for 2 hours at 37°C and aggregation was scored on a scale 0-5.

PMA-stimulated RL-5 cell aggregation requires intracellular signalling events and metabolic energy and is completely inhibited by pre-treatment of cells with Staurosporin or sodium azide. RL-5 and BJ/873 cells were pre-treated with Staurosporin or sodium azide (described section 2.7.3). The effects of this treatment on aggregation induced by PMA, or mAb BJ2/343 and BJ3/41 were compared (table 7.4). Untreated RL-5

cells aggregated following incubation with PMA, BJ2/343 and BJ3/41, but not medium alone or control mAb. Untreated BJ/873 cells did not aggregate when incubated with PMA or control mAb, but aggregation was induced by BJ2/343 and BJ3/41. Treatment with sodium azide or Staurosporin completely inhibited PMA-stimulated RL-5 cell aggregation although mAb-induced aggregation proceeded as normal. Treated BJ/873 cells aggregated in the presence of mAb to the same degree as untreated cells. These data strongly suggest that, unlike PMA-induced aggregation, BJ2/343 and BJ3/41-induced aggregation of rabbit T cell lines does not result from activation of PKC.

	Aggregation	
Treatment	**RL-5**	**BJ/873**
No stimulus	0	0
PMA	4	0
198	0	0
BJ3/41	5	3
BJ2/343	5	2
Staurosporin	1	1
Sodium azide	0	1
PMA + Staurosporin	1	1
BJ3/41 + Staurosporin	5	3
BJ2/343 + Staurosporin	5	2/3
PMA + sodium azide	1	1
BJ3/41 + sodium azide	5	3
BJ2/343 + sodium azide	5	2/3

Table 7.4. Effects of Staurosporin and sodium azide on mAb-induced aggregation. Cells were pre-treated with Staurosporin or sodium azide before adding 50ng/ml PMA or 25μg/ml mAb. Cells were incubated for 2 hours at 37°C and aggregation was assessed and scored on a scale of 0-5.

To determine whether mAb induced aggregation is temperature sensitive, the cell lines were incubated with medium, BJ2/343, BJ3/41 or PMA, as a positive control, in parallel at 4°C or 37°C (table 7.5). Low temperature completely inhibited cell aggregation, but returning the cells to 37°C allowed aggregation to proceed at a normal rate. Thus mAb-induced aggregation is temperature dependent.

Treatment	RL-5	BJ/873
None	0	0
PMA	3	0
BJ3/41	5	3
BJ3/41 at 4°C	0	0
BJ3/41 at 4°C, → 37°C	5	3
BJ2/343	5	2
BJ2/343 at 4°C	0	0
BJ2/343 at 4°C, → 37°C	5	2

Table 7.5. Temperature requirements of mAb-induced aggregation of RL-5 and BJ/873 cells compared with PMA-stimulation of RL-5 cells. Aggregation was induced by incubating cells with 50ng/ml PMA or 25μg/ml mAb as described and aggregation was assessed after 2 hours and scored on a scale of 0-5.

The role of cations in mAb BJ2/343 and BJ3/41-induced aggregation was investigated, using the methods described in section 2.7, and compared with PMA-stimulated RL-5 cell aggregation. The results are summarised in table 7.6. As expected, pre-treatment with EDTA completely inhibited PMA-stimulated RL-5 cell aggregation. Incubation of EDTA pre-treated RL-5 and BJ/873 cells with mAb BJ2/343 or BJ3/41 allowed only partial cell aggregation.

Treatment	RL-5	BJ/873
None	0	0
PMA	3	0
BJ3/41	5	3
BJ2/343	5	2
EDTA	0	0
EDTA + PMA	0	0
EDTA + BJ3/41	3	1
EDTA + BJ2/343	3	1

Table 7.6. Summary of the cation requirements of mAb-induced aggregation of RL-5 and BJ/873 cells compared with PMA-stimulation of RL-5 cells. Aggregation was induced by adding PMA (50ng/ml) or mAb (25μg/ml). Cells were incubated at 37°C for 2 hours and aggregation was scored on a scale of 0-5.

The ability of anti-CD11/CD18 mAb to inhibit mAb-induced aggregation were investigated and compared with inhibition of PMA-induced RL-5 cell aggregation. RL-5 cells were pre-incubated with anti-CD11 or CD18 mAb before being stimulated with control mAb, PMA, BJ2/343 or BJ3/41 (figure 7.4). Full aggregation of the RL-5 cells was seen following incubation with PMA, BJ2/343 or BJ3/41. PMA-induced aggregation was inhibited by the anti-CD18 mAb L13/64, 7E4, R15.7 and the anti-CD11a mAb NR185. The anti-CD18 mAb L13/74 and RCN1/21, and the anti-ICAM-1 mAb RR1/1, did not inhibit PMA-induced aggregation. mAb L13/64, 7E4 and R15.7 also completely inhibited BJ2/343 and BJ/41-induced aggregation. mAb NR185 partially blocked aggregation suggesting that mAb-induced aggregation is CD11a/CD18-dependent. Interestingly, mAb L13/74, which had no effect on PMA-induced aggregation, completely inhibited BJ2/343 and BJ3/41-induced aggregation. This inhibition was not the result of L13/74 preventing BJ2/343 or BJ3/41 binding to CD11a/CD18 since pre-incubation of RL-5 cells with L13/74 did not block BJ3/41-FITC binding (figure 7.5). These data suggest that different regions of CD11a/CD18 are involved in PMA-induced and mAb-induced aggregation.

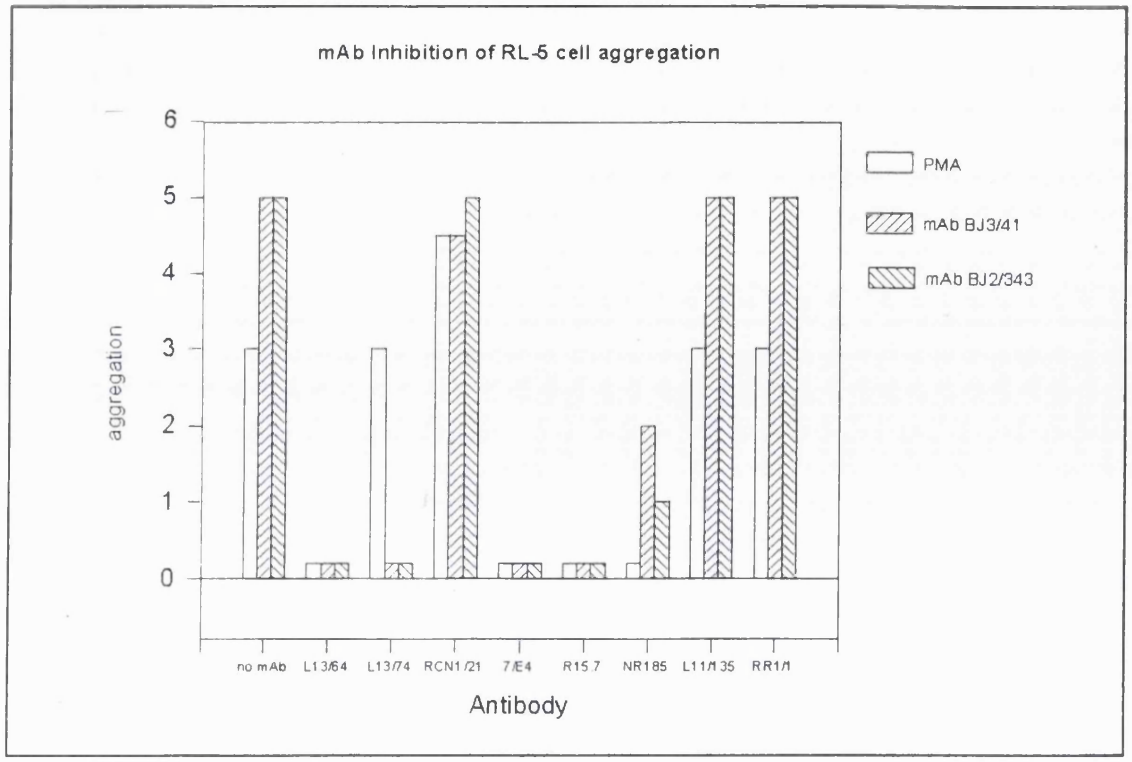

Figure 7.4. Inhibition of PMA and mAb-induced RL-5 cell aggregation with anti-CD11/CD18 mAb. Cells were pre-incubated with the anti-CD18 mAb L13/64, L13/74, RCN1/21, 7E4 and R15.7, and with NR185 (anti-CD11a), L11/135 (anti-CD43) and RR1/1 (anti-ICAM-1), for 30 minutes before aggregation was induced on the addition of 50ng/ml PMA or 25μg/ml BJ2/434 or BJ3/41. Aggregation was assessed after 2 hours and scored on a scale of 0-5.

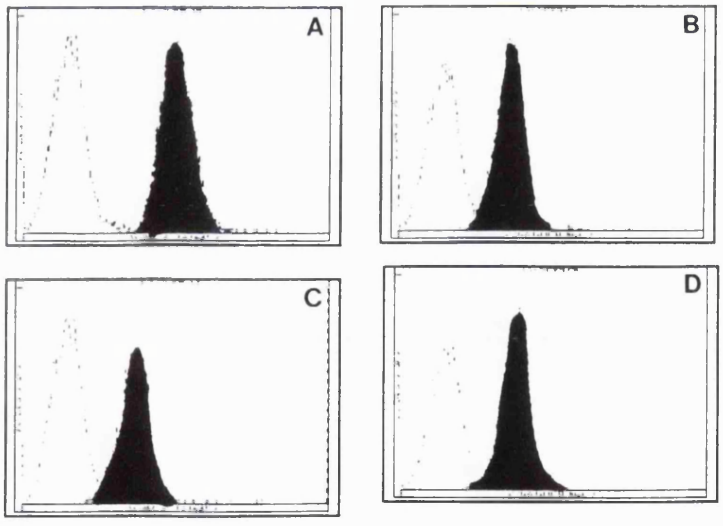

Figure 7.5. mAb L13/74 does not inhibit binding of BJ3/41 to CD11a/CD18. RL-5 cells were stained for flow cytometry with 10μg/ml mAb T1/7 (negative control, open hiostogram), (A) L13/64 and (B) L13/74 and mAb binding was detected with goat anti-mouse-FITC. Cells were stained with (C) BJ3/41-FITC and (D) L13/74 and then with BJ3/41-FITC.

7.2.3. mAb BJ3/22 promotes aggregation of rabbit T cell lines

In the previous chapter, mAb BJ3/22 was shown to recognise rabbit CD11c. In experiments to determine whether CD11c/CD18 was involved in PMA-induced aggregation of RL-5 cells, cells were pre-incubated for 30 minutes with 25μg/ml of control mAb or mAb BJ3/22 as described in section 2.7.3. Cells were stimulated with PMA (50ng/ml) and aggregation was assessed after two hours. RL-5 cells pre-incubated with control mAb formed into irregular aggregates containing about 60% of the cells, while those which had been pre-incubated with mAb BJ3/22 formed into tight spherical aggregates containing approximately 80% of the cells (figure 7.6. D). Aggregation was re-assessed at 18 hours and, while PMA-stimulated cells had dis-aggregated, cells stimulated with PMA and BJ3/22 remained aggregated. Treatment of the RL-5 cells with mAb BJ3/22 alone did not induce cell aggregation (figure 7.6. C).

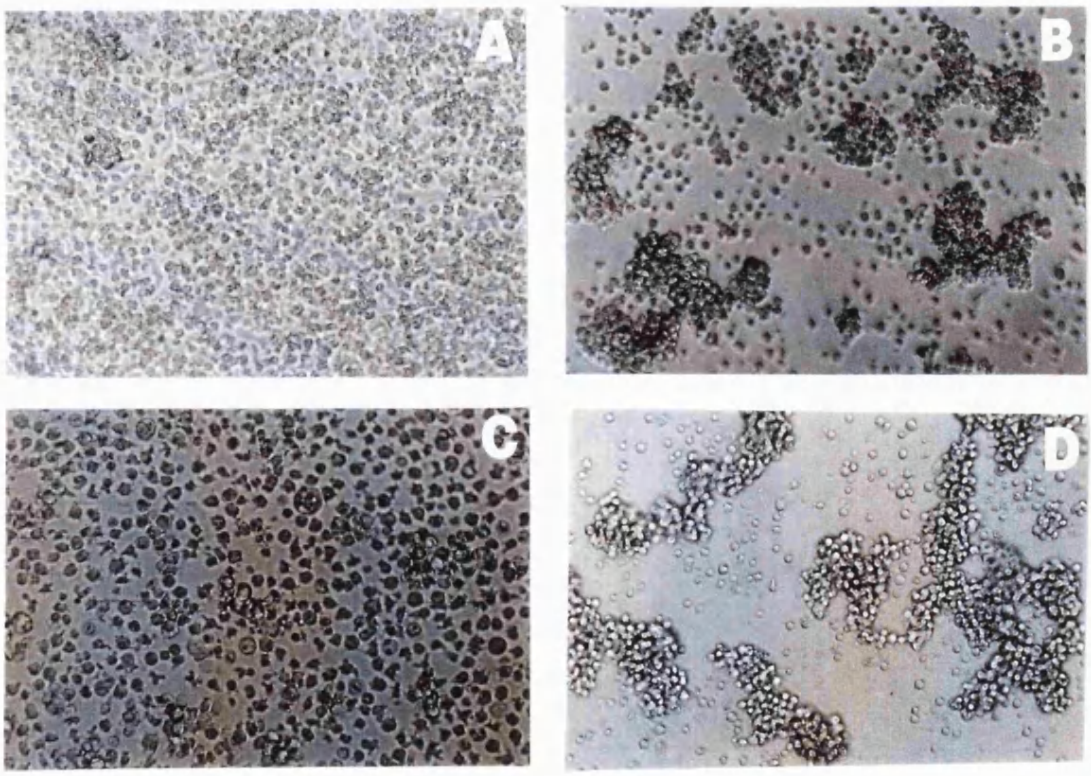

Figure 7.6. RL-5 cells were incubated with (A) medium alone, (B) 50ng/ml PMA, (C) 25μg/ml mAb BJ3/22 and (D) 25μg/ml BJ3/22 and 50ng/ml PMA. Aggregation was assessed after 2 hours.

The effects of mAb BJ3/22 on the BJ/873 cell line were investigated (figure 7.7, table 7.7). BJ/873 cells do not aggregate in the presence of PMA. However, incubation of the BJ/873 cells with BJ3/22 caused cellular aggregation (figure 7.7.B). Typically aggregation began at 30 minutes, with cells forming into small aggregates of 4-8 cells, and by 2 hours several large aggregates containing between 80-90% of the total cells were seen. To ensure that BJ3/22-induced aggregation was not the result of passive cross-linking of cells by bivalent antibody, $F(ab')_2$ and Fab fragments of mAb BJ3/22 were generated according to the methods described in section 2.2.12. The absence of contaminating whole or partially digested antibody in $F(ab')_2$ and Fab fragment preparations was shown by SDS-PAGE (figure 7.8). Maximal BJ/873 cell aggregation was induced by both BJ3/22 $F(ab')_2$ and Fab fragments (figure 7.7.C and D). Titration experiments showed that whole BJ3/22 antibody, $F(ab')_2$ and Fab fragments were able to produce maximal levels of aggregation at concentrations above 0.8μg/ml.

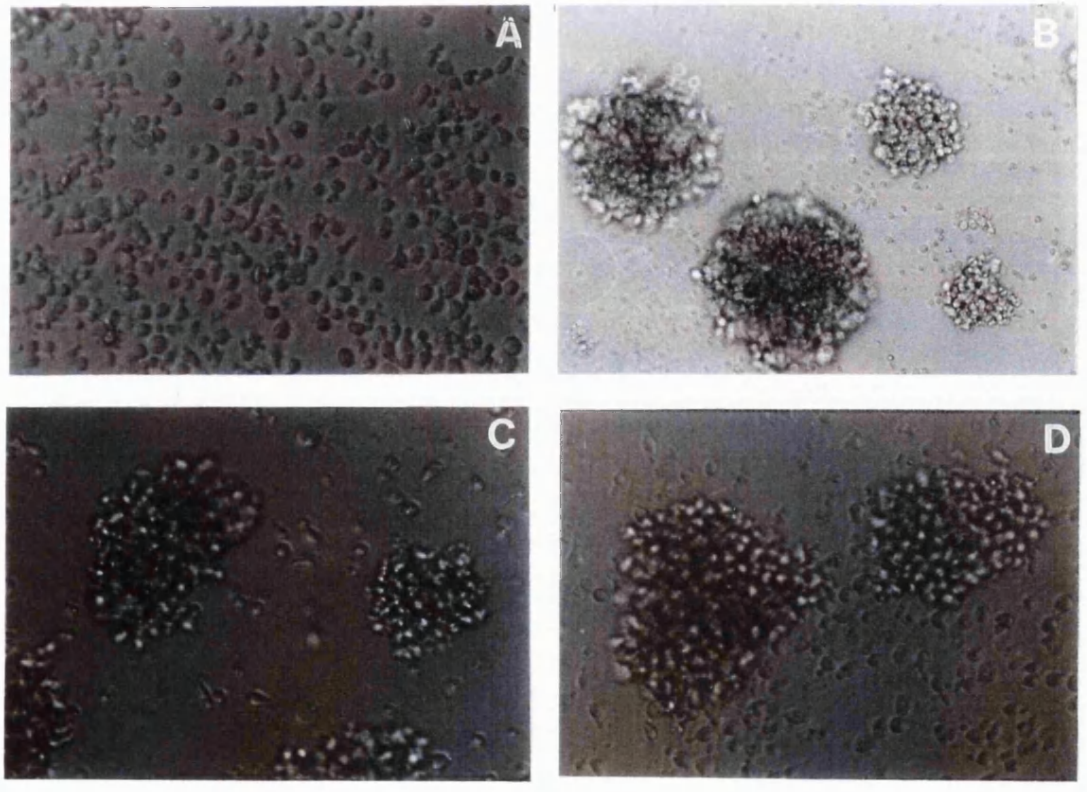

Figure 7.7. BJ3/22-induced aggregation of BJ/873 cells. Cells were incubated with (A) medium, (B) 25μg/ml BJ3/22, (C) 25μg/ml BJ3/22 $F(ab')_2$ and (D) 25μg/ml BJ3/22 Fab. Aggregation was assessed after 2 hours and scored on a scale of 0-5.

