STUDIES OF CD38

IN

CHRONIC LYMPHOCYTIC LEUKAEMIA

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

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CONTENTS

ABSTRACT

This Thesis consists of 4 experimental Chapters preceded by a General Introduction and followed by a brief section dealing with conclusions and future.

In Chapter 1, the prognostic value of CD38 and the relationship between surface expression of this molecule and IgVH mutation are considered. It is shown that surface CD38 expression on CLL cells is highly predictive of non-hypermutated IgVH gene mutation status, while CD38 negativity lacks such predictive value, since IgVH mutated and unmutated cases occur with approximately equal frequency in this subgroup. It is also shown, in agreement with the literature, that CD38 expression is associated with shorter survival and male preponderance.

Abundant data are available on the intracellular expression of CD38 in other cell types, but not in CLL. Therefore, Chapter 2 deals with intracellular CD38 in CLL cells and with the different molecular forms expressed in sCD38+ and sCD38- CLL clones. Using a number of different techniques, it is shown that CD38 is expressed intracellularly in all CLL cells irrespectively of the surface expression of the molecule. In keeping with these findings, CD38 mRNA was found in both sCD38+ and sCD38- CLL clones at comparably low levels. It is documented by Western blotting and immunoprecipitation that CLL cells express, in addition to the 45 kD CD38 monomer, other molecular forms of 27, 60 and 205 kD. Among these, the high molecular weight molecule probably represents a tetrameric form of CD38 and is described in CLL for the first time. A main difference found between the sCD38+ and sCD38- clones was the almost complete absence of the 45 kD monomeric form in the sCD38- clones. An unexpected and interesting finding was a surface immunoreactivity with the anti-CD38 antibody Ab-4 on CLL cells previously classified as "CD38 negative" with the HB-7 antibody.

In Chapter 3, the topology of different molecular forms of CD38 is studied further and their enzymatic functions are examined. By subcellular fractionation it is demonstrated that the tetrameric form of CD38 is most abundant in the membrane fraction. Surface radioiodination of CLL cells indicated, for the first time that the main forms of CD38 on the surface of CLL cells are the 45 kD and the ~205 kD molecules. The 205 kD molecule was demonstrable on both sCD38+ and sCD38- cells, while the 45 kD molecule was present only on sCD38+ cells. Surface forms of CD38 on CLL cells were characterised further by enzymatic assays performed before and after protease digestion of surface proteins. Results indicated that surface forms are the major sources of CD38 enzymatic activity in CLL cells. Total cyclase and hydrolase activities were also compared in sCD38+ and sCD38- CLL cells and it was shown that both cell types possess these enzymatic functions, although activities were lower in sCD38- cells. It seems likely that the 45 kD molecule (probably present as non-covalently linked dimers) is responsible for the greater enzymatic activity of sCD38+ clones. Enzymatic assays performed after recovering proteins from polyacrylamide gels indicated that eluates from the HMW (>116 kD) section of the gels containing the putative CD38 tetramers have both cyclase and hydrolase activities.

Chapter 4 addresses the question of why CD38-positive CLL is characterised by progressiveness and poor outcome. Since CD38 has a well-documented role in cell cycle regulation and cell proliferation in other cell types, it was hypothesized that a similar linkage might be also present in CLL, contributing to the the adverse clinical outcome. To test this hypothesis, CLL cells were co-stained for surface CD38 and nuclear Ki-67 following a saponin-based permeabilization procedure. CLL clones classified as CD38+ expressed significantly higher levels of Ki-67 than did sCD38- clones. Clones classified as Ki-67+ (>5%) expressed significantly higher levels of CD38 than did Ki-67- ones. Relating surface CD38% and nuclear Ki-67% revealed a linear correlation between these two parameters. Also, the Ki-67+ subpopulation within a given CLL clone expressed significantly higher CD38 values than did the Ki-67- subpopulation. Furthermore, larger CLL cells showed significantly higher Ki-67 values than small CLL lymphocytes. Finally, CD38 immunoreactivity in CLL lymph node sections was found to be strongest in the proliferation centres. These data therefore indicate, for the first time, that surface CD38 expression is a marker of cell cycling activity in CLL and help to explain the adverse prognosis of CD38+ CLL and CLL/PL.

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DECLARATION

All the data presented in this thesis represent my own work with the following exceptions:- Dr. P.D. Sherrington performed the mRNA studies in Chapter 2, and Drs A.S. Kamiguti and K.J. Till contributed to the radioiodination and immunohistochemical experiments of Chapters 3 and 4, respectively.