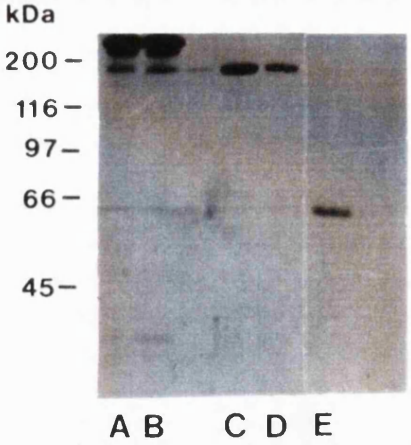

Figure 7.8. Preparation of F(ab')$_2$ and Fab fragments of mAb BJ3/22. F(ab')$_2$ and Fab fragments were prepared according to the methods described in section 2.2.12 and analysed on 10% SDS-PAGE gels under non-reducing conditions. Lanes (A and B) 20μg whole Ig, (lanes C and D) 2μg F(ab')$_2$ and (lane E) 1.5μg Fab fragments.

Treatment	BJ/873	RL-5
none	0	0
BJ3/22	5	0
BJ3/22 + PMA	-	4
BJ3/22 F(ab')$_2$	4/5	-
BJ3/22 Fab	4/5	-

Table 7.7. Stimulation of cell aggregation with mAb BJ3/22. Aggregation was induced on the addition of 25μg/ml whole Ig, F(ab')$_2$ or Fab. RL-5 cells were stimulated with 50ng/ml PMA. Aggregation was assessed at 2 hours and scored on a scale of 0-5, (- not done).

To determine whether CD11c-induced aggregation involved intracellular signalling events, RL-5 and BJ/873 cells were pre-treated with Staurosporin or sodium azide as described in section 2.7.3. Cell membrane integrity was maintained following these treatments as assessed by Trypan blue exclusion. Incubation with sodium azide or Staurosporin inhibited PMA-stimulation of RL-5 cells. However, pre-treated BJ/873 aggregate to the same extent as untreated cells following stimulation with BJ3/22 (figure 7.9; table 7.8). These data suggest that BJ3/22-induced aggregation is exclusively an extracellular event which must involve the interaction of CD11c with its ligand.

The cation dependence of CD11c-induced aggregation was investigated and compared with PMA-stimulated RL-5 aggregation (table 7.8). Cells were pre-incubated in medium containing 5mM EDTA, as described in section 2.7.3, and were stimulated in Ca^{2+} and Mg^{2+}-free medium. No aggregation was seen for BJ/873 cells incubated with BJ3/22 (figure 7.9. F) or RL-5 cells incubated in PMA in the absence of cations. The epitope recognised by mAb BJ3/22 is not cation sensitive, as shown by immunofluorescence staining of cells in the presence of EDTA (figure 7.10).

Treatment	**BJ873**	**Treatment**	**RL-5**
none	0	none	0
BJ3/22	5	PMA	3
NaN_3	0	NaN_3	0
NaN_3 + BJ3/22	5	NaN_3 + PMA	0
Staurosporin	1	Staurosporin	1
Staurosporin + BJ3/22	5	Staurosporin + PMA	1
EDTA	0	EDTA	0
EDTA + BJ3/22	0	EDTA + PMA	0

Table 7.8. *Effects of Staurosporin, sodium azide and EDTA on BJ3/22-induced aggregation of BJ/873 cells compared with PMA-induced aggregation of RL-5 cells. Cells were pre-treated with Staurosporin, sodium azide or EDTA before adding 25μg/ml BJ3/22 or 50ng/ml PMA. Aggregation was assessed after 2 hours and scored on a scale of 0-5.*

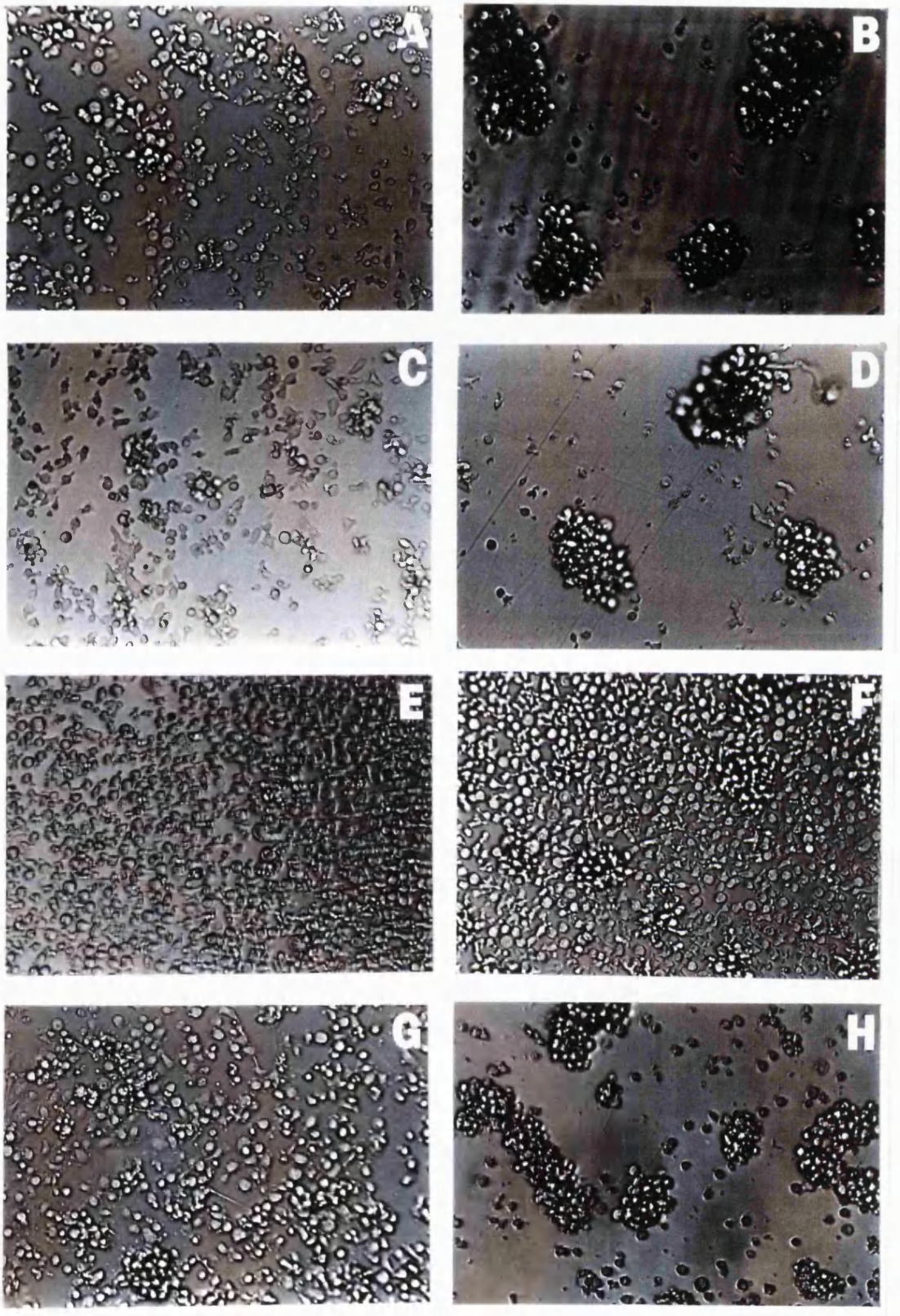

Figure 7.9. *mAb BJ3/22-induced aggregation of BJ/873 cells. Cells were incubated with (A) medium alone, (B) 25μg/ml BJ3/22, or were pre-treated with (C and D) NaN_3, (E and F) EDTA and (G and H) Staurosporin before aggregation was induced (D, F and H) with 25μg/ml BJ3/22.*

The temperature dependence of anti-CD11c mAb-induced aggregation was compared with PMA-stimulated RL-5 cell aggregation. BJ/873 cells were incubated with BJ3/22 and RL-5 cells with PMA at 4°C or 37°C, as described in section 2.7.3. The results are shown in table 7.9. As expected, PMA stimulation of RL-5 cells was inhibited at 4°C, while cells incubated at 37°C aggregated. Similarly, BJ/873 cells incubated with BJ3/22 at 4°C did not aggregate, while cells incubated at 37°C aggregated. Returning cells to 37°C, from 4°C, allowed aggregation to proceed at a normal rate. The epitope recognised by BJ3/22 is not temperature sensitive, as shown by immunofluorescence staining of cells at 4°C (figure 7.10).

Treatment	BJ3/22-induced	PMA-induced
none	4/5	3
4°C	0	0
4°C, → 37°C	4/5	3

Table 7.9. *Summary of the physiological requirements of BJ3/22-induced aggregation compared with PMA-induced RL-5 cell aggregation. Cells were incubated with 25μg/ml BJ3/22 or 50ng/ml PMA at 4°C or 37°C for 2 hours. Aggregation was scored on a scale of 0-5.*

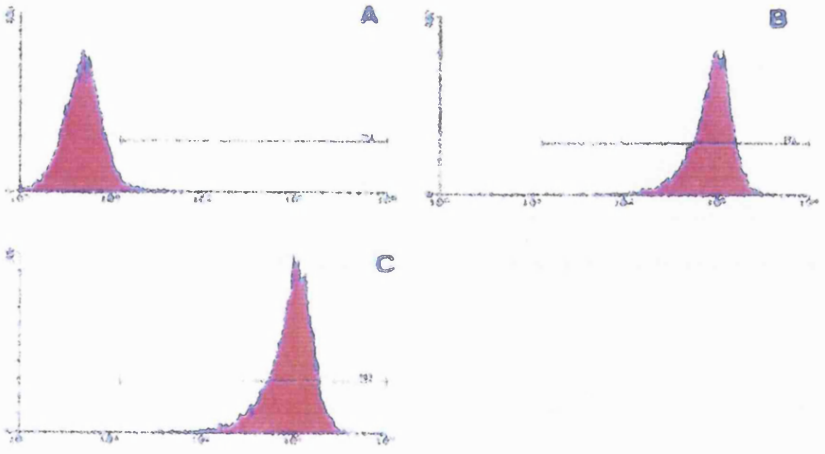

Figure 7.10. *mAb BJ3/22 stains BJ/873 cells brightly at 4°C and in the absence of divalent cations. (A) BJ/873 cells stained with FITC-conjugated rabbit anti-mouse F(ab')$_2$ at 4°C, (B) BJ/873 cells stained with BJ3/22 at 4°C and (C) BJ/873 cells stained with BJ3/22 in the absence of divalent cations.*

BJ/873 cells were incubated with mAb which recognise various cell surface molecules prior to stimulation with BJ3/22 (figure 7.11). mAb NR185 (anti-CD11a) and 198 (anti-CD11b) had no inhibitory effect. The anti-rabbit CD18 mAb L13/64 and the anti-human CD18 mAb 7E4 and 6.5E inhibited aggregation. Aggregation was not inhibited by the anti-CD18 mAb L13/74 or the anti-CD11a mAb NR185. It has been reported that ICAM-1 acts as a ligand for CD11c/CD18 (Diamond *et al.*, 1993; De Fougerolles *et al.*, 1995). The possibility of BJ3/22-induced aggregation involving CD11c/CD18-ICAM-1 interactions was explored. Pre-incubation with the anti-ICAM-1 mAb, RR1/1, inhibited BJ3/22-induced aggregation of BJ/873 cells. Titration experiments showed that the inhibitory effects of RR1/1 were lost at concentrations below 6.0μg/ml. mAb Rb2/1 recognises rabbit ICAM-1, although it has not been shown to functionally block ICAM-1-dependent interactions. mAb Rb2/1 was unable to inhibit BJ3/22-induced aggregation.

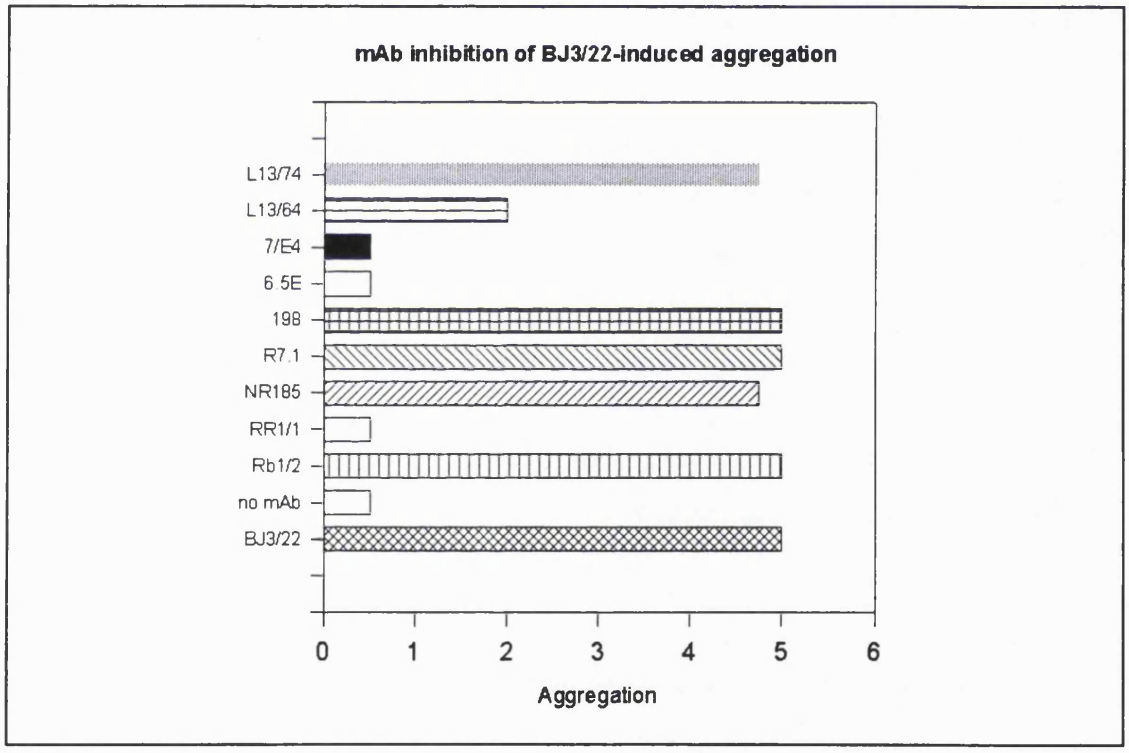

Figure 7.11. *Inhibition of BJ3/22-induced RL-5 cell aggregation with anti-CD11/CD18 mAb. Cells were pre-incubated for 30 minutes with the anti-CD18 mAb L13/64, L13/74, 6.5E and 7E4, the anti-CD11a mAb NR185 and R7.1, 198 (anti-CD11b) and the anti-ICAM-1 mAb RR1/1 and Rb2/3. Aggregation was induced on the addition of 25μg/ml mAb BJ3/22. Aggregation was assessed after 2 hours and scored on a scale of 0-5.*

The response of the rabbit HTLV-1 transformed T cell lines RHK/30, RH/K34, SPL66A, Thy-1, Thy-2 and Thy66A (Sawasdikosol *et al.*, 1993) to mAb BJ3/22 was assessed. RH/K34 cells gave a significant aggregatory response. RH/K34 cells grow as a mixture of free cells and loose aggregates (figure 7.13. E). While the addition of the anti-CD43 mAb L11/135 did not alter the level of aggregation, incubation with mAb BJ3/22 resulted in the formation of tighter aggregates with fewer free cells in the background (figure 7.13. F).

The expression of CD18, CD11a, CD11b, CD11c and ICAM-1 on RL-5, BJ/873 and HTLV-1 cell lines was determined (figure 7.12). Only BJ/873 and RH/K34 cells expressed CD18, CD11a, CD11c and ICAM-1 and aggregated in the presence of mAb BJ3/22. RL-5 cells and the other HTLV-1 cell lines were shown to express CD11a, CD11c and CD18, but only low levels of ICAM-1 and did not aggregate in the presence of BJ3/22 alone. The relationship between ICAM-1 expression and degree of aggregation in response to BJ3/22 is shown in table 7.10. BJ/873 and RH/K34 cells were pre-incubated with L13/64 (anti-CD18) or RR1/1 (anti-ICAM-1) prior to stimulation with BJ3/22 (figure 7.13). BJ3/22-induced aggregation of BJ/873 cells (figure 7.11 C and D) and RH/K34 cells (figure 7.13 G and H) was inhibited by mAb L13/64 and RR1/1.

Cell line	CD11c expression	ICAM-1 expression	BJ3/22-induced aggregation
BJ/873	+++	+++	+++
RH/K34	+++	++	++
RL-5	++	+	-

Table 7.10. Correlation of BJ3/22-induced aggregation with expression of ICAM-1. Cells were stained for flow cytometry with 10μg of mAb BJ3/22 (anti-CD11c), or mAb RR1/1 (anti-ICAM-1). Aggregation of cell lines with mAb BJ3/22 was assessed 2 hours after adding 25μg/ml BJ3/22.

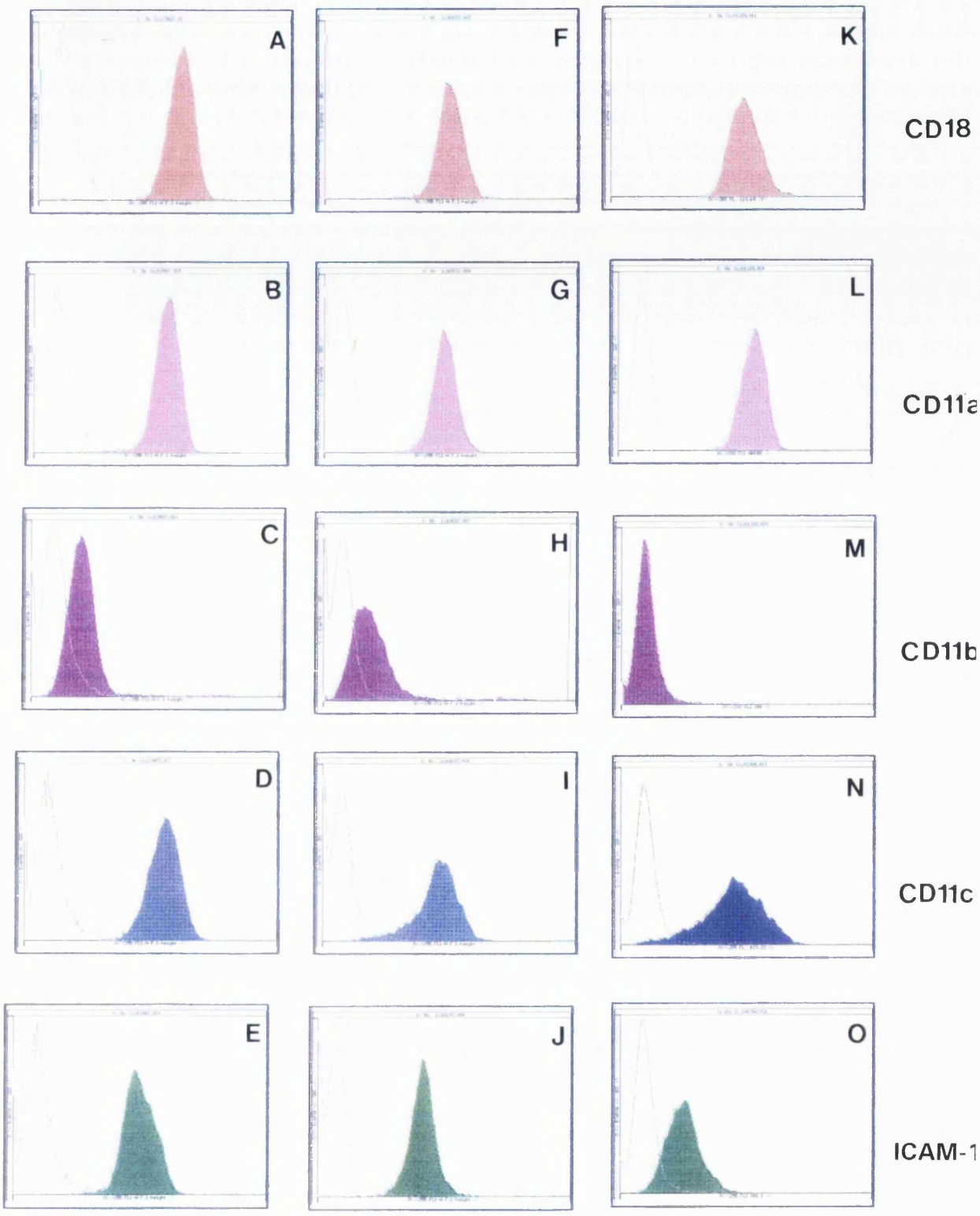

Figure 7.12. *Expression of CD11/CD18 molecules and ICAM-1 on rabbit T cell lines: (A-E) BJ/873 cells, (F-J) K34 cells and (K-O) RL-5 cells.*

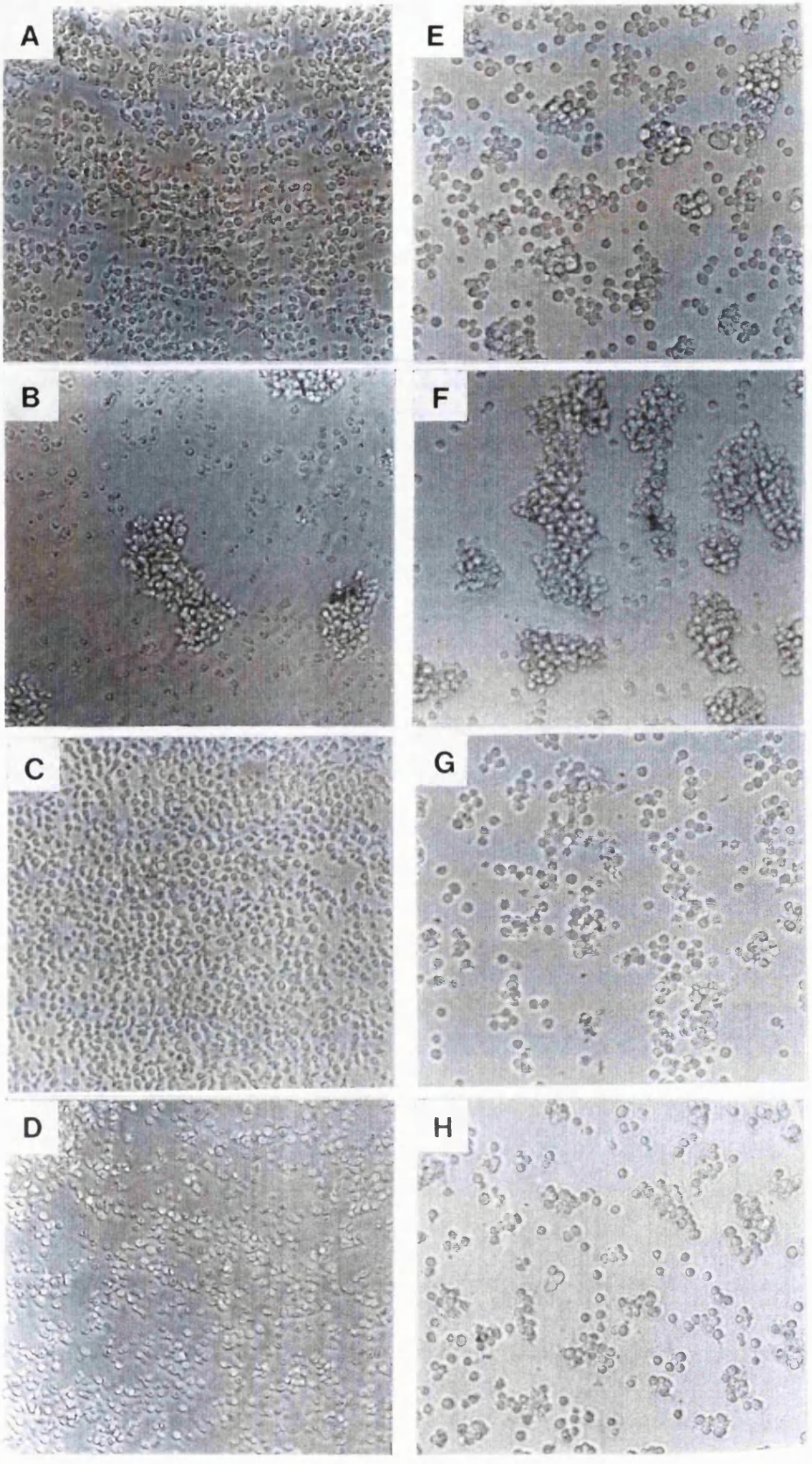

Figure 7.13. *Inhibition of mAb BJ3/22-induced BJ/873 (A - D) and RH/K34 (E - H) cells. Cells were incubated in (A and E) medium alone, (B and F) 25μg/ml mAb BJ3/22, or were pre-incubated for 30 minutes with (C - G) L13/64 (anti-CD18) and (D and H) RR1/1 (anti-ICAM-1), before aggregation was induced by adding 25μg/ml mAb BJ3/22. Aggregation was assessed after 2 hours.*

7.2.4. Anti-CD11c mAb BJ6/112 and BJ7/208-induce BJ/873 cell aggregation

The ability of the anti-CD11c mAb BJ6/112 and BJ7/208 to induced BJ/873 aggregation was assessed. Both mAb induced aggregation of the BJ/873 cells. The physiological requirements of this aggregation were compared to BJ3/22-induced BJ/873 aggregation and the results are summarised in table 7.11. Like BJ3/22-induced aggregation, BJ6/112 and BJ7/208 induced aggregation was inhibited by anti-CD18 mAb, but not CD11a or CD11b mAb (table 7.12). These data suggest that all three anti-CD11c mAb induce a conformational change in CD11c/CD18 probably by binding to the same activation epitope on CD11c.

		mAb-induced aggregation		
Treatment	**Cells**	**BJ2/33**	**BJ6/112**	**BJ7/208**
mAb	1	4	4	4
4°C	0	0	0	0
37°C	1	4	4	4
4°C, → 37°C	1	4	4	4
NaN_3	1	4	4	4
EDTA	0	0	0	0
Staurosporin	1	4	4	4

Table 7.11. Comparison of the physiological requirements for BJ/873 cell aggregation induced by anti-CD11c mAb. Cells were pre-treated with Staurosporin, EDTA or NaN_3 and aggregation was induced on the addition of 25μg/ml mAb. Aggregation was assessed after 2 hours and scored on a scale of 0-5.

			mAb-induced BJ/873 cell aggregation		
Antibody	**Specificity**	**Cells**	**BJ3/22**	**BJ6/112**	**BJ7/208**
L11/135	CD43 (R)	0	5	5	5
L13/74	CD18 (R)	0	4	5	3
BJ3/120	CD18 (R)	0	0	1	0
7E4	CD18 (H)	0	3	2	1
6.5E	CD18 (H)	0	1	1	1
NR185	CD11a (R)	0	5	5	5
198	CD11b (R)	0	5	5	5

Table 7.12. Inhibition of anti-CD11c mAb-induced aggregation with anti-CD18, CD11a and CD11b mAb. Cells were pre-incubated for 30 minutes with 25μg/ml mAb before aggregation was induced by adding 25μg/ml of BJ3/22, BJ6/112 or BJ7/208. Aggregation was assessed after 2 hours and scored on a scale of 0-5, R = anti-rabbit and H = anti-human mAb).

7.2.5. Appendix to chapter seven: mAb NR185 blocks neutrophil migration *in vivo*

The ability of the anti-CD11a mAb, NR185, to inhibit leucocyte adhesion *in vivo* was studied in collaboration with Dr. Martyn Robinson and the Department of Inflammation Biology, Celltec Ltd. in an *in vivo* model of inflammation. It has been reported that in the rabbit, the oedema associated with intradermal injection with fMLP is dependent on leucocyte migration (Wedmore and Williams, 1981), while intradermal injection with bradykinin produces oedema which is leucocyte migration-independent. Oedema formation can be monitored by measuring the leakage of i.v. injected ^{125}I-labelled albumin from the vasculature at the site of injection. The effects of prior injection with inhibitory mAb NR185 (anti-CD11a), 198 (anti-CD11b) and 6.5E (anti-CD18), on oedema formation were assessed. NR185 (i.v. 0.5mg/kg) inhibited fMLP-induced oedema, but had no effect on bradykinin-induced oedema. Similarly, pre-treatment of rabbits with mAb 6.5E (i.v. 0.24mg/kg) totally abolished fMLP-induced oedema, but did not alter bradykinin-induced oedema. mAb 198 (i.v. up to 2.3mg/ml), did not prevent fMLP-induced or bradykinin-induced oedema. To ensure that the inhibition of oedema in rabbits pre-treated with NR185 or 6.5E was associated with changes in the pattern of neutrophil migration, at 0.5, 1, 1.5, 2 and 3 hours after injection of fMLP, skin sites were removed and examined histologically for neutrophil accumulation. Neutrophil accumulation was completely inhibited in rabbits pre-treated with NR185 or 6.5E, while large numbers of neutrophils were seen in the vessels and tissues at fMLP-injection sites in untreated animals. Examination of fMLP injection sites in rabbits pre-treated with 198, showed that neutrophil accumulation had occurred. Interestingly, neutrophils in the 198 pre-treated animals, 30 minutes after fMLP injection, were predominantly associated with blood vessels, in contrast to control animals in which cells were mainly at extravascular sites. At later time points, no differences between control rabbits and 198-treated rabbits could be observed. These studies show that in the rabbit, binding of neutrophils to endothelium and subsequent transmigration to extravascular sites is CD11a/CD18-dependent. Although blocking CD11b/CD18 function did not prevent neutrophil accumulation and endothelial binding, it seemed to slow the rate of transmigration. This suggests that CD11b/CD18 is involved in transmigration, but that other receptor ligand pairs, including CD11a/CD18, are also involved.

7.3. Discussion

Homotypic aggregation of leucocytes, originally reported to be induced by phorbol esters, represents a suitable model for identifying receptor-ligand interactions involved in leucocyte adhesion. The response of a number of rabbit T cell lines to stimulation with the phorbol ester PMA has been studied. The $CD4^+$ cell line, RL-5, aggregated in the presence of PMA, and this response was shown to be CD11a/CD18-dependent. While screening anti-rabbit CD11/CD18 mAb for their ability to inhibit RL-5 cell aggregation, several mAb were found to have the somewhat unusual property of being able to cause homotypic aggregation of rabbit T cells in the absence of PMA. The receptor/ligand interactions involved in this aggregation have been defined and the mechanisms by which these mAb induce cellular adhesion studied.

Incubation of RL-5 cells with PMA resulted in cellular aggregation which was rapid, temperature sensitive, cation dependent and involved intracellular signalling events. It was completely inhibited by anti-CD18 and CD11a mAb, although the anti-ICAM-1 mAb RR1/1, which has previously been shown to functionally block ICAM-1-dependent adhesion, had no effect on RL-5 cell aggregation. PMA-induced aggregation of RL-5 cells is therefore CD11a/CD18-dependent and ICAM-1-independent. In the human, CD11a/CD18 binds to ICAM-1 (Rothlein *et al.*, 1986), ICAM-2 (Staunton *et al.*, 1989) and ICAM-3 (Vazeux *et al.*, 1992; Fawcett *et al.*, 1992; De Fougerolles and Springer, 1992). No reports have yet described the rabbit equivalent of ICAM-2 or ICAM-3, although they probably exist. These data suggest that in the rabbit, CD11a/CD18 is able to bind to other ligands which may be ICAM-2 or ICAM-3.

mAb BJ3/41 and BJ2/343 recognise CD18 and CD11a respectively (chapter six). Incubation of RL-5 cells with these mAb, either as tissue culture supernatant or purified protein, induced cellular aggregation. The effects produced by mAb BJ3/41 and BJ2/343 are not the result of a passive cross-linking event because isotype-matched cell surface binding mAb were unable to form such aggregates and $F(ab')_2$ and Fab fragments of mAb BJ3/41 and BJ2/343 were able to stimulate aggregation. Transmembrane signalling events are not involved since aggregation occurs in the absence of metabolic energy and protein kinase activity. Several anti-human CD18

mAb have been described which are able to induce CD11a/CD18 and CD11b/CD18-dependent cell aggregation (Robinson *et al.*, 1992; Andrew *et al.*, 1993; Keizer *et al.*, 1988). Binding of these mAb has been shown to alter the conformation of CD11a/CD18 or CD11b/CD18 and it is believed that this change modulates the affinity of the integrin molecule for its ligands. Presumably, binding of mAb BJ3/41 or BJ2/343 promotes a similar conformational change in rabbit CD11a/CD18 resulting in cell aggregation. BJ3/41 and BJ2/343-induced aggregation was inhibited by pre-incubation of RL-5 cells with anti-CD18 or anti-CD11a mAb, confirming that CD11a/CD18 is the adhesive receptor involved. The anti-ICAM-1 mAb, RR1/1 did not inhibit aggregation and the nature of the CD11a/CD18 ligand in this system remains unknown, but is likely to be either ICAM-2 or ICAM-3.