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Lin K, Sherrington PD, Dennis M, Matrai Z, Cawley JC and Pettitt AR (2002) Relationship between p53 dysfunction, CD38 expression, and IgV(H) mutation in chronic lymphocytic leukemia. *Blood,* 100(4):1404-9.

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Leukemia & Lymphoma, 44(5):797-800.

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ABBREVIATIONS

ADP	Adenosine diphosphate
AML	Acute myeloid leukaemia
ALL	Acute lymphoblastic leukaemia
ATM	Ataxia teleangiectasia mutated
APAAP	Alkaline phosphatase-anti-alkaline phosphatase
BCR	B-cell receptor
BSA	Bovine serum albumin
cADPR	Cyclic adenosine diphosphoribose
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CLL	Chronic lymphocytic leukaemia
CLL/PL	CLL with >10% prolymphocytes
DSSB	Double-strength sample buffer
ECL	Enhanced chemiluminescence
εNAD	$1,N^6$-etheno NAD
FACS	Fluorescence-activated cell sorter
FCS	Fetal calf serum
FITC	Fluorescein isothyocyanate
FMLP	Formyl-methionyl-leucyl-phenylalanine
HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cells
IgVH	Immunoglobulin heavy chain variable region
IH	Immunohistochemistry
IP	Immunoprecipitation
LDH	Lactate dehydrogenase
mAb	Monoclonal antibody
MFI	Mean fluorescence intensity
MW	Molecular weight
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NAADP	Nicotinic acid adenine dinucleotide phosphate

NGD	Nicotinamide guanine dinucleotide
NHD	Nicotinamide hypoxanthine dinucleotide
PBS	Phosphate-buffered saline
PCL	Plasma-cell leukaemia
PE	Phycoerythrin
PE-Cy5	Phycoerythrin - cyanin 5.1
PMSF	Phenylmethylsulfonyl fluoride
RFI	Relative fluorescence intensity
RT-PCR	Reverse-transcriptase polymerase chain reaction
SDS	Sodium-dodecyl sulfate
PAGE	Polyacrylamide gel electrophoresis
sCD38+	Surface CD38-positive (by HB-7 mAb)
sCD38-	Surface CD38-negative (by HB-7 mAb)
SME	Syringe maceration extraction
TBST	TRIS-buffered saline with Tween
WB	Western blotting
WBC	White blood count

AIMS AND SCOPE OF THESIS

When this Thesis was started, there was much controversy concerning the prognostic value of CD38 in CLL and whether or not cell surface CD38 can serve as a surrogate marker for IgVH mutation. The initial aim of the work was to clarify these issues. Therefore, the relationship between IgVH mutation and surface CD38 and their prognostic significance were examined (Chapter 1).

Having done this, it seemed important to investigate the biology of CD38 in CLL. Since it is known in other cell types that there are different molecular forms of CD38 at different intracellular locations, the next aims were to investigate what molecular forms are present in CLL cells (Chapter 2) and where they are located (Chapter 3). Also, since CD38 is an enzyme, the enzymatic functions of CD38 in CLL cells were examined (Chapter 3).

The last aim of the Thesis was to determine why CD38+ CLL has a poor prognosis. On the basis of work with other cell types, it was hypothesised that surface CD38 might be a marker of proliferating cells. In Chapter 4, it is shown that this is so for CLL, thus providing an explanation of why CD38 positivity is associated with an adverse outcome.

GENERAL INTRODUCTION

CLL

AN OVERVIEW OF CLL

Chronic lymphocytic leukaemia (CLL) is characterized by the monoclonal expansion of mature-looking, CD5+ malignant B-cells in the blood and lymphoid organs. CLL is the most frequent form of leukaemia in the Western world affecting mostly elderly individuals. The diagnosis is made from the morphology and immunophenotype of the leukaemic cells in patients presenting often with lymphadenopathy, fatigue or with elevated white blood count on routine laboratory examinations performed for unrelated causes. CLL is a heterogeneous condition with some patients having a lifespan of normal individuals, others dying in a few years despite aggressive therapy. Identification of progressive cases early in the course of the disease by prognostic markers like CD38 has paramount importance and will be discussed in detail later. In the course of CLL, the relentless enlargement of tumour bulk leads to progressive bone marrow failure; patients are usually dying of infections aggravated by the immune deficiency accompanying the disease.