The presence of divalent cations, particularly Mg^{2+}, has been shown to be essential for binding of CD11a/CD18 to its ligands (Marlin and Springer, 1987; De Fougerolles *et al.*, 1994). The requirement for divalent cations in RL-5 cell aggregation induced by PMA or mAb BJ3/41 and BJ2/343 was investigated. Cells were pre-treated with EDTA and aggregation was induced in medium containing 5mM EDTA. Under these conditions, PMA failed to induce RL-5 cell aggregation, although some aggregation was seen when cells were incubated with BJ3/41 or BJ2/343. Petruzzelli *et al.*, (1995), recently described mAb CBR LFA-1/2 which recognises human CD18. CBR LFA-1/2 induce CD11a/CD18-dependent adhesion of JY cells to immobilised ICAM-1 at a level comparable to that seen with phorbol esters. Adhesion of JY cells to ICAM-1 could be induced by mAb CBR LFA-1/2 at considerably lower concentrations of Mg^{2+} than required for phorbol ester-induced adhesion, and this was interpreted as resulting from CBR LFA1/2-activated CD11a/CD18 having increased affinity for ICAM-1 compared with PMA-activated CD11a/CD18. In the presence of cations, the level of cellular aggregation induced by treatment with BJ3/41 or BJ2/343 is greater than that induced by PMA, presumably because mAb-activated CD11a/CD18 has a higher affinity for its ligand than PMA-activated CD11a/CD18. It is possible that EDTA treatment of RL-5 cells lowered the concentration of Mg^{2+} in the cell system enough to inhibit PMA-induced RL-5 cell aggregation, but that sufficient Mg^{2+} was still available to allow the higher affinity mAb-induced aggregation to proceed.

The anti-CD18 mAb L13/64, 7E4 and R15.7 were able to inhibit both PMA and mAb-induced RL-5 cell aggregation, presumably because they have epitopes near to, or overlapping, the CD11a/CD18-ligand binding site. mAb L13/74 did not inhibit PMA-induced aggregation but completely inhibited mAb-induced aggregation. From this, it was assumed that L13/74 prevented binding of mAb BJ3/41 and BJ2/343 to CD11a/CD18 and thus indirectly inhibited mAb-induced aggregation, rather than blocking the CD11a/CD18-ICAM interaction. However, flow cytometry experiments showed that in the presence of L13/74, BJ3/41 was still able to bind to its epitope. Thus PMA-induced and mAb-induced aggregation involved binding through different regions of CD11a/CD18. It has been proposed that CD11a/CD18 can acquire conformations that selectively bind to ICAM-1, ICAM-2 and ICAM-3 (Binnerts *et al.*, 1994; Ortlepp *et al.*, 1995) with these different ligands binding to different regions of CD11/CD18 (Landis *et al.*, 1994). The data presented here suggest that PMA-activated CD11a/CD18 interacts with a different ligand from that of BJ3/41 and BJ2/343-activated CD11a/CD18. Presumably these different ligands bind to overlapping regions of CD11a/CD18.

Incubation with PMA did not induce BJ/873 cell aggregation, although mAb BJ3/41 and BJ2/343 induced partial aggregation of the BJ/873 cells. This difference in behaviour between RL-5 and BJ/873 cells may be explained by the differences in the levels of CD11a/CD18 expression. Immunoprecipitation with the anti-CD11a mAb NR185, showed that BJ/873 cells express lower levels of CD11a/CD18 at the cell surface compared with RL-5 cells (chapter six). Consequently, following stimulation with PMA, BJ/873 cells may not become "sticky" enough to support cell aggregation, while the high affinity interaction between CD11a/CD18 and its ligand which appears to be induced by mAb BJ3/41 and BJ2/343 is able to support some intercellular adhesion. It has been proposed that PMA-induced aggregation of RL-5 cells involves adhesion of CD11a/CD18 to a different ligand from that involved in BJ3/41 and BJ2/343-induced aggregation. If this hypothesis is correct, the inability of PMA to induce BJ/873 cell aggregation may be the result of a lack of ligand expression.

The anti-CD11c mAb, BJ3/22, produced during this study, induced homotypic aggregation of BJ/873 cells. This aggregation was rapid, temperature-sensitive and cation-dependent, which is typical of integrin mediated adhesion. It occurred following treatment of cells with Staurosporin and sodium azide and is not dependent on transmembrane signalling events. Aggregation was induced by $F(ab')_2$ and Fab fragments and thus does not result from cell cross-linking by bivalent antibody or Fc receptor interactions. Combined, these data suggest that aggregation induced by BJ3/22, and the other anti-CD11c mAb BJ6/112 and BJ7/208, is a cell surface associated event. Presumably binding of these mAbs to their epitope on CD11c modifies the affinity of CD11c/CD18 for its ligand. Several mAb, described here and elsewhere, have been shown to stimulate CD11a/CD18 or CD11b/CD18 dependent adhesion, but not CD11c/CD18-dependent adhesion.

In experiments to determine the adhesion molecules involved in this anti-CD11c mAb-induced aggregation, cells were incubated with anti-CD18 mAb and the anti-CD11a mAb, NR185. These mAb had previously been shown to inhibit CD18/CD11a-dependent adhesion of RL-5 cells following stimulation with PMA, BJ3/41 or BJ2/343. Aggregation was completely inhibited by the anti-CD18 mAb, but not mAb NR185, which confirms that CD11c/CD18 is indeed the adhesive receptor involved. Aggregation was also inhibited by the anti-ICAM-1 mAb, RR1/1, which indicates that, in this system, ICAM-1 is the ligand for CD11c/CD18. This observation was supported by experiments with other cell lines. RL-5 cells express CD11c/CD18, but only low lecels of ICAM-1 and did not show an aggregatory response when incubated with BJ3/22. Like BJ/873 cells, the HTLV-1 transformed cell line, RH/K34, expresses significant amounts of CD11c/CD18 and ICAM-1 and aggregated following incubation with BJ3/22. Thus, expression of both CD11c/CD18 and ICAM-1 on the same cell is necessary for BJ3/22-induced aggregation to occur.

Purified functional CD11c/CD18 heterodimers immobilised on plastic bind endothelial cells stimulated for 18 hours with IL-1β (Stacker and Springer, 1991). ICAM-1 is unregulated on endothelial cells by IL-1β, and expression is maximal at 18 hours. Thus it was proposed that ICAM-1 may act as the ligand in this interaction. In agreement with this, chinese hamster ovary cells transfected with CD11c/CD18 have been shown

to weakly bind to immobilised ICAM-1 (Diamond *et al.*, 1993). The interaction of CD11c/CD18, expressed on native cells, with ICAM-1 has not been reported. The data presented here confirm that activated CD11c/CD18 on T lymphocytes can function as a lymphocyte adhesion molecule by binding to ICAM-1. This finding suggests that the contribution of CD11c/CD18 to leucocyte adhesion in other systems should be given careful consideration.

CHAPTER EIGHT

Cloning of Rabbit CD18

8.1 Introduction

As shown in the previous chapter, a number of mAb have been described which are able to induce CD11a/CD18 and CD11b/CD18 dependent adhesion in the absence of intracellular signalling or metabolic energy (Robinson *et al.*, 1992; Andrew *et al.*, 1993 Petruzzelli *et al.*, 1995). The anti-rabbit CD11c mAb BJ3/22, BJ6/112 and BJ7/208, described in this study, are able to stimulate CD11c/CD18-dependent aggregation of rabbit T cell lines. Aggregation is completely inhibited by the anti-ICAM-1 mAb RR1/1 suggesting that CD11c/CD18 functions as a receptor for ICAM-1 in the rabbit.

Our understanding of CD11/CD18-dependent cell adhesion has been advanced by cloning of cDNA molecules encoding the subunits of the CD11/CD18 antigens and expressing them, as functional surface receptors, in mammalian cells which do not express CD11/CD18 integrins. For example, transfection of CD11a/CD18 and CD11b/CD18 in COS and chinese hamster ovary (CHO) cells has yielded functional integrins which are able to bind to the same ligands as these integrins on their native cells, including ICAM-1 and iC3b (Larson *et al.*, 1990; Diamond *et al.*, 1993). A cDNA clone which contained the complete coding information for human CD11c, isolated by Corbi *et al.*, (1987), has been co-expressed with CD18 (Kishimoto *et al.*, 1987; Law *et al.*, 1987) on the surface of CHO and COS cells. Studies using these transfectants have demonstrated CD11c/CD18 binding to immobilised heparin and IgM-iC3b-coated erythrocytes, although binding to iC3b required heterologous expression of human CD11c with chicken CD18 (Bilsland *et al.*, 1994; Diamond *et al.*, 1995). Wild-type CD11c/CD18 expressed on CHO cells weakly binds immunoaffinity immobilised ICAM-1, following stimulation of the cells with PMA, and this binding is completely inhibited by CD11c/CD18 mAb (Diamond *et al.*, 1993).

The interaction of CD11c/CD18 with ICAM-1 is controversial. The human erythroleukaemic cell line K562, which does not normally express CD11/CD18 proteins, was stably transfected with cDNA encoding CD18 and CD11c. Treatment of these transfectants with the adhesion-promoting mAb KIM127 and KIM185, stimulated CD11c/CD18-dependent adhesion to plastic coated with a number of proteins including ICAM-Ig fusion proteins, BSA, and serum proteins (Ortlepp *et al.*, 1995). Cells bound equally well to each of the proteins suggesting that in this situation CD11c/CD18-ICAM-1 interactions may not be specific.

To further investigate the interactions of CD11c/CD18 with ICAM-1 using the rabbit system it is necessary to work with cells which express CD11c/CD18, but not other cell adhesion molecules. Attempts were therefore made to isolate cDNA clones encoding rabbit CD11c and CD18. This work was carried out under the supervision of Dr Bishop Hague at the Laboratory of Immunogenetics, NIAID, Bethesda, USA.

8.2. Results

8.2.1. Amplification of CD18 cDNA fragments from a rabbit spleen cDNA library

A rabbit spleen cDNA library, made under contract by Invitrogen, which had been used to clone rabbit CD4 (Hague *et al.*, 1992) was used for these experiments. Staining of rabbit lymphoid tissues with anti-CD18 and CD11c mAb showed that spleen cells express high levels of CD18 and intermediate levels of CD11c (section 6.2.7) and it was decided that this library would be an appropriate source of CD18 and CD11c cDNA clones. The library was constructed using size selected (>2.5 kb) cDNA, which was modified with *Bst*XI adaptors and ligated into the pcDNAI plasmid vector. The recombinant plasmids were used to transform *E. coli* MC1061/P3 cells.

Using peptide mass-fingerprinting techniques, the sequence of the rabbit CD18 protein was shown to be homologous with human and mouse sequences (section 6.2.8). On this basis, four oligonucleotides were designed from the aligned sequences of mouse (Zeger *et al.*, 1990; Wilson *et al.*, 1989) and human (Law *et al.*, 1987; Kishimoto *et*

al., 1987) CD18 cDNA sequences, (5'→3' oligonucleotides CD18-1 cctggtgccagaagctgaacttc, CD18-3: gcgttcaacgtgacctccgg, 3'→5' CD18-2 gaccagtagaccttccggggact and CD18-4 ggactttcagtggatactgaggaagg). These oligonucleotides were used to amplify fragments of DNA from a spleen cDNA template by PCR (Saiki *et al.*, 1985; Mullis *et al.*, 1986; Saiki *et al.*, 1988) according to the methods described in section 2.9.2. PCR products were analysed by electrophoresis on agarose gels (section 2.8.3). Five products were amplified and these are shown in figure 8.1 and detailed in table 8.1.

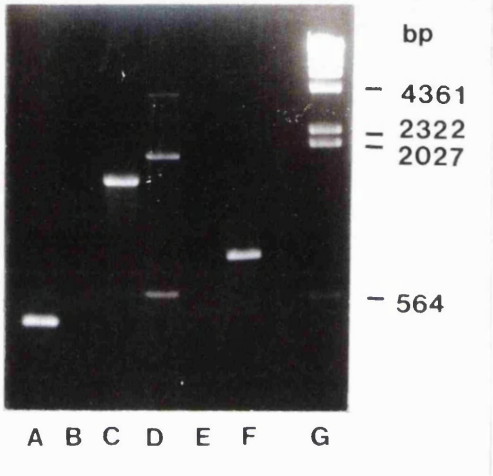

Figure 8.1. *Amplification of rabbit spleen cDNA by PCR. Lane (A) product 1, (lane B) control (oligonucleotide 1 and 2, no template), (lane C) product 2, (lane D) products 3 and 4, (lane E) control (oligonucleotides 3 and 4, no template), (lane F) product 5 and (lane G) λDNA.HindIII marker. PCR products were run on a 1.2% agarose/TBE gel at 60v for 1.5 hours.*

Oligonucleotide	PCR Product
1 and 2	1 (450 bp)
1 and 4	2 (1.7 kb)
3 and 2	3 (1.95 kb)
3 and 2	4 (564 bp)
3 and 4	5 (750 bp)

Table 8.1. *PCR Products obtained from reactions with rabbit CD18 oligonucleotides and rabbit spleen cDNA template*

8.2.2 Cloning of the rabbit CD18 PCR products for sequencing using the TA cloning® system

To determine whether rabbit CD18 was amplified during the PCR, the DNA fragments were sequenced and compared to known CD18 sequences. Before sequencing, the PCR products were cloned using the TA Cloning® system. Cloning of PCR products serves two purposes: (i) cloned products are easily sequenced and (ii) large quantities of PCR products can be obtained from cultures of transformed cells using basic plasmid preparation methods. For each PCR product, one cloning experiment was performed according to the methods described in sections 2.9.3 and 2.9.4.

8.2.2.i. The TA Cloning® system

The system (figure 8.2) is based upon the ability of T*aq* polymerase to add a single deoxyadenosine residue to the 3' ends of PCR products thus producing 3' A-overhangs. The pCR™II plasmid vector, supplied with the kit as a linearised molecule, has 3' T-overhangs which allows the PCR product to ligate with the plasmid forming a recombinant molecule which is then used to transform bacterial cells by the heat shock method. Bacterial colonies which contain recombinant plasmid molecules, i.e. transformed cells, are identified using antibiotic resistance and blue/white screening.

The host strain cells, in this case *E.coli* (One Shot™ cells, supplied with the kit) are normally ampicillin and kanamycin sensitive and do not grow in the presence of these antibiotics. The pCR™II plasmid carries ampicillin and kanamycin resistance genes. On transformation with pCR™II, the host cells acquire ampicillin and kanamycin resistance and can grow on nutrient agar plates containing these antibiotics.

Linearised plasmid may self-ligate and be taken up by bacterial cells during transformation allowing bacteria which do not contain the PCR insert to form colonies on antibiotic plates. To ensure that these non-transformed cells are not selected, a second method for identifying transformed cells is employed. β-galactosidase is an enzyme found in *E. coli* which hydrolyses lactose into galactose and glucose allowing

E. coli to use lactose as its sole source of carbon. The genome of the *E. coli* host cells supplied with the TA-Cloning system are specifically deleted so that they code only for the carboxy-terminal region of β-galactosidase. The pCR™II plasmid carries a segment of DNA derived from *E. coli* which codes for the regulatory sequences and the first 146 amino acids of the β-galactosidase gene (*Lac*Z). Alone, the β-galactosidase fragments encoded by the host cell genome and plasmid genome are enzymatically inactive, however the two fragments can associate, a process known as α-complementation, to form a functionally active enzyme. Cells containing plasmid form blue colonies in the presence of the chromogenic enzyme substrate 5-bromo-4-chloro-indolyl-β-D-galactoside (X-gal). Ligation of foreign DNA into the multiple cloning site of pCR™II, which is located within the *Lac*Z gene, disrupts the reading frame of the gene and α-complementation can no longer occur. Therefore cells containing recombinant plasmids produce functionally inactive β-galactosidase and form white colonies in the presence of X-gal.

8.2.3. Analysis of cloned PCR products

Several white colonies from each transformation experiment were selected and grown in liquid culture overnight. Plasmid DNA was isolated from each of the cultures the next day, as described in section 2.8.1, and digested with *Eco*R1 to release the inserts (figure 8.2).

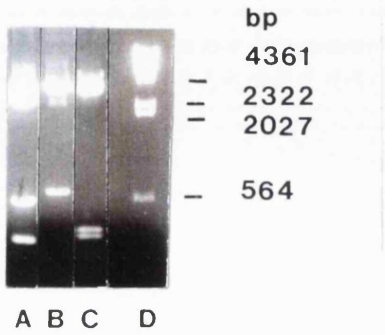

*Figure 8.2. Recombinant DNA was isolated from cells transformed with PCR products 1, 4 and 5. Recombinant DNA molecules were digested with Eco*R1 *and analysed by gel electrophoresis on a 1.2% agarose/TBE gel. Lane (A) PCR product 5, (B) product 4, (C) product 1 and (D) λDNA.Hin*dIII *marker.*

PCR products 2 and 3 were not successfully cloned. Many blue colonies were present on the culture plates which suggests that there was a problem with the ligation of PCR product into plasmid. It has been shown that as insert size increases, the efficiency of ligation decreases and in turn this lowers the transformation efficiency. This may have been a contributing factor in the unsuccessful cloning of products 2 and 3 which were 1.7 kb and 1.95 kb respectively.

8.2.4. Sequencing of the rabbit CD18 PCR products

The PCR product inserts were sequenced using the Sequenase® Version 2.0 System (section 2.9.5). The system is based upon the chain termination method first described by Sanger *et al.*, (1977). This method involves the synthesis of a new DNA strand by DNA polymerase from a single stranded DNA template. DNA synthesis is primed by oligonucleotides which anneal to a site within the DNA template. Nucleotides (deoxyribonucleoside 5' triphosphates, dNTP's) are continually added to the 3'-OH terminus of the new strand until further extension is inhibited by incorporation of a

nucleotide analog (di-deoxynucleoside 5'-triphosphates, ddNTP's) in place of a nucleotide. The nucleotide analogs lack the 3' hydroxy-terminus needed to form the next phophodiester bond and DNA synthesis is terminated. By using a mixture of three dNTP's and one ddNTP, DNA synthesis will be inhibited where the ddNTP can be incorporated. Hence, a mixture of DNA chains of varying lengths are produced which all have the same ddNTP at the 3' terminal. Four sets of reactions, one for each of the four nucleotides, are performed and complete sequence information is gained following high resolution electrophoresis and autoradiography.

The sequences of the PCR products were compared with CD18 from other species using the Macvector program. The sequence of PCR product 4 did not align to CD18 cDNA sequences from other species. However, the sequences of PCR products 1 and 5 were homologous to the known CD18 sequences (figure 8.4). This confirmed that rabbit CD18 DNA was amplified during the original PCR. These PCR inserts were used as rabbit CD18 probe 1 (PCR product 1) and probe 2 (PCR product 5).

Bacteria transformed with PCR product 1 or product 5 were grown overnight, recombinant DNA was isolated and digested with *Hin*dIII and *Not*I (as described in section 2.8). The digest was analysed by gel electrophoresis and the PCR insert, released as a single fragment, was isolated using Geneclean II (section 2.8.3). To confirm recovery and purity, Geneclean II samples were analysed by agarose gel electrophoresis (figure 8.3). The PCR inserts (50ng) were randomly labelled with [α^{32}P]dCTP using the ^{T7}Quick Prime Kit to generate the rabbit CD18 probes 1 and 2.

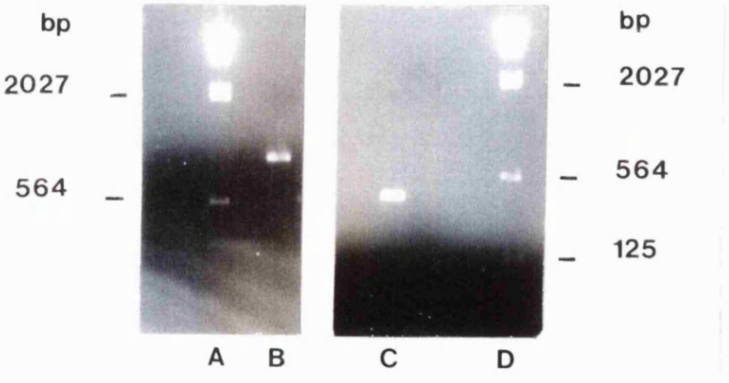

Figure 8.3. *Analysis of Geneclean products by gel electrophoresis. Lanes (A and D) λDNA.HindIII marker, lane (B) rabbit CD18 PCR product 1 and lane (C) rabbit CD18 PCR product 5. Samples were analysed by agarose gel electrophoresis on a 1.2% agarose/TAE gel.*

Rabbit CD18 probe 1

```
      797
5'      C   CTGGACGCCC  CTGAGGTGGG  CTGGACGCAT  GATGCAGGTC  Bovine CD18
         -  ----------  ----------  ----------  ----------  PCR Product

                                                      886
GCCGCGTGCC  CGGAGAAATC  GGCTGGCCGA  ATGTCACGCG  GCTGCTGGTG ..3'
----T-----  ----------  -------GC-  ----------  .....
```

Rabbit CD18 probe 2 (SP6 primer)

```
    446
5' GCGTTCA  AACGTGACCT  TCCGACGGGC  CAAGGGCTAC  CCCATCGACC  Bovine CD18
   -------  ----------  -----G----  -----CG---  -----T----  PCR Product

                                                    546
TGTACTACCT  GATGGACCTC  TCCTATGGTG  GATGACCTCG  TCAACGTCAA ...3'
----------  ---------   -------C-C  --C------A  ---------G
                                               ctcca
```

Rabbit CD18 probe 2 (T7 primer)

```
            1290
3'          ACAGAAGGAG  TCGTAGGTGA  CTTTCAGGGT  GTCAGGGAGG  Bovine CD18
            G---------  ----------  G---G----A  ------C---  PCR Product

GTGCTGTGAT  CCAGGAAGAC  TCTGGAGGAC  AGTTTATTGT  AGGCTTCTTG  Bovine CD18
-C------C-  ----------  C-G---C---  -----GC---  ----G-----  PCR Product
                                          1147
ATAAGCTCCA  CACGTTCCTG  GAGACTCAGA  CAGCTCC ....5'          Bovine CD18
---G---G--  ------G---  ---T----AG  ------- ....           PCR Product
```

Figure 8.4. Partial nucleotide sequence of rabbit CD18 probes. PCR products were cloned into the pCR™ II plasmid and sequenced using the UBS Sequenase system. Product 1 (450 bp) was sequenced using the T7 primer and product 5 (750 bp) was sequenced using both SP6 and T7 primers. Primer sequence is shown in bold. Sequencing reactions were run on a 6% poly-acrylamide sequencing gel, the gel was stopped when the first dye front reached 2cm from the end of the gel.

8.2.5. Screening the rabbit spleen cDNA library

After screening 26 000 colonies of a rabbit spleen cDNA library (as described in 2.10), with a rabbit CD18 probe 1, and 39 000 colonies with probe 2, 16 hybridising colonies were selected. Following three rounds of re-screening a single positive clone, 32.2, was isolated. This was grown overnight and the next day plasmid DNA was purified and digested with *Eco*RI or *Bst*XI (section 2.8.2). The digest was analysed by gel electrophoresis (figure 8.5). Two fragments of 1.8 kb and 300 bp were released following digestion with *Eco*RI and a single fragment of 2.1 kb was released following digestion with *Bst*XI.

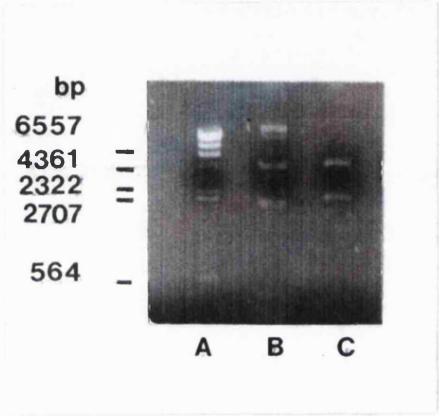

Figure 8.5. *Digestion of plasmid from clone 32.2 with Bst*XI. *Lane (A) λDNA.Hin*dIII *marker, (B) uncut 32.2 plasmid DNA, (C) clone 32.2 plasmid DNA cut with Bst*X1. *Digests were analysed by agarose gel electrophoresis on 1%TBE/gels.*

8.2.6. Rabbit CD18 probe hybridises to the plasmid DNA from clone 32.2

To confirm that the CD18 probe specifically hybridised to clone 32.2, plasmid DNA from clone 32.2 was digested with *Bst*XI and the digest was run on a 1.2% agarose/TAE gel. The DNA was transferred to a nylon membrane overnight (section 2.10.5). The Southern blot was probed with rabbit CD18 probe 1 which had been digoxigen-labelled. The probe annealed to DNA from clone 32.2, but not to control bacterial DNA (figure 8.6). The cDNA insert from clone 32.2 was sequenced on a ABI automated sequencer using SP6, T7 and internal sequence primers by Dr. Bishop Hague, Laboratory of Immunogenetics, NIAID, Bethesda, USA.

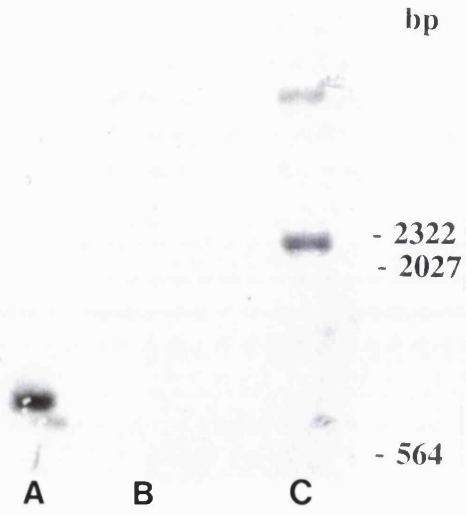

*Figure 8.6. Hybridisation of rabbit CD18 DIG-labelled probe to clone 32.2 plasmid DNA. Lane (A) unlabelled rabbit CD18 probe 2, (lane B) control bacterial DNA and (lane C) clone 32.2 digested with BstX*I.

8.2.7. Rabbit CD18 is homologous to CD18 of other species

Clone 32.2 contained a 2.1 kb insert and was sequenced in both directions. Alignment of rabbit CD18 cDNA sequence with human and bovine sequences, shows that the rabbit sequence lacks the 5' untranslated region and the first 480 bp of the 5' coding region, but covers 1835 bp of open reading frame ending with a stop codon at bp 1835, followed by 240 bp of 3' non-coding sequence. The partial rabbit nucleotide sequence shows a high level of sequence identity with the available CD18 sequences (figure 8.7).

The nucleotide sequence translates to an amino acid sequence of 610 residues. This was compared with the murine, bovine, human and chicken sequences (figure 8.8). The partial rabbit protein sequence shares 82%, 81%, 84% and 58% homology with human (Kishimoto *et al.*, 1987; Law *et al.*, 1987), murine (Wilson *et al.*, 1989; Zeger *et al.*, 1990), bovine (Shuster *et al.*, 1992) and chicken CD18 (Bisland and Springer, 1994), respectively. In the transmembrane domain rabbit CD18 shares 91% sequence identity with human CD18 and 87% identity with bovine and murine CD18. This high level of sequence identity continues into the cytoplasmic domains with rabbit, bovine, murine and human CD18 sharing 93% homology.

The extracellular domain of human CD18 protein contains 56 cysteine residues and these are conserved in the murine, bovine and chicken proteins. The rabbit sequence contains 55 conserved cysteine residues lacking the cysteine at position 447. However, the rabbit sequence contains two extra cysteine residues at positions 662 and 680 (bovine alignment). There are 6 potential N-glycosylation sites in human CD18 and of these, 5 are conserved between murine, bovine and chicken CD18. The chicken sequence has a sixth potential N-glycosylation site (residue 362, figure 8.8). The partial rabbit CD18 sequence contains three of these conserved residues. The first two conserved N-glycosylation sites are located at residues 50 and 116 in the bovine sequence and this region is absent from the partial rabbit sequence.