CLL cells have a characteristic immunophenotype, being CD19+, CD5+, CD23+, FMC7- and expressing weak, clonally restricted surface immunoglobulin. Most clones express surface IgM and/or IgD, a minority of cases IgG. Light chains are expressed in a clonally restricted pattern, with 60% of the cases using κ and 40% λ chains. In addition to the immunoglobulin molecule, the B-cell receptor (BCR) complex on the CLL cell is composed of two accessory proteins, CD79a and CD79b, both required for intracellular assembly, surface expression and signal transduction of the BCR.

The normal cellular counterpart of CLL is unknown. However, there is strong evidence that its cell of origin might be a mature, antigen-experienced B-cell. Firstly, gene array studies showed that all CLL clones - both mutated and unmutated ones - resemble memory B-cells (Rosenwald et al, 2001). Secondly, the phenotype of CLL reflects previous cell activation (expression of CD23, CD25, CD69, CD71), presumably deriving from prior BCR engagement by antigen. Moreover, all CLL cells

express the memory-cell marker CD27. On the basis of CD5 positivity and the frequent autoreactivity of CLL clones, B1 cells were also suggested as possible cells of origin (Chiorazzi et al, 2003).

PROGNOSTIC FACTORS IN CLL

Different prognostic indicators that have been identified are listed in Table 1. Among these, IgVH mutation and CD38 have attracted particular attention and are central to this Thesis. They will therefore be discussed separately and in some detail.

		Prognosis		
		favourable	adverse	reference
Clinical/laboratory	Gender	female	male	Catovsky et al, 1989
	Atypical morphology	absent	present	Criel et al, 1997
	Prolymphocytes	<10%	>10%	Melo et al, 1987
	BM histology	nodular	diffuse	Rozman et al, 1984
Tumour bulk	Stage (Rai)	0	I,II,III, IV	Rai et al, 1975
	Stage (Binet)	A	B,C	Binet et al, 1981
	β2-microglobulin	normal	increased	Keating et al, 1985
	LDH	normal	increased	Lee et al, 1987
Clonal instability	Karyotype	13q14 del	17pdel; 11qdel;12+	Kroeber et al, 2002
	p53/ATM mutations	absent	present	Lin et al, 2002
Cell proliferation rate	Lymphocyte doubling time	>1 year	<1 year	Montserrat et al, 1986
	Thymidine kinase	normal	increased	Hallek et al, 1999
	Ki-67	low	high	Cordone et al, 1992b
	sCD23	normal	increased	Sarfati et al, 1996
Differentiation/activation	IgVH mutation status	mutated	unmutated	Hamblin et al, 1999
	CD38 positivity	<30%	>30%	Damle et al,1999
	ZAP-70 positivity	low	high	Orchard et al, 2004

Table 1. Prognostic factors in CLL.

IgVH mutation

IgVH mutation status in CLL: the evolution of a concept

CLL lymphocytes had originally been thought to originate from CD5+ mantle-zone naive B-cells, and early data on IgVH genes in CLL found them to be in germline configuration (Kipps et al, 1989). In 1994, however, Schroeder and Dighiero reported that almost half of the 75 cases studied showed >2% divergence from the germline sequence and that there is biased usage of IgVH genes within the mutated vs unmutated subgroup. These findings were confirmed by Fais et al (1998). The first clinical implications of IgVH mutation in CLL were documented by Oscier et al

(1997), who found that trisomy 12 occurs predominantly in unmutated CLL, while 13q14 del is a feature of the mutated subgroup. The same investigators reported in 1999, on a larger cohort of patients, that patients with mutated IgVH genes do remarkably better, with survival times of 24 years as compared to 9 years in unmutated cases (Hamblin et al, 1999). Damle et al reported a similar association in the same issue of Blood, and also called attention to the suitability of CD38 as a surrogate marker for IgVH mutation and its role as an independent prognostic marker (see later). Since then, the prognostic significance of IgVH mutation has been confirmed by many other groups (Maloum et al, 2000; Kroeber et al, 2002; Lin et al, 2002). Although there has been a controversy on the optimal cutoff for defining "mutated" cases, - most reports using values between 2-5% – there is a general agreement in that mutated and unmutated CLL cases have very different clinical and biological characteristics. Our data on IgVH mutation in CLL are presented in Chapter 1.