Partial cDNA Sequence of Rabbit CD18

```
                                                        527
ATCACCGAGT  CCGGCCGTGT  CGGCTTCGGG  TCCTTCGTGG  ACAAGACGGT  Rabbit
----------  -G-----CA-  T--T------  ----------  ----------  Bovine
----------  -T-----CA-  ------T---  --G--T----  ----------  Mouse
----------  -------CA-  T---------  ----------  -------C--  Human
-C---ACCC-  -TC----CA-  A--T--T--T  -----T----  -------T--  Chick

GCTGCCCTTC  GTCAACACGC  ACCCCGAGAA  GCTGAGGAAC  CCCTGTCCCA  Rabbit
---C------  ----------  ----------  ----C-----  -----C----  Bovine
------T--T  --T-----C-  -T--T-----  ----------  --A-------  Mouse
------G---  -G--------  ----T--T--  ----C-A---  --A--C----  Human
A-----T--T  -------A--  -T--T-----  -----A----  --T--C----  Chick

                                                        627
ACAAGGAGAA  GGAGTGCCAG  GCGCCCTTTG  CCTTCCGGCA  CGTGCTGAAG  Rabbit
----------  ----------  C-C--G--C-  -----A----  ----T-----  Bovine
----------  --CC------  C-C--A----  ----T-----  ------C---  Mouse
----------  A-------C-  C-C-------  -----A----  ----------  Human
-------C-G  TA-T------  C-T-----C-  -----AA---  -A-C--CTCA  Chick

CTGACCAGCA  ACTCCGAGCA  GTTCCGGACC  GAGGTTGGCA  AGCAGCTGAT  Rabbit
--C--TGA--  -----A-A--  ----GA---A  --A--C--G-  ----------  Bovine
T-A---GA--  -----A-C--  ---T-A---A  -----C----  ----A-----  Mouse
-------AGA  -----A-C--  ---T-A----  ----C--G-  ----------  Human
-----TGA--  -TG----GA-  ---TGA--GT  --A--G--G-  -----T-C--  Chick

                                                        727
CTCCGGGAAC  CTGGACGCCC  CCGAGGGCGG  GCTGGACGCC  ATGATGCAGG  Rabbit
---G------  ----------  -T-----T--  ----------  ----------  Bovine
T-----A---  ----------  -T-----T--  ------T---  --A-----A-  Mouse
T-----A---  -----T--A-  -------T--  ----------  ----------  Human
---A------  -----T----  -A-----A--  ------T---  ----------  Chick

TCGCCGTGTG  CCCGGAGGAA  ATCGGCTGGC  GCAATGTCAC  GCGGCTGCTG  Rabbit
-G----C---  ----------  ----------  ----------  CA--------  Bovine
-T--T-CA--  T---------  --T-------  ----------  -A--------  Mouse
------CC--  ----------  ----------  ----C-----  ----------  Human
CG---A----  TGGA--CTTG  --T-------  -------G--  C--AT-----  Chick
```

```
                                                        827
GTGTATGCCA  CCGACGACGG  CTTCCACTTT  GCTGGTGATG  GGAAGCTGGG  Rabbit
----TC----  -G-----T--  G---------  --G--C----  -A--------  Bovine
----T-----  -A-----T--  ----------  ----------  -C--A-----  Mouse
----T-----  -T--T-----  -------T--  --G--C--C-  -A--------  Human
--------A-  -T--T--T--  ---------C  ----------  -C-----C--  Chick

CGCCATCCTG  ACCCCCAATG  ACGGCCAATG  CCACCTGGAG  GGAAACATGT  Rabbit
T--------C  ----------  ------GC--  ---------A  -AC---C---  Bovine
T---------  ----------  -T----GC--  ----------  -AT-------  Mouse
----------  --------C-  ------GC--  T---------  -AC---T---  Human
G-G---TT--  ----------  -T-----G--  ----T-----  --C-------  Chick

                                                        927
ACCGGCGCAG  CACCGAATTC  GACTACCCGT  CGGTGGGGCA  GCTGGCGCAC  Rabbit
--AAAA----  --A------T  --------A-  -------C--  ------A---  Bovine
--AA-A-G--  --AT--G---  --------A-  -C-----T--  ------A---  Mouse
--AA-A-G--  --A-------  --------A-  -------C--  ----------  Human
--AAAAA---  --AT--G--T  -----T--T-  -T--T--C--  -----TC--G  Chicken

AAGCTGGCCG  AGAACAACAT  CCAGCCCATC  TTCGCTGTGA  CCAAGAAGAT  Rabbit
--A-----A-  -A-G------  ----------  --T--A--A-  ----------  Bovine
--A--TT---  ---G------  ----------  --T--G----  -A--------  Mouse
--------T-  -A--------  ----------  -----G----  ---GT-G---  Human
--A--T--T-  -A--------  T--------T  --T-----C-  ---GC-----  Chicken

                                                       1027
GGTGAAGACC  TATGAGAAAC  TCACGGAGAT  CATCCCCAAG  TCGGCCGTCG  Rabbit
------A--G  --C-----G-  -G--A-----  ----------  --T--A----  Bovine
------A--G  ----------  ----------  ----------  --A--A--G-  Mouse
----------  --C-------  ----C-----  ----------  --A-----G-  Human
----G-TGTT  --CA-A----  ---GT--T--  G-----A---  --A--A--A-  Chicken

GGGAGCTGTC  TGAGGACTCC  AGCAACGTGG  TGCAGCTCAT  CAAGGACGCC  Rabbit
----------  ---A--T---  --G-------  --G----T--  ----A-T---  Bovine
----A-----  ---C------  ----------  ----------  ----A-T---  Mouse
----------  ----------  -----T----  -C--T-----  T---A-T--T  Human
-----T--AA  C---------  -------AC-  -TG-A-----  -C---TG---  Chicken

                                                       1127
TACAGCAAAC  TGTCGTCCCG  GGTCTTCCTG  GAGCACAGCG  CCCTGCCTGA  Rabbit
----AT----  ----C---A-  A---------  --T------A  ----C-----  Bovine
---TAT----  -C--C--TA-  A---------  --C------A  ----C--G--  Mouse
----AT----  -C--C---A-  ----------  --T----A--  ----C--C--  Human
----AT--C-  -C--T--A--  -A--A-----  --C---TC-A  -------A--  Chicken
```

```
CTCCCTGAGG  GTCACCTACG  ACTCCTTCTG  CAGTAACGGG  AGATCGCTCC  Rabbit
A------AA-  ----------  ----------  ----------  -A-----AGG  Bovine
A------AA-  --------T-  ----------  ------T--A  GC----AGTA  Mouse
-A--------  ----------  ----------  ---C--T---  T------A-A  Human
TGT----GAT  ----AA--T-  -----A----  --AT---AAC  -C--G-AG--  Chick

                                                      1227
TGGGCCAGCG  CAGAGGGGAC  TGTGACGACG  TGCAGATCAA  CGTCCCGGTC  Rabbit
---A-----C  ----------  --C----G--  -C--------  -------A--  Human
-A---A-ATC  -C-T------  -----T-G--  -A--------  -AA-------  Mouse
G-AA-----C  ------T---  -----T-G--  ----------  T------A--  Human
AA-AATGAA-  -------C-G  --C----AT-  -TA-------  T-ATGA----  Chick


ACCTTCCAGG  TGAAGGTCAC  AGCCTCCGAG  TGCATCCAGG  AGAAGTCGTT  Rabbit
----------  ----------  ----A---T-  ---------C  --C----C--  Bovine
----------  -A-------T  G--T------  --T-------  --C----C--  Mouse
----------  ----------  G-A-CA----  ----------  --C-------  Human
------A-A-  ----------  ---AAAT---  ------A-AA  GCC----C--  Chick

                                                      1327
TGTCATCCGG  GCCCTGGGCT  TCACCGACAC  GGTGACGGTG  CGGGTCCTCC  Rabbit
CAC-------  --G-------  ----G-----  ------C---  ----------  Bovine
----------  --A-----T-  ----T--T--  A-----C---  -A-----GT-  Mouse
----------  --G-------  ----G----T  A-----C---  -A---T----  Human
CAC-------  C---------  ----A-----  -C-C--T---  -ACC-GGA-A  Chick


CCCTGTGTGA  GTGCCGGTGC  CGGGACCAGA  GGCAGGAGCG  TGGCCTGTGC  Rabbit
---A---C--  -----AA---  ------GCC-  -CAG---CG-  CA--A-C---  Bovine
---A------  ---T-A----  ----------  -T-G-----A  GA-T--C--T  Mouse
---A------  ----------  ----------  -CAGA--C--  CA----C---  Human
G-TAC-----  C--TGAC---  A-A--G----  -C-GT--TCA-  AACTGCC---  Chick

                                                      1427
GGGGGCAAGG  GCTCCATGGA  GTGCGGCGTC  TGCAGGTGCG  ACGCCGGCTA  Rabbit
--C----GA-  ----G-----  ----------  --------T-  ----------  Bovine
--A--------  -AGT------  ---T--TA--  --------T-  -GT-T-----  Mouse
CAT-------  ---T-T----  -------A--  --------T-  --A-T-----  Human
A-T--A--T-  --AAGG-C-T  C--T--GA--  -----T---A  -TTTGA----  Chick


CACCGGGAAG  AACTGTGAGT  GCCAGACGCA  GGGCAGGAGC  AGCCAGGAGC  Rabbit
--T-------  -----C----  ----------  ----C-----  ----------  Bovine
--TT-----A  ----------  -------T--  ---TC-----  ----------  Mouse
--TT-----A  ----------  -------A--  ----C-----  ----------  Huamn
---G------  ----------  -TG-C--C--  A----A--C-  ---A-A----  Chick
```

```
                                                          1527
TGGAGGGAAG  CTGCCGGAAG  GACAACAGCT  CCATTGTCTG  CTCGGGGCTG  Rabbit
-------C--  ------C---  ----------  ----CA----  ----------  Bovine
-----A---A  ---T------  -----T--T-  ----C--G--  ---A-----T  Mouse
----A-----  ----------  -------A--  ----CA----  ---A------  Human
-------C--  ----------  ----------  --G-CA----  ---A------  Chick

GGGGACTGCA  TCTGCGGGCA  GTGCGTGTGC  CACACCAGCG  ACAACCCCAA  Rabbit
----------  ----------  ----------  -----G----  --GTG-----  Bovine
----------  ----T-----  ---T--A---  --T-----T-  --GT------  Mouse
--------TG  ----------  ----G----C  ----------  --GT----GG  Human
---------G  -G--T-----  ------C---  --------T-  --GTA--TGG  Chick

                                                          1627
CAAGCAGATC  TTCGGGCGGT  ACTGCGAGTG  CGACAACATG  AACTGTGAGC  Rabbit
----A-----  -A---C-A--  T---------  -------G-C  -----C--A-  Bovine
---AG-----  --T----AA-  ----------  TG----TG-C  ---------A  Mouse
-----T---A  -A-----G--  ----------  T----C---C  ----------  Human
---GG-----  -AT--CACC-  T------C--  T---------  -----C---T  Chick

GCTACGACGG  CCAGGTCTGC  GGTGGCAAAA  AGAGGGGGTC  CTGCTCCTGC  Rabbit
----------  ---A------  --G---G---  --------CT  -----T----  Bovine
-A--TA--A-  ---A------  ------TC-G  -TC----T--  ----AA---T  Mouse
-----A----  ----------  --C---GCCG  G-------CT  -----T----  Human
TTC--A----  -TCAC-G--T  ------G-GG  --C-T--ACG  A---GA---T  Chick

                                                          1727
GGCCAGTGCC  ATTGCAACGA  CGGCTTCGAG  GGTTCGGCGT  GCCAGTGCAA  Rabbit
---ACC---A  GG---G----  GCAG-AT---  --C-----A-  --------CT  Bovine
---A-A--TA  G------GCC  ---T-A----  --CT----C-  -------TC-  Mouse
--GA------  GC---CA-CC  G-----T---  --C--A----  --------G-  Human
--TG-----A  -G----CACC  -AAG-A----  -GCAGT--C-  ----A-----  Chick

GAAGTCCACG  GACGGCTGCC  TGGATGCGCA  GGGCGTGGAG  TGCAGCGGCC  Rabbit
C--------T  C-G-------  -CA-CTT-G-  C-----C---  ----------  Bovine
--G------C  ACG-----T-  --A----A-G  -CTG--A---  -----T----  Mouse
--G-A----T  --G-------  --A-CC---G  TC-T--T---  --T--T--T-  Human
------G--T  --T-----TA  G-A-CAGC-G  -CAAAAT--A  ------CTG-  Chick

                                                          1827
GTGGACAGTG  CCGTTGCAAC  GTGTGTGTGT  GCAAGAAGGG  GTACCAGCCG  Rabbit
-C--C-GA--  ---C-----T  -----CCA--  --G-CCCC--  C---------  Bovine
----C--C--  --AA------  AG---CA-A-  -TG-CGAA--  C--------A  Mouse
----C-G---  ---C------  --A--C-A--  --C-TTCA--  C-------T-  Human
----CTCC--  --CA------  CGC--CA---  --CGAGG---  C--------C  Chick
```

```
CCCATGTGCC  AGGAGTGCCT  GGGCTGCACC  TCGCCCTGCG  GCCAGTACAT  Rabbit
---C-----A  GC-------C  -------C--  GT------T-  CGGGCTT-GC  Bovine
--G-----TG  ----T--T-C  CA----TGG-  ---A----A-  -GG-CA--CA  Mouse
--TC------  ---------C  C------C--  --A-----T-  --A-------  Human
---T-C---G  -------T-C  T-----TGC-  --T-A--C-T  -TGGCAGGCA  Chick

                                                      1927
CTCCTGCATT  GAGTGCCTGA  AATTCGACAA  GGGCCCCTTT  GGGAAGAACT  Rabbit
-C------CA  ----------  -G--------  ---------C  -CC-------  Bovine
-A---CTTG-  ----------  -G--T--T--  ------T---  -A--------  Mouse
-------GCC  ----------  -G-----A--  ----------  ----------  Human
-ATT-C-TGC  ------AA-T  C----A-T-G  T--G--AC-G  -CA-------  Chick

GCAGCACCCA  GTGCAAAAAC  CTGCAGCTGG  TGKCCACTGC  GGGGCAAGAC  Rabbit
-----G-AGC  ----GGGC-G  AC-A-----C  --T---GCC-  --T--CC-G-  Bovine
-T--TGTT--  ---TGCTGGT  A--AC----C  A-A-T-TCC-  TTT-A-GA-A  Mouse
-----G-GGC  G--TCCGGG-  ---------T  C-AA--ACC-  C-T-A-G-G-  Human
--TCTGTGGC  C----CC-G-  A-C-------  ---TG-GCC-  GGGCAGG-GT  Chick

                                                      2027
TGCAAGTGCA  AGGAGCAAGA  CTCGGAGGGC  TGCTGGATGG  CCTTCACCCT  Rabbit
C---------  ------GC--  ---C------  ---------A  ---A------  Bovine
AAGCCC----  -----A-G--  ------A---  --T-----AA  -T-A---TT-  Mouse
A-G-CC----  -----AGG--  ---A------  -----CG---  ---A---G--  Human
C-GC------  -----A-G--  ---T--GAA-  --------CT  -T---TATA-  Chick

GTGGCAGCGC  CAAGGGATGG  ACAACTATGA  TGTCCACGTG  GAGGACAGCC  Rabbit
-GT-------  G-C---CG--  ---GA--C--  C--G------  --C----TG-  Bovine
-CA----AAG  G-C--A-G-A  ---TT--CA-  CA----T---  --------T-  Mouse
-GA-----AG  G-C-------  --CG---CCT  CA--T-T---  --T--G----  Huamn
-GCC--AGAT  G-T--AGA-A  -G-TG--CAC  C---ACT--T  --CCCT-AGA  Chick

                                                      2127
GAGAGTGTGT  GCAGGGCCCC  AACATCGCTC  CCATCGTCGG  GGGCACCGTG  Rabbit
TC--------  -A--------  --------C-  -------G--  ----------  Bovine
T---------  -A--------  --TG-G--T-  -------A--  ----------  Mouse
----------  ----------  --------C-  ----------  ----------  Human
A------CCC  AG--CCT---  --------GC  TG-----A--  CA-----A-T  Chick

GCGGGCGTTG  TGCTCATCGG  CGTCCTCCTG  CTGGTCATCT  GGAAGGCACT  Rabbit
-G------C-  -----G----  -A--------  ----------  -------C--  Bovine
-TA--T--C-  -A--G--T--  T--------C  ----------  -------C--  Mouse
--A---A-C-  ----G-----  -A-T------  ----------  -------T--  Human
--C--T--G-  CT-----T--  -C-G--G--C  ---C-G-C--  --CG--TCT-  Chick
```

```
                                                        2227
GACCCACCTG  AGTGACCTCC  GGGAGTACAG  GCGCTTTGAG  AAGGAGAAGC  Rabbit
---A------  --C------A  --------CA  T---------  ----------  Bovine
----------  -C-------A  ----------  ----------  --------A-  Mouse
--T-------  --C-------  ----------  ----------  ----------  Human
---AG-GA-C  TT-----G--  -A--A---C-  CA-G------  --------AT  Chick

                                                        2277
TCAAGTCCCA  GTGGAACAAT  GATAACCCGC  TTTTCAAGAG  TGCCACCACC  Rabbit
----------  ---------C  --------T-  ----------  ---------G  Bovine
----------  A---------  --C-----C-  -C--------  ---T--G--A  Mouse
----------  ----------  ----T---C-  ----------  C---------  Human
C----G--A-  -------GC-  -----T--T-  -G--------  ----------  Chick

                                    2310
ACGGTCATGA  ACCCGAAGTT  TACCCAGAGT  TAG   Rabbit
--A-------  ----T-----  -G--G-----  ---   Bovine
----------  ----C-----  -G-TG-A--C  ---   Mouse
----------  ----C-----  -G-TG-----  ---   Human
--C-------  -T--C-GA--  -GATGG-CAA  ---   Chick
```

Figure 8.7. *The partial nucleotide sequence of the rabbit CD18 subunit cDNA compared with the sequences for bovine (Shuster et al., 1992), murine (Wilson et al., 1989; Zeger et al., 1990) human (Kishimoto et al., 1987; Law et al., 1987) and chicken (Bilsland and Springer, 1994) CD18. The transmembrane region is double underlined. The numbering system is taken from the bovine sequence (Shuster et al., 1992).*

Partial amino-acid sequence of rabbit CD18

```
                                                                  202
      EITE  SGRVGFGSFV  DKTVLPFVNT  HPEKLRNPCP  NKEKECQAPF  Rabbit
      ----  ---I------  ----------  ----------  -------P--  Bovine
      ----  ---I------  ----------  ----------  -------P--  Mouse
      ----  ---I------  ----------  --D-------  -------P--  Human
      -T-P  -R-I------  ----------  -----K----  --DSN--P--  Chick

                                                                  252
AFRHVLKLTS  NSEQFRTEVG  KQLISGNLDA  PEGGLDAMMQ  VAVCPEEIGW  Rabbit
---------D  --K--E----  ----------  ----------  --A-------  Bovine
---------D  --N--Q----  ----------  ----------  --A-------  Mouse
---------N  --N--Q----  ----------  ----------  --A-------  Human
--K-I-S--D  -A-K-ES---  --F-------  ----------  A---GLD---  Chick

                                                                  302
RNVTRLLVYA  TDDGFHFAGD  GKLGAILTPN  DGQCHLEGNM  SRRSTEFDYP  Rabbit
----------  ----------  ----------  --R----D-L  YKS--N----  Bovine
----------  ----------  ----------  --R----D--  YK---N----  Mouse
----------  ----------  ----------  --R----D-L  YK---N----  Human
----------  ----------  ----G-----  --Q----D--  YKK--N----  Chick

                                                                  352
SVGQLAHKLS  ENNIQPIFSV  SKKMVKTYEK  LTEIIPKSAV  GELSEDSSNV  Rabbit
---------A  -S------A-  T---------  ----------  ----------  Bovine
----------  -S------A-  T---------  ----------  ----D-----  Mouse
---------A  --------A-  TSR-------  ----------  ----------  Human
-----VQ--A  --------A-  TS---DV-K-  -SDM------  ---N-----I  Chick

                                                                  402
VQLIKDAYSK  LSSRVFLEHS  ALPDSLRVTY  DSFCSKG.TL  LGQRRGDCDD  Rabbit
-E---N--N-  -------D--  T---T-K---  -----N-.-Q  VD-P-----G  Bovine
-----N--Y-  -------D--  T---T-K---  -----N-ASS  I-KS-----G  Mouse
-H---N--N-  -------D-N  T---T-K---  -----N-V-TH  RN-P-----G  Huamn
IE--QV--NN  ----II-D--  T---V-D-K-  --I-NNNG-A  KNEA--Q--N  Chick
```

```
                                                                452
VQINVPVTFQ  VKVTASECIQ  EKSFVIRALG  FTDTVTVRVL  PLCEYRCRDQ  Rabbit
------I---  -----T----  QQ--T-----  ----------  -Q--CQ---A  Bovine
------I---  ---M------  Q--V------  -------Q-R  -Q--CQ----  Murine
------I---  -----T----  -Q--------  ---I---Q--  -Q--C-----  Human
-K--DE---K  -----NE--K  SQS-T-----  ----L--HLD  SI-DCD--E-  Chick

                                                                502
RQERGLCGGK  GSMECGVCRC  DAGYTGKNCE  CQTQGRSSQE  LEGSCRKDNS  Rabbit
SRDGSI---R  ----------  ----I-----  ----------  ----------  Bovine
SR-QS-----  -V----I---  -S--I-----  ----------  --RN------  Murine
SRD-S--H--  -FL---I---  -T--I-----  ----------  ---------N  Human
-PDPTA----  -KVV--I-S-  NLS-------  -D-K-KT-K-  ----------  Chick

                                                                552
SIVCSGLGDC  ICGQCVCHTS  DNPNKQIFGR  YCECDNMNCE  RYDGQVCGGK  Rabbit
--I-------  ----------  -V---K-Y-Q  F-----V---  ---------E  Bovine
----------  ----------  -V---E----  ------V---  --NS-----S  Murine
--I-------  -----L----  -V-G-L-F-Q  -----TU---  --N------P  Human
-VI-------  V---------  -V-G-E-Y-T  F-D-------  FHN-SL---E  Chick

                                                                602
KRGSCSCGQC  HCNDGFEGSA  CQCKKSTDGC  LDAQGVECSG  RGQCRCNVCV  Rabbit
---L-F--T-  R-DEQY----  ---L---Q--  -NLD------  --R------Q  Bovine
D----N--T-  S-KP-Y----  ---QR--T--  -N-RL-----  --H-Q--R-I  Murine
G--L-F--T-  R-HP------  ---ERT----  -NPRR-----  --R------E  Human
E--R-D--E-  K-TPKY----  ----------  RNQRQN---L  --S-P--R-Q  Chick

                                                                652
CKKGYQPPMC  QECLGCTSPC  .GQYISCIECL  KFDKGPFGKN  CSTECKNLQL  Rabbit
-DP-----L-  S--P--PV--  .AGFAP-T---  -------A--  --AA-GQTK-  Bovine
-DE-------  ED-PS-GSH-  RDNHT--A---  -------E--  --VQ-AGMT-  Murine
-HS---L-L-  ---P--P---  .K-----A---  --E-------  --AA-PG---  Human
-RG-----F-  E--P--P---  .GRH---V--K  S-NS--LA--  --VA-TSI--  Chick

         *                      *                      702
VDVALRGKTC  KTCKEQDSEG  CWMAFTLCQR  QGMDNYDVHV  EDSRECVQGP  Rabbit
LSSPVP--GR  -K---R----  ---TY--V--  D-R-R-----  D-ML---K--  Bovine
QTIP-----K  -P---R----  --ITY--Q-K  D-RNI-NI--  ---L---K--  Murine
--SNNPV--G  R----R----  --V-Y--E-Q  D---R-LIY-  DE-----A--  Huamn
A--EP--AGS  RQ---K---N  --IS-YMA-D  D-EEM-T-T-  DPKK--PEP-  Chick
```

```
                                                                752
NIAAIVGGTV  AGVVLIGVLL  LVIWKALTHL  SDLREYRRFE  KEKLKSQWNN  Rabbit
----------  G----V-I--  ----------  ---H------  ----------  Bovine
-V--------  V---------  ---------T  ----------  ----------  Murine
----------  --I----I--  -------I--  ----------  ----------  Human
-L---S-IA-  -A-I-L---L  T-RL--EIF-  R---------  -S-AK--EDN  Chick

                  769
DNPLFKSATT  TVMNPKFTQS  Rabbit
----------  -------AE-  Bovine
----------  -------AE-  Murine
----------  -------AE-  Human
----------  -----R-DGQ  Chick
```

Figure 8.8. *The deduced amino acid sequence of rabbit CD18 compared with bovine, murine, human and chicken sequences. The conserved cysteine residues are shown in bold type, cysteine residues marked with*, are found in the rabbit sequence only and potential N-glycosylation sites are underlined. The transmembrane region is double underlined. The numbering system is taken from the bovine sequence (Shuster et al., 1992).*

8.2.8. Preparation of rabbit CD11c probes

To produce a probe which would hybridise to rabbit CD11c, two oligonucleotides primers (oligonucleotides CD11c 1: 5'→3' attgtcatcactgatgg and CD11c 2: 3'→5' gtcataaaacccgtccg) were prepared based upon the amino-acid sequence of rabbit CD11c tryptic peptides (section 6.2.6). A second set of oligonucleotide primers were designed from the transmembrane and metal-binding domains of the human CD11c sequence. In other integrin CD11 sub-units, these regions have been shown to be highly conserved between species (oligonucleotides CD11c 3: 5'→3' tggagaaggtggtggtgtgat and 4: 3'→ 5' ctcacggttcgtccttgtctgg).

Oligonucleotides 1 and 2 were used to amplify DNA fragments from rabbit spleen cDNA by PCR as described in section 2.9.2. Figure 8.9 shows the results of the PCR. A major product of approximately 1000 bp (product CD11c 1) was obtained. In addition, a product of approximately 830 bp (product CD11c 2) was also amplified, although the amount of obtained was considerably less than product 1. To increase the specificity of the PCR, the annealing temperature was increased from 52°C to 54°C. At this temperature, the reaction was inhibited.

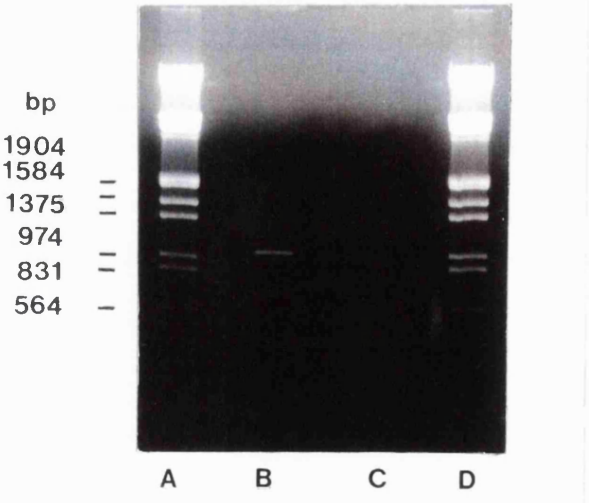

*Figure 8.9. Amplification of DNA fragments from a rabbit spleen cDNA library using oligonucleotides CD11c 1 and 2. Lane (A) λDNA.Hin*dIII,*Eco*RI *marker, (lane B) PCR CD11c products 1 and 2, (lane C) control PCR (oligonucleotides CD11c 1 and 2, no template) and (lane D) λDNA.Hin*dIII,*Eco*RI *marker, 9μl of PCR product, mixed with 1μl loading buffer was analysed on a 1% agarose/TAE gel.*

The two rabbit CD11c PCR products were gel purified (section 2.8.4) and ligated into pCR™II plasmid and the recombinant molecules were used to transform competent cells (sections 2.9.3 and 2.9.4). Transformants were selected and plasmid DNA was isolated from overnight cultures, digested with *Eco*RI and analysed by agarose gel electrophoresis (figure 8.10). The PCR products were successfully cloned. Plasmid DNA from the transformed cells was sequenced using the T7Sequencing system. The sequence obtained did not match to the human CD11c sequence.

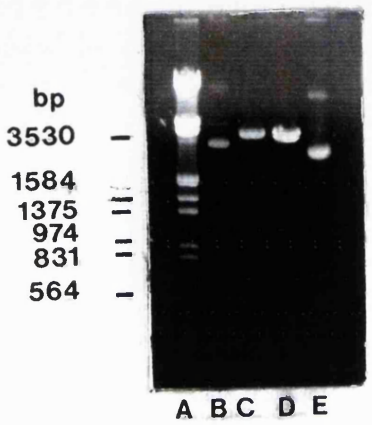

Figure 8.10. *PCR products CD11c 1 and 2 were cloned using the TA-Cloning system. Plasmid DNA was isolated from cells transformed with (lane B) PCR product 1, uncut, (lane C) PCR product 1, digested with EcoRI, (lane D) PCR product 2, digested with EcoRI and (lane E) PCR product 2, uncut, 9μl of PCR product, mixed with 1μl loading buffer was analysed on a 1% agarose/TAE gel. Lane (A) λDNA.HindIII,EcoRI marker.*

Oligonucleotides CD11c 3 and 4 were used to amplify CD11c cDNA fragments from human spleen cDNA and rabbit spleen cDNA (figure 8.11). A single product of 1.9 kb was amplified from the human (product H1) and rabbit (product R1) templates. These products were cloned using the TA cloning® kit and transformed cells were plated out and grown overnight (section 2.9.3 and 2.9.4). Several white colonies were selected from each transformation and grown overnight in liquid culture. Plasmid DNA was isolated using standard methods (section 2.8.1) and digested with *Eco*RI. Clones H1 and H2, from cells transformed with PCR product H1, contained inserts of 1.8 kb as did clones R1 and R2, from cells transformed with PCR product R1. The inserts were sequenced using the Sequenase® System Version 2.0. The results of this

experiment are shown in figure 8.12. The sequence obtained from clone H1 aligned to the human CD11c cDNA and was used as a CD11c probe to screen a rabbit CD11c library. Only plasmid sequence was obtained from sequencing clones R1 and 2, suggesting that there was a problem with the transformation experiment.

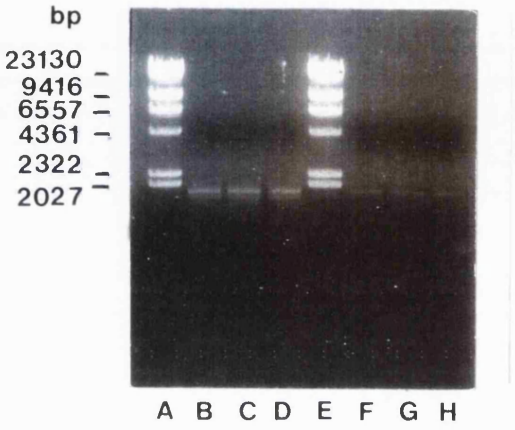

Figure 8.11. Amplification of DNA fragments from (lanes B-D) human spleen cDNA and (lanes F-H) rabbit spleen cDNA using rabbit CD11c oligonucleotides 3 and 4, 9μl of PCR product, mixed with 1μl loading buffer was analysed by agarose gel electrophoresis on a 1.2%/TAE gel. Lanes (A and E) λ.HindIII.DNA marker.

Clone H1 aligned to human CD11c cDNA

```
3230
GTCCGCCAGA  TATTGCAGAA  GAAGGTGTCG  GTCGTGAGTG  TGGCTGAAAT  Human CD11c
 ---------  ----------  ----------  ----------  ----------  Clone H1

TACGTTCGAC  ACATCCGTGT  ACTCCCAGCT  TCCAGGACAG  GAGGCATTTA  Human CD11c
----------  ----------  ----------  -----.----  ----------  Clone H1

TGAGAGCTCA  GACGACAACG  GTGCTGGAGA  AGTACAAGGT  CCACAACCCC  Human CD11c
----------  ----------  ----------  ----------  ----------  Clone H1
      3389
ACCCCCCTCA  Huamn CD11a
-------     Clone H1
```

Figure 8.12. The insert from clone H1 was sequenced using the Sequenase® System version 2.0 and the sequence was compared with the human CD11c cDNA (Corbi et al., 1987).

8.2.9. Screening of Rabbit spleen cDNA library with a human CD11c probe

Clone H1 was grown in culture overnight, plasmid DNA was isolated and digested with *Eco*RI and the insert was gel purified (described in section 2.8) and randomly labelled with [α^{32}P]dCTP using the ^{T7}Quick Prime kit to generate the probe. After screening 30 000 colonies from a rabbit spleen cDNA library with the human CD11c probe four hybridising colonies were found. However, the CD11c probe did not hybridise to these colonies during a second round of screening. Insufficient time was available to continue screening the rabbit spleen cDNA library.

8.3. Discussion

After screening a rabbit spleen cDNA library with a rabbit CD18 probe, a single hybridising clone was isolated and shown to have an insert of 2.1 kb. This was sequenced and compared with CD18 sequences from other species. The clone codes for 1835 bp of open reading frame and 240 bp of 3' untranslated sequence but lacks the 5' untranslated sequence and the first 480 bp of 5' coding sequence. Re-screening the spleen library using the insert from clone 32.2 as a probe may identify a full-length clone. An alternative approach might be to use a known full-length CD18 cDNA as a probe. Previously, clones encoding the complete cDNA sequence of murine and chicken CD18 were isolated using a probe derived from the complete human CD18 cDNA sequence, and clones encoding bovine CD18 cDNA were isolated using the full-length murine CD18 cDNA as a probe.

The predicted protein sequence of the partial CD18 cDNA clone contains 610 amino acids. The human and bovine CD18 cDNA sequences translate into a product of 769 amino acids (Kishimoto *et al.*, 1987; Law *et al.*, 1987; Shuster *et al.*, 1992). The mouse sequence codes for an extra amino acid at residue 623 and therefore contains 770 residues (Wilson *et al.*, 1989; Zeger *et al.*, 1990), while the chicken CD18 sequence contains only 748 residues (Bilsland and Springer, 1994). The partial rabbit sequence shares 82% identity with the human sequence and this supports the previous finding that a number of anti-human CD18 mAb bind to rabbit CD18.

The mature human protein contains 56 cysteine residues and these are conserved in the bovine, murine and chicken sequences. Thirty-seven of these cysteine residues are found within a cysteine rich region which stretches from residues 449-628 in the human sequence (Kishimoto *et al.*, 1987). This region is also conserved in the β_1 and β_3 subunits in the human (Kishimoto *et al.*, 1987). The rabbit sequence lacks cysteine 447, but contains two novel cysteine residues at 662 and 680. The cysteine residues present in CD18 are thought to be important for tertiary structure. Although it is possible that rabbit CD18 tertiary structure differs from that in other species, given the overall level of sequence conservation, any differences are likely to be minimal and do not seem to affect the ability of mAb raised against human CD18 to block the function of rabbit CD18 *in vitro* or *in vivo* (Galea-Lauri *et al.*, 1993; Nourshargh *et al.*, 1989). Chicken CD18 has been expressed as a functional heterodimer with human CD11a and CD11c on the surface of COS cells (Bilsland *et al.*, 1994). If a full-length rabbit CD18 cDNA is obtained, it will be interesting to determine whether rabbit CD18 can associate with human CD11 proteins.

The transmembrane and cytoplasmic domains of CD18 are highly conserved across species. Mutational studies using human CD18 have shown that the conserved cytoplasmic domain of CD18 is involved in the regulation of CD11a/CD18 binding to ICAM-1 (Hibbs *et al.*, 1991a; Hibbs *et al.*, 1991b). Deletion of a phenylalanine at position 677 and three threonines from 758-760 inhibits CD11a/CD18 and ICAM-1 interactions. These four residues are conserved in the rabbit sequence.

A full-length cDNA clone which encodes human CD11c has previously been isolated and sequenced (Corbi *et al.*, 1987). Recently genomic clones which encode for the complete CD11c protein have been isolated and used to determine the genomic organisation of the CD11c gene (Corbi *et al.*, 1990). Sequence data for CD11c from other species has not been described although amino acid sequencing of tryptic peptides obtained from immunoaffinity purified rabbit CD11c revealed sequence identity between human and rabbit CD11c (figure 6.14). Oligonucleotides designed from human CD11c cDNA were able to amplify products of identical length by PCR from rabbit and human templates and the human product was sequenced and aligned to CD11c. Despite the positive PCR result, a clone encoding rabbit CD11c was not isolated. Time restrictions

limited the number of colonies screened to approximately 30 000. It is likely that by screening more colonies a CD11c cDNA clone will be identified. Other workers have isolated human CD11c cDNA from libraries constructed from PMA-stimulated HL-60 cells (Corbi *et al.*, 1987) or cDNA prepared from PMA-stimulated U937 cells (Ortlepp *et al.*, 1995). Stimulation of these cells with phorbol ester results in high surface expression of both CD11b/CD18 and CD11c/CD18 which is associated with increased mRNA expression and protein synthesis. Since only 50% of spleen cells express CD11c/CD18, a more productive approach to cloning rabbit CD11c may have been to construct a library from BJ/873 cells which constitutively express high levels of CD11c.

CHAPTER NINE

GENERAL DISCUSSION

Cells of various haemopoietic lineages, or at different developmental stages within a lineage, express distinct patterns of antigens at the cell surface. The development of monoclonal antibodies which recognise these surface antigens has been of immense importance for defining leucocyte subsets, for functional studies and for biochemical characterisation of the antigen. More recently, the techniques of molecular biology have resulted in the determination of the complete primary structure of many of these antigens and allowed their function to be assessed in different cellular contexts. Over one hundred human leucocyte antigens have been characterised, and many of the murine and rat equivalents of these identified. The immune system of the pig has emerged as a useful model for human transplantation biology and a panel of anti-porcine mAb have been developed (Pescovitz *et al.*, 1984; Pescovitz *et al.*, 1985; Hildreth *et al.*, 1989). Similarly, the discovery of bovine and canine forms of leucocyte adhesion deficiency (Muller *et al.*,1994; Giger *et al.*, 1987) has prompted the development of mAb which react with cattle (Splitter and Morrison, 1991) and dog (Danilenko *et al.*, 1992; Danilenko *et al.*, 1995) leucocytes. Despite the potential use of the rabbit as an experimental animal for studying many diseases, few rabbit leucocyte surface antigens have been identified. During this study, a panel of anti-rabbit leucocyte mAb have been produced and their antigens characterised. Several of these mAb react with cell adhesion molecules and block leucocyte adhesion function both *in vitro* and *in vivo*.

One well characterised model of a human disease process is antigen induced arthritis in the rabbit, initiated by injection of ovalbumin into the knee joint of sensitised rabbits, which resembles human rheumatoid arthritis (RA) in terms of immunopathology, disease chronicity and histological appearance. This model has been shown to be dependent on a cellular immune response (Hunneyball, 1984). Initial work was directed towards an investigation of the role of T lymphocytes in the rabbit RA model, attempts were made to generate mAb which could be used to distinguish T lymphocyte subsets and interfere with T lymphocyte function *in vivo*. Molecules of particular interest were CD4 and CD8. mAb R9/96 was raised against the RL-5 cell line and shown to

recognise the rabbit CD4 homologue. At the same time, Kotani *et al.*, (1993) described the production of a second anti-rabbit CD4 mAb, Ken-4. Both mAb immunostained frozen sections of rabbit tissue and could be used in immunohistochemical investigations to determine the localisation of $CD4^+$ T lymphocytes in inflamed synovial tissue. Rabbit models of AIDS (Varnier and Kindt, 1992) and adult T cell leukaemia (ATL) (Seto *et al.*, 1988) have been developed and mAb R9/96 can be expected to be useful for studying the infection of rabbits with the retroviruses HTLV-1 and HIV-1, which preferentially infect T cells expressing CD4. The ability of mAb R9/96 to inhibit $CD4^+$ T lymphocyte functions, such as cell proliferative response to allogenic cells, has not been determined in this study and so is not clear whether R9/96 would be useful for *in vivo* investigations concerned with blocking $CD4^+$ T lymphocyte function. mAb R9/96 is an IgM class antibody and may possibly be used to deplete $CD4^+$ lymphocytes from mixed leucocyte pools by complement-mediated killing. Alternatively, pure populations of $CD4^+$ lymphocytes could be isolated using magnetic beads coated with mAb R9/96. In further experiments to elicit an immune response to rabbit CD8, eight mice were immunised with the $CD8^+$ BJ/873 cell line and approximately 3000 fusion wells were screened. Antibodies reactive with the CD8 homologue were not detected. This was not a reflection of the screening protocol because any hybridomas which showed reactivity with $CD8^+$ BJ/873 cells, but not $CD4^+$ RL-5 cells, were investigated. It is more likely to be because CD8 is not highly immunogenic and thus does not induce a strong humoral immune response in mice.