IgVH mutation in normal B-cell development

The central process in the normal development of B-cells is to equip the cell with a functioning, antigen-specific B-cell receptor (BCR). The expression of an antigen-specific immunoglobulin (Ig) in the BCR enables the B-cell to react specifically with antigens. B-cells obtain the huge diversity of Ig-s required in the recognition of diverse antigenic challenges by the processes of Ig gene rearrangement and somatic hypermutation. In the course of the former, diversity is brought about by recombination of the V, D and J segments of Ig heavy chain genes (IgVH). Further diversity is introduced by the enzyme terminal deoxytransferase (TdT) introducing nucleotides in a random manner into the Ig gene. These processes occur in the bone marrow; subsequent development occurs in the peripheral lymphoid organs and is driven by antigen. In the periphery, lymphocytes alter their DNA in two further ways to change the function and affinity of their immunoglobulin. These two processes are class switch recombination and somatic hypermutation. Most CLL cells have not undergone class switch recombination and therefore this process will not be considered further. During somatic hypermutation, base pair changes are introduced at high frequency and these alter the affinity of the BCR for antigen. Cells with high-affinity BCR are positively selected, while those with low affinity undergo apoptosis.

The process of VH hypermutation normally occurs in germinal centers under the influence of antigen-presenting cells, CD40-ligand expressing T cells and cytokines. However, it is known that somatic hypermutation can occur in the absence of CD40L and germinal centres (de Vineusa et al, 2000). This extrafollicular process is important for T-independent antigens and may well be relevant for CLL cell development, which is thought often to be driven by T-independent autoantigens (Chiorazzi et al, 2003).

After undergoing somatic hypermutation, B cells then become either recirculating memory cells or migrate to the bone marrow (and other sites) and become plasma cells. These stages of differentiation are under the control of cytokines and CD40-ligand.

IgVH mutation in CLL: biological implications

The fact that around half of all CLL cases have somatic hypermutation in their IgVH genes led to the concept that CLL might be two diseases – one derived from mutated post-germinal center memory B-cells, the other from unmutated pregerminal center B cells (Hamblin et al, 1999). This theory has been largely ruled out by the discovery that the gene expression profiles of both mutated and unmutated cells are very similar and resemble that of normal memory B cells (Klein et al, 2001; Rosenwald et al, 2001) The idea that CLL is a tumour of memory B cells is supported by the fact that all cases express the memory-cell marker CD27 and show a range of activation markers, such as CD23, CD25, CD69 and CD71. It remains unclear, why unmutated cells have not acquired somatic hypermutations but it may be they have responded to T-independent antigens outside the germinal center.

Routes of development of CLL clones and the role of antigen

Since CLL cells are CD5+, it is important to consider the relationship between CLL cells and normal CD5+ B-cells - the so-called B1 cells. B1 cells are now known to be positively selected by interaction with particular types of self antigen during fetal and neonatal development. Although in adults B1 cells are predominantly peritoneal B-cells, a similar type of cell is also found among marginal zone B-cells (Dammers et al, 2000). It is tempting therefore to suggest that CLL cells are memory cells derived from B1 cells stimulated by autoantigen.

Mutated CLL clones might have responded to T-dependent antigens in the germinal center reaction, but have failed to undergo class switch recombination. However, given that VH mutation can occur independently of T-cells (see above), it is not clear, whether or not this suggestion is correct. Nevertheless, what is certainly true is that the biased use of Ig gene segments in both mutated and unmutated CLL often results in the creation of a BCR with a broad specificity and relatively low affinity for T-independent antigens. It therefore seems likely that the autoantigens involved in the pathogenesis of CLL are often T-independent. Recent work from this Department and Southampton showed that mutated CLL clones are usually unresponsive to BCR crosslinking (Allsup et al, 2002; Lanham et al, 2003). In the context of the above considerations, this may mean that mutated CLL cells have interacted with autoantigen and thus entered the state of functional hyporesponsiveness. In contrast, unmutated CLL which retain ability to signal through the BCR possibly reflect a different type of autoantigenic stimulaton. It seems likely that the type of antigen and the extent of antigenic stimulation are the principal factors determining the phenotype of a particular CLL clone.

IgVH mutation: clinical implications

While all CLL cases seem to be memory-cell related, the two subtypes of unmutated- and mutated CLL show very different biological features. Unmutated cases are characterised by a higher proliferative rate (Magnac et al, 2003), extensive cycling history showed by telomere length (Hultdin et al, 2003; Caligaris-Cappio, 2004), progressive disease, atypical morphology (Hamblin et al, 1999), bad-prognosis karyotypes (Kroeber et al, 2002), CD38-and ZAP-70 expression (Damle et al, 1999; Orchard et al, 2004), p53 mutation (Lin et al, 2002) and BCR responsiveness (Lanham et al, 2003). In contrast, mutated cases show a lower proliferative rate, less extensive cycling history, stable disease, typical morphology, good-prognosis karyotypes, are negative for CD38 and ZAP-70, have normal p53 and show BCR unresponsiveness. Thus, the subclassification of CLL cases on the basis of VH mutation does seem to define subgroups with clinically relevant differences.

The extensive associations of unmutated CLL with other bad prognostic features such as CD38 or p53 partly explain why these patients have a poor outcome. However, other biological characteristics of this subgroup could also have a role. For example, the higher turnover rate observed in unmutated CLL might be related to a

permanent restimulation of the cells by antigen via the BCR (Lanham et al, 2003). These signals, in turn, might be further potentiated by the the tyrosine kinase ZAP-70, overexpressed in this subgroup (Chen et al, 2002).

An advantage of IgVH mutation status as a clinical prognostic marker, that, unlike CD38 or p53, it is stable over time (Hamblin et al, 2002). On the other hand, the fact that it is not available as a routine laboratory test, necessitated the search for substitutes.

It is clear from the published studies on IgVH mutation in CLL that it is better in predicting good prognosis (mutated) cases, than bad prognosis (unmutated) cases, since these latter ones comprise a very heterogeneous group prognostically, containing both cases with very poor and intermediate prognosis. For example, unmutated cases with CD38 positivity and/or p53 mutation do much worse than those without these features (Lin et al, 2002). These considerations emphasize the need to evaluate multiple prognostic factors before determining prognosis and management in the clinical setting.

The optimal cutoff for the distinction of somatically mutated and unmutated immunglobulin genes has been a matter of controversy and different groups suggested 2% (Hamblin et al, 1999; Damle et al, 1999), 4% (Kroeber et al, 2002) or 5% (Lin et al, 2002) as optimal cutoffs. Lin et al found that cases with 2-5% IgVH mutation have a similar worse prognosis than have <2% cases and therefore suggested that a 5% cutoff might be better than 2%. Despite of this controversy, it has been generally agreed that cases with extensively mutated IgVH genes have better prognosis than those with relatively unmutated IgVH genes.

CD38 as a prognostic marker

Since this matter is discussed in detail in Chapter 1, the subject is considered only briefly here.

It was Damle et al (1999), who introduced CD38 into the diagnostics of CLL on the basis that it can serve as a surrogate for IgVH mutation and also has prognostic significance. They examined the relationship between VH mutation and CD38 because this antigen is differentially expressed during B-cell differentiation and they assumed that the extent of VH mutation is related to differentiation. Subsequently, Hamblin et al confirmed that CD38 has prognostic significance, but failed to

demonstrate the relationship with IgVH mutation. This was the stage at which the studies detailed in Chapter 1 were performed in order to clarify the relationship between CD38 expression and IgVH mutation in CLL.

CELL PROLIFERATION IN CLL

For a long time, CLL has been widely regarded as an accumulative, indolent condition in which the slow increase of the tumour bulk is a consequence of defective apoptosis rather than the rapid proliferation of the cells. Increased levels of anti-apoptotic proteins such as BCl-2 seemed to support this concept (Pepper et al, 1998). However, recent data clearly indicate that CLL clones can have a surprisingly high turnover rate. Thus, Messner et al (2002) determined leukemic cell turnover in vivo by a non-radioactive, stable isotopic labeling technique and found cell turnover rates between 1% and 10% per week. These unexpectedly high values indicate that the B-CLL clone is undergoing substantial replication.

Different reports indicate a greater cycling activity in unmutated CLL clones compared to mutated ones. Thus, higher thymidine kinase values were found in unmutated CLL (Magnac et al, 2003). Also, Damle et al recently showed that unmutated cases have a significantly longer cycling history as shown by their shorter telomere length (Damle et al, 2004). These data on the higher cycling rate of unmutated CLL clones help to explain the worse outcome and greater clonal instability of this subset.

CD38

THE HISTORY OF CD38

CD38 was originally described by antibody typing as a surface antigen on thymocytes (Reinherz et al, 1980). Subsequently, it was also detected on the surface of B-cells, monocytes and NK cells (Malavasi et al, 1992). In 1990, Jackson et al isolated a cDNA for CD38, the sequence of which predicted an unusual 30 kD polypeptide with a short N-terminal cytoplasmic tail and a carboxy-terminal extracellular domain carrying the four potential N-linked glycosylation sites.

A turning point in the research of CD38 came when, in 1992, States et al discovered the 30% amino acid sequence similarity between CD38 and the soluble mollusk enzyme Aplysia. This latter enzyme has a MW of 29 kD and its enzymatic product, cyclic-ADP-ribose (cADPR) is a potent Ca^{++} mobilizer second messenger (Lee et al, 1991). It soon became clear that CD38 has similar enzymatic properties, but it has both cyclase and hydrolase activites (Howard et al, 1993) in contrast to the Aplysia, which only has cyclase function. Subsequently, CD38 was found in different human and non-human tissues, both at the cell membrane and intracellularly (Lee HC, 2000).

In 1994, another homologue of CD38, BST-1 (bone marrow stromal cell antigen 1) was identified on the surface of human bone marrow stromal cell lines derived from patients with rheumatoid arthritis (Kaisho et al, 1994). The GPI-linked molecule, which has both cyclase and hydrolase activities and is coded on the same chromosome as CD38, was clustered as CD157; it is expressed in myeloid precursors, monocytes and also in AML, but not in ALL (Ishihara et al, 2000; Todd et al,1985). The protein has very low levels of enzyme activity as compared with those of CD38 (Hussain et al, 1998).

More recently, it has become clear that the CD38/cADPR/Ca^{++} second messenger system is essential in vital cellular functions, such as cell proliferation, motility, activation and signalling. Due to its stage-specific expression, CD38 has also become a valuable tool as a differentiation marker in lymphoid and myeloid development (Konopleva et al, 2000).

THE CD38 GENE AND ITS TRANSCRIPTIONAL REGULATION

The CD38 gene maps to chromosome 4p15 and CD38 is encoded by a >80 kb gene, of which >98% is composed of intronic sequences (Ferrero et al, 2000). The gene has eight exons and exon 1, the second largest, encodes the intracytoplasmic, transmembrane and the membrane proximal 33 amino acids of the extracellular region. Two main transcripts have been reported: a full length, and an alternatively spliced variant (CD38alt) (Nata et al, 1997), the protein product of which is unknown at present. A change of the C→G nucleotides at bp 184 of intron 1 gives rise to a polymorphism (CD38A), present in a minority of the population (Ferrero et al, 2002).

The transcriptional regulation of CD38 has been extensively reviewed (Mehta K, 2000) and will not be discussed in detail here. Retinoids and interferons are particularly well-known inducers of transcription and an interferon-responsive element in the regulatory region of the CD38 gene has been identified (Ferrero et al, 1997). Alpha-and gamma interferons induce CD38 expression in normal resting B-cells and CLL cells (Stoeckler et al, 1996; Beavouis et al, 1999), and recently, IL-2 has been shown to upregulate CD38 on CLL cells (Deaglio et al, 2003).

STRUCTURE

CD38 is composed of 300 amino acids and has a MW of 45 kD. It is a transmembrane type II glycoprotein with a long ectocellular domain harbouring its enzymatic active site. It has a 25-30% sequence homology with the Aplysia cyclase and with CD157 (Lee HC, 2000). The most remarkable among the conserved amino acids are the 5-disulfide-forming 10 cysteine array of the Aplysia cyclase - the protein signature of this superfamily. CD38 contains 2 more cysteines, which are responsisble for its additional hydrolase function (Lee HC, 2000). The Aplysia cyclase and CD38 share another feature distinguishing them from CD157 - a six amino acid sequence (Thr144-Leu-Glu-Asp-Thr-Leu149, TLEDTL) that contains one of the critical glutamates for enzymatic activity (Graeff et al, 2001).

In CD38, the ectocellular part (257 amino acids) contains the C-terminus and the intracellular domain the N-terminus (21 amino acids); in CD157, the orientation is opposite and the molecule is linked to the membrane by a GPI-anchor (Kaisho et al, 1994). A short 23 residue hydrophobic sequence forms the transmembrane portion. (Jackson et al, 1990)

X-ray crystallographic analysis showed that the Aplysia cyclase is a dimer with a central cavity, the size of which corresponds well to that of cADPR, the enzymatic product of the cyclase (Fig.1/A.). This structure was confirmed by documenting that, in solution, the cyclase forms a stable homodimer (Munshi et al 1988) linked by non-covalent bonds. Based upon the amino acid homology, the structure of CD38 was predicted to form a similar, but membrane-spanning dimer (Fig.1/B.)

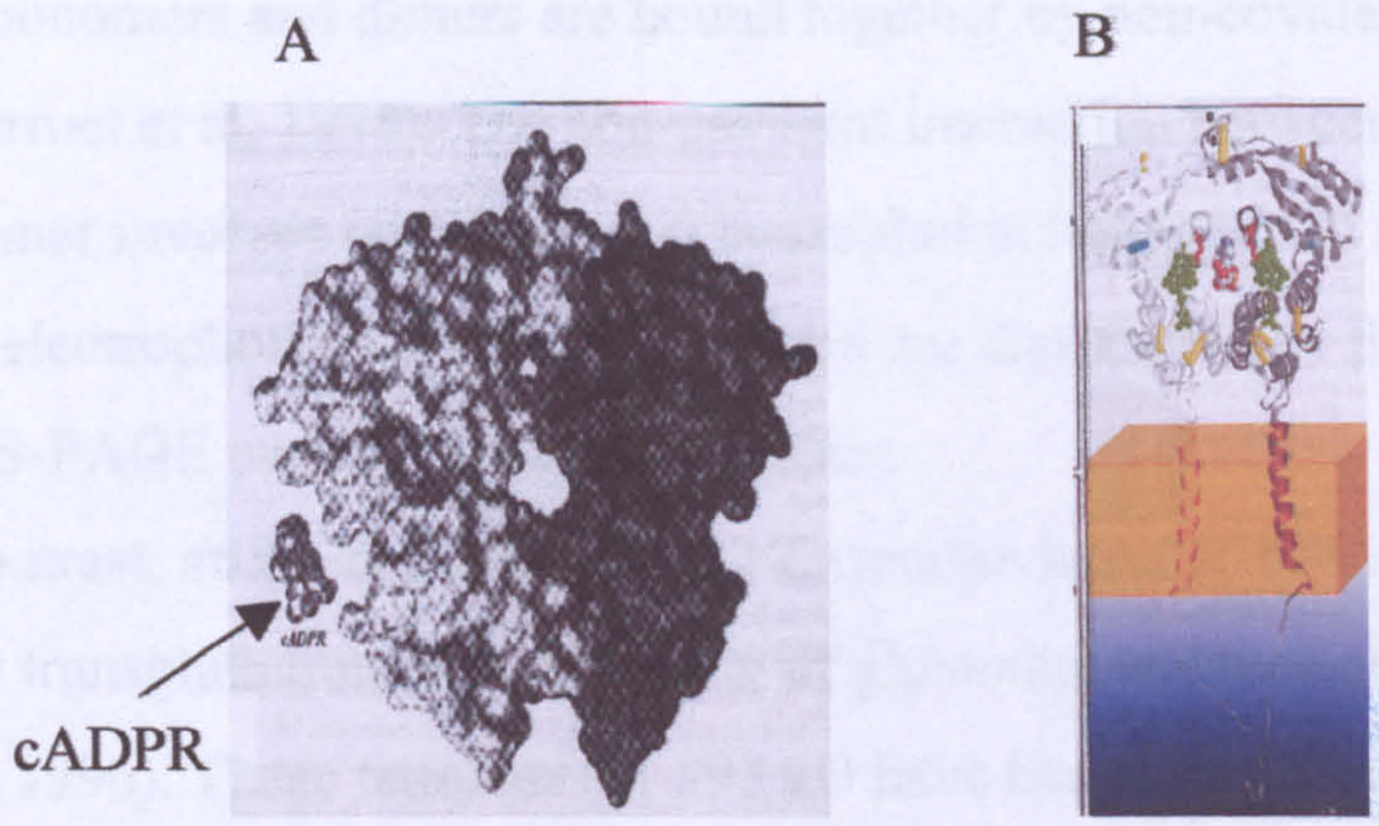


Fig.1. The homodimeric structure of the ADP-ribosyl cyclase (A) and CD38 (B). The size of the central cavity corresponds to that of cADPR. (from: Lee CH, 2000)

Subsequently, many studies confirmed the propensity of this molecule to form dimers and multimers (Bruzzone et al, 1998, Umar et al, 1996, Khoo et al, 1998). Our data on CD38 multimer formation in CLL cells are considered in Chapters 2 and 3.

There are 4 glycosylation sites, all on the ectocellular part of the molecule; without these side chains, the MW of CD38 is 34 kD (Jackson et al, 1990). The de-glycosylated protein loses its enzymatic function (Chidambaram et al, 1998a).

Since cADPR is generated from NAD^+ at the ectocellular enzymatic site, how it reaches its intracellular targets for Ca^{++} mobilization – the so-called topological paradox of CD38 - has been the subject of controversy (De Flora et al, 2000). It has been shown that there are different mechanisms capable of transporting the generated cADPR into the cell interior, thereby mobilizing Ca^{++} (de Flora, 1997). These include a cADPR transport channel formed by dimers (Franco et al, 1998), and internalization of CD38 multimers after oligomer formation (Zocchi et al, 1999). This clearly underlines the importance of multimer formation in the function of CD38.

Dimer and tetramer formation of CD38

As discussed above, both the Aplysia cyclase and CD38 are predisposed to form multimers. It has been shown that in CD38-transfected HeLA cells, CD38 forms a mixture of catalytically active monomers, dimers and tetramers (Bruzzone et al, 1998; Moreno-Garcia, 2004). As already mentioned, CD38 dimers and tetramers are

functional prerequisites for the transport of cADPR. In the case of reversible multimer formation, monomers and dimers are bound together by non-covalent or disulfide linkages (Berruet et al, 1998). The con-covalent interaction between two molecules of CD38 monomer involves both ionic and hydrophobic linkages and are disrupted by SDS during electrophoresis. Disulfide linkages are dissociated by β-mercaptoethanol used for SDS-PAGE under reducing conditions.

In contrast, an irreversible tetramer formation has also been described, produced by transglutaminase crosslinking of glutamate residues of CD38 dimers (Umar et al, 1996). These tetramers of 190 kD have been shown to have increased cyclase activity as compared to the 45 kD monomer.

Other molecular forms of CD38

Molecular forms of CD38 other than dimers or tetramers have also been described. Thus, a 39 kD soluble form of CD38, presumably generated by proteolytic cleavage of surface forms were found in the body fluids of patients with myeloma and other malignancies (Funaro et al, 1996). In the congenital immunodeficiency, X-linked agammaglobulinaemia, a 78 kD soluble form was found in the plasma of such patients, probably originating from CD38 tetramers by proteolytic cleavage (Mallone et al, 1998). A recombinant, 30 kD soluble form has also been engineered, lacking the transmembrane and intracellular portions but with preserved cyclase and hydrolase activities (Fryxell et al, 1995).

Lateral associations of CD38

It has been postulated that CD38 evolved by the association of the soluble cyclase with the cell membrane, thereby enabling the molecule to perform both receptorial and signalling functions (Ferrero et al, 2002). Due to its short intracytoplasmic tail, CD38 depends on other cell-surface molecules in order to signal. Thus, direct lateral associations have been documented between CD38 and the TCR in T-cells, (Morra et al, 1998), with the BCR in B-cells (Lund et al, 1996) with CD16 in NK-cells (Deaglio et al, 2002) and with the Fc gamma receptors in monocytes (Inoue et al, 1997). Indeed, Lund et al showed that CD38 is able to transmit signals to the cell interior without its intracellular and transmembrane region. Another form of lateral

association is its close association with lipid rafts (Zubiaur et al, 2001; Deaglio et al, 2003).

LOCALIZATION

Tissue distribution and intracellular CD38

In addition to its typical membrane location, CD38 has also been found intracellularly in a number of human-and non-human tissues. Thus, its presence has been documented in the endoplasmic reticulum (Sun et al, 2002), mitochondria (Ziegler et al, 1997), nuclear envelope (Adebanjo et al, 1999) and cytosol (Guse et al, 1999). Intracellular CD38 has not been studied in CLL – the subject forms part of Chapters 2 and 3.

Because of its intracellular expression, CD38 is able to utilize intracellular NAD^+ stores for cADPR generation and Ca^{++} mobilisation, thus not relying upon the usually low concentrations of extracellular NAD+ (Sun et al, 2002).

Outside haematopoietic tissues, CD38 has been found at various locations, including the eye (Khoo et al, 1999), liver (Ziegler et al, 1997; Khoo et al, 2000), pancreas (Okamoto et al, 2000), brain (Yamada et al, 1997), cardiac muscle (Chidambaram et al, 1998b), lung (Khoo et al, 1998) and the prostate (Kramer et al, 1995).

Expression on haematopoietic cells

CD34+ haematopoietic stem cells capable of multilineage differentiation and self-renewal are CD38-. Cells with this phenotype constitute less than 10% of all CD34+ stem cells in adults (Campana et al, 2000). However, after cytokine stimulation, cells become CD38+ in parallel with entry to the cell cycle (Holm et al, 1999).

On myeloid cells, CD38 is mainly expressed on precursors while mature neutrophils and monocytes show only weak expression. CD38 is expressed on erythrocytes (Zocchi et al, 1993) and platelets (Ramaschi et al, 1996).

CD