During the course of these experiments mAb reactive with the rabbit equivalents of CD43 (BJ4/55), CD44 (R5/23, R9/380, R9/483) and CD45 (R5/151) have been produced. In the human, alternative forms of CD44 and CD45 arise through alternative splicing of their respective genes, and CD43 shows heterogeneity due to variable glycosylation patterns. The anti-CD44 mAb produced during this study recognised the same major isoform as another anti-rabbit CD44 mAb, RPN3/24. However, BJ4/55 and R5/151 appeared to recognise alternative forms of CD43 and CD45 expressed by rabbit T cell lines when compared to the previously described anti-CD43 mAb L11/135 and the anti-CD45 mAb L12/201. These newly defined mAb are potentially useful for studying the expression of these alternative forms of CD43 and CD45 in the rabbit. The exact role of CD43 is unclear though it may be involved in T lymphocyte

stimulation and activation (Park *et al.*, 1991) and cell adhesion (Rosenstein *et al.*, 1991). mAb BJ4/55, together with L11/135, may prove to be useful for studying the functions of CD43 in an animal model.

A tentative assignment of rabbit CD28 to the antigen recognised by R9/17 was suggested based on the results of biochemical studies and mass-peptide fingerprint analysis. Time restrictions and a lack of resources limited investigations to characterise this antigen further. CD28 provides an obligate co-signal for antigen-dependent T lymphocyte proliferation and cytokine production. In the human it is expressed by T lymphocyte populations and plasma cells, though R9/17 stains most rabbit mononuclear cells to different degrees. Targeting CD28 ligand interactions *in vivo* using a murine model has experimentally blocked graft-vs-host-disease, a major complication of bone marrow transplantation (Renner *et al.*, 1994). R9/17 may prove to be valuable in the study of T lymphocyte activation in the rabbit and for developing novel therapies targeting CD28.

As the project developed a number of anti-rabbit CD11a, CD11c and CD18 mAb with interesting functional properties were prepared and the study of rabbit leucocyte adhesion became the main focus of the project. Rabbit CD11b was previously characterised by Smet *et al.*, (1986) using mAb 198. Results from experiments using these newly defined CD11a, CD11c and CD18 mAb suggest that the expression of the leucocyte integrins in the rabbit follows that demonstrated in the human and other species with three α-chains associated with a common β_2-chain. Molecular cloning of rabbit CD18 showed that it shares a high level of sequence identity with human CD18. No evidence for a fourth α-chain, α_d, was found during the course of this investigation. However, α_d has been reported to be a macrophage associated CD11/CD18 integrin and this study has focused primarily on T lymphocytes, thus the expression of α_d by rabbit macrophages cannot be ruled out.

When stimulated with PMA, RL-5 cells exhibited homotypic aggregation. Aggregation was completely inhibited with the anti-rabbit CD11a mAb, NR185, or anti-CD18 mAb and is thus a CD11a/CD18-dependent event. Previously, Argenbright *et al.*, (1991) using intravital microscopy techniques to visualise the microcirculation of the rabbit

mesentery, showed that anti-human cross-reactive CD11a/CD18 and ICAM-1 mAb blocked C5a-induced leucocyte adhesion to the endothelium demonstrating the interaction of rabbit CD11a/CD18 with ICAM-1. It was presumed therefore, that PMA-induced homotypic aggregation would involve a CD11a/CD18-ICAM-1 interaction. However, the RL-5 cells express low levels of ICAM-1 and functionally blocking ICAM-1 with mAb RR1/1 did not inhibit aggregation. Thus, as in the human, rabbit CD11a/CD18 can interact with other ligands, presumably ICAM-2 or ICAM-3. Clearly, activation of CD11a/CD18 is required for adhesion to these ligands and this is in accordance with the findings of Binnerts *et al.*, (1994) who showed that human T cells activated with PMA, Mn^{2+} or by cross-linking CD3, bind to ICAM-1, ICAM-2 and ICAM-3, while non-activated T cells do not. Anti-human ICAM-2 and ICAM-3 mAb were identified by screening for mAb which had the ability to inhibit PMA-stimulated, CD11a/CD18-dependent, ICAM-1-independent leucocyte aggregation (De Fougerolles and Springer, 1992; De Fougerolles *et al.*, 1991). The RL-5 homotypic aggregation described in this study could be used to identify novel rabbit ICAMs.

The anti-CD11a mAb, BJ2/343, and the anti-CD18 mAb, BJ3/41, have been characterised during this study. Incubation of RL-5 cells with BJ2/343 and BJ3/41 promoted cell aggregation. This aggregation was shown to be a cell surface event and it is probable that on binding, BJ2/343 and BJ3/41 caused a conformational change in CD11a/CD18 revealing a ligand binding site. Like PMA-induced aggregation, BJ2/343 and BJ3/41-induced aggregation involved the interaction of CD11a/CD18 with unidentified ligands.

mAb BJ2/343 and BJ3/41-induced aggregation was compared with PMA-induced aggregation. The results of these experiments suggest that mAb-induced aggregation involved high affinity CD11a/CD18-ligand interactions, while PMA-induced aggregation involved intermediate affinity CD11a/CD18-ligand interactions. In these different affinity states, CD11a/CD18 appears to interact with different ligands. ICAM-2 and ICAM-3 are both expressed on lymphoid cell lines (De Fougerolles *et al.*, 1991; De Fougerolles *et al.*, 1994). Studies by Binnerts *et al.*, (1994) showed that incubation of human T lymphocytes with activating mAb directed against CD18 induced binding to transfectants expressing ICAM-3, whereas human T lymphocytes incubated with PMA

failed to bind to ICAM-3$^+$ cells. It seems reasonable to assume that PMA-induced aggregation of RL-5 cells is dependent on CD11a/CD18-ICAM-2 interactions, while mAb-induced aggregation of RL-5 cells is dependent on CD11a/CD18-ICAM-3 interactions. The differential expression of ligands acts as a mechanism to regulate CD11a/CD18 interactions, another possible mechanism may be the generation of different CD11a/CD18 affinity states. Further studies using these cell adhesion systems may lead to a greater understanding of the mechanisms regulating the affinity of the CD11/CD18 integrins for different ligands.

mAb BJ3/22, BJ6/112 and BJ7/208 were shown to recognise rabbit CD11c/CD18. In the rabbit CD11c/CD18 is expressed by monocytes and subpopulations of leucocytes from peripheral blood, lymph nodes, spleen, bone marrow and thymus. Interestingly, rabbit neutrophils do not express CD11c; in this the rabbit is like sheep, but differs from human and cattle. Species differences such as these need to be considered when using the rabbit as a model of human disease.

The anti-CD11c mAb were selected for their ability to induce homotypic aggregation of rabbit T cell lines. Anti-CD11c mAb induced aggregation was found to be a cell surface event and the CD11c/CD18 ligand in this system was confirmed as ICAM-1. The anti-ICAM-1 mAb, RR1/1, completely inhibited CD11c mAb-induced aggregation suggesting that other CD11c/CD18 ligands are not involved. Although human CD11c/CD18 expressed on the surface of COS cells has been shown to support binding to purified ICAM-1, cell-cell adhesion mediated by CD11c/CD18-ICAM-1 interactions has not previously been demonstrated.

The biological roles of CD11a/CD18 and CD11b/CD18 have received considerable attention since their discovery, while less is known about those of CD11c/CD18. Cellular immune responses in the skin are initiated by the presentation of antigen by cutaneous Langerhans cells to memory CD4$^+$ T lymphocytes. Functionally blocking CD11c on Langerhans cells inhibited antigen presentation to CD4$^+$ T lymphocytes (Meunier *et al.*, 1994). This suggests that CD11c/CD18 is required for presentation to T lymphocytes and is thus important in the initiation of cellular immune responses. Accordingly, CD11c/CD18 is expressed by macrophages and dendritic cells (Springer

et al., 1986; Freudenthal and Steinman, 1990) which can also present antigens to lymphocytes. The results of this investigation suggest that an interaction between CD11c/CD18 and ICAM-1 could stabilize the APC-T lymphocyte interaction.

It is clear that migration of lymphocytes from the blood stream to secondary lymphoid tissues or the migration of leucocytes to sites of infection is essential for host defence, and that inappropriate accumulation and activation of leucocytes results in many acute and chronic allergic, autoimmune or inflammatory diseases. Many rabbit models of such human diseases are available including those for rheumatoid arthritis (Jasin *et al.*, 1992), bacterial meningitis (Tuomanen *et al.*, 1989) and reperfusion-ischaemia injury (Linsley and Ledbetter, 1993). These models have been investigated *in vivo* with cross reactive anti-human CD18 mAb and, in all cases, the specific recruitment of leucocytes and associated tissue pathology has been inhibited to some degree, suggesting anti-adhesion therapy as a possible method of anti-inflammatory therapy. Therapy targeting adhesion molecules would need to be tightly controlled to prevent an induced form of LAD developing leaving patients susceptible to infection. Determining whether different CD11/CD18 integrins mediate leucocyte migration to different inflammatory sites and understanding the regulation of integrin activation and ligand interactions *in vivo* will allow anti-adhesion therapies to be more specifically targeted. The rabbit should prove to be an important model for such studies.

APPENDIX I List of reagents

Cell Culture and Antibody Production	
Aminopterin	Sigma
Azaserine/Hypoxanthine	Sigma
Condimed H-1	Boehringer Mannheim
DMEM	Gibco BRL
DMEM (methionine free)	Flow Laboratories
DMSO (Dimethyl Sulphoxide)	Sigma
Fungizone	Gibco
Foetal Calf Serum (FCS)	Gibco BRL
Glutamine	Gibco BRL
Hypoxanthine	Sigma
Hypoxanthine/Azaserine/Thymidine	Sigma
Penicillin/Streptomycin solution	Gibco BRL
RPMI-1640	Gibco BRL
Sodium pyruvate	Imperial Labs
Thymidine	Sigma
Trypsin	Sigma
Immunofluorescence	
$F(Ab')_2$ Rabbit-anti-mouse Ig	Serotec
Fluorescein Isothiocyanate	Sigma
Goat-anti-mouse IgG-FITC	Capell
Nycoprep™ Animal separation solution	Nycomed
Paraformaldehyde	BDH
Streptavidin-phycoerythrin	Becton Dickinson
Antibody Purification and Isotyping	
Anti-mouse IgG (whole molecule)	Sigma
Isotyping kit	Serotec
Protein A-Sepharose CL-4B	Sigma
ELISA and Immunoblotting	
Acetic acid	BDH
Coomassie Brillant blue R250	Sigma
3,3'-Diaminobenzidine	Sigma
Gelatin, type II (swine)	Sigma
Glutaraldehyde	BDH
Goat-anti-mouse IgG HRP	Bio-Rad
H_2O_2	Sigma
HPLC Methanol	BDH
Nitrocellulose membrane	Millipore
O-phenylene diamine (OPD)	Sigma
Poly-L-Lysine	Sigma
Problot membrane	Applied Biosystems

Immunoprecipitation

ECL Detection kit	Amersham
ECL-Hybond nitrocellulose	Amersham
ECL molecular weight markers	Amersham
Hyperfilm-ECL	Amersham
NHS-LC-biotin	Pierce
NP-40	Sigma
Phenylmethylsulphonylfluoride (PMSF)	Sigma
Streptavidin-biotinylated HRP mAb	Amersham
^{35}S-methionine	Amersham
X-ray film	Fuji

SDS-PAGE

Acrylamide	BioRad
Amomium persulphate	BioRad
Glycerol	BDH
Glycine (aminoacetic acid)	BDH
Iodoacetamide	Sigma
β-mercaptoethanol	BDH
N,N'-Methyene-bis-acrylamide	BioRad
Protein molecular weight standards	Bio-Rad/Sigma
Sodium dodecylsulphate (SDS)	Sigma
TEMED	BioRad
Trizma Base (Tris[hydroymethyl]aminomethane)	Sigma
Trizma HCl	Sigma

Protein Purification and Sequencing

Cyanogen-bromide activated beads	Pharmacia
3-cyclohexylamino-1-propanesulphonic acid	Sigma
Mercaptoacetic acid	Sigma

Aggregation Assays

EDTA	BDH
HEPES	BDH
Phorbol myristate acetate	Sigma
Staurosporin	Sigma

General Use

Bovine Serum Albumin	Sigma
Ethanol	BDH
Methanol	BDH
Phosphate Buffered Saline	Gibco BRL
Potassium chloride (KCl)	BDH
Potassium dihydrogen orthophosphate ($KH2PO_4$)	BDH
Sodium acetate	BDH
Sodium azide (NaN_3)	Sigma
Sodium Chloride (NaCl)	BDH
Sodium hydrogen carbonate ($NaHCO_3$)	BDH
di-Sodium hydrogen orthophosphate (Na_2HPO_4)	BDH
Sodium hydroxide (NaOH)	BDH

Tween 20	BDH
Triton x100	BDH

Molecular Biology

Agar	Oxoid
Agarose	Sigma
Low melting point agarose	Sigma
Ampicillin	Sigma
Dig System for Nucleic Acid Analysis	Boehringer Mannheim
Dimethylformamide (DMF)	Sigma
DNA markers	Gibco
Genclean II	BIO 101 Inc.
Hybridsol	Oncor
Kodak XAR-5 film	Kodak
Nitrocellulose discs (82mm)	Dupont
Nitrocellulose membrane	Boehringer Mannheim
PCR reagents (*Taq* polymerase, dNTPs, 10x PCR buffer)	Boehringer Mannheim
Rabbit Spleen cDNA library	Invitrogen
Restriction enzymes	Promega
Sequenase™ Version 2.0	Amersham International
^{T7}Sequencing ™ kit	Pharmacia Biotech
SP6 Sequencing primer	Invitrogen
T7 Sequencing primer	Invitrogen
[^{35}S]dATPαS	Amersham
Sequagel	National Diagnostics
TBE Sequencing	National Diagnostics
TA cloning kit®	Invitrogen
T7 Quickprime kit	Pharmacia
TE solution	Sigma

Molecular Biology Grade chemicals

Ammonium acetate	Sigma
Chloroform: isoamyl alcohol (24:1)	Sigma
EDTA	Sigma
Ethidium bromide	Sigma
GTE solution	Sigma
Lauri-Bertani medium	Sigma
Lauryl sulphate (sodium salt) SDS	Sigma
Lithium chloride (LiCl)	Sigma
N-lauroylsarcosine	Sigma
Maleic acid	Sigma
Magnesium chloride ($MgCl_2$)	Sigma
Mineral oil	Sigma
Phenol	Sigma
Potassium acetate	Sigma
RNase	Sigma
Sodium hydroxide (NaOH)	Sigma
SSC (20x powder)	Sigma
Tris-(hydroxymethyl) methylamine	Sigma

APPENDIX II List of Abstracts and Poster Presentations

1. Blackford, J. and Wilkinson, J.M. (1993) A Monoclonal Antibody which Recognises Rabbit CD11a and which Inhibits Homotypic T Cell Aggregation. *Biochem. Soc. Trans. 21: 198S.*
2. Blackford, J., Shock, A., Minion, T., Howat, D., Robinson, M.K. and Wilkinson, J.M. (1993) Inhibition of Rabbit Leucocyte Adhesion Functions by a Monoclonal Antibody to CD11a. *BSI Spring meeting, Liverpool.*
3. Blackford, J. (1994) A Monoclonal Antibody Recognising Rabbit CD11c and its Effects on Rabbit T Lymphocytes. *BSI Spring meeting, Southampton.*
4. Blackford, J., Hague, B.F., Pappin, D.J.C. and Wilkinson, J.M. (1995) cDNA cloning of rabbit CD18. *BSI Spring meeting, Birmingham.*
5. Blackford, J., Reid, H.W., Pappin, D.J.C., Wilkinson, J.M. (1995) ICAM-1 is the Ligand for CD11c/CD18 in the rabbit. *9th International Congress of Immunology, San Francisco.*

List of Publications

1. Galea-Lauri, J., Blackford, J. and Wilkinson, J.M. (1993) The expression of CD11/CD18 molecules on rabbit leucocytes: Identification of Monoclonal Antibodies to CD18 and Their Effects on Cellular Adhesion Processes. *Mol.Immunol. 30: 529-537*
2. Rutter, J., James, T.J., Howat, D., Shock, A., Andrew, D., De Baestelier, P., Blackford, J., Wilkinson, J.M., Higgs, G., Hughes, B. and Robinson, M.K. (1994) The In Vivo and In Vitro Effects of Antibodies against Rabbit β_2-Integrins. *J.Immunol. 153: 3724-3733*
3. Blackford, J. Reid, H.W., Pappin. D.J.C., Bowers, F.S. and Wilkinson, J.M. (1996) A Monoclonal Antibody, 3/22, to Rabbit CD11c which Induces Homotypic T Cell Aggregation: Evidence that ICAM-1 is a ligand for CD11c/CD18. *Eur. J. Immunol. 26: 525-531*

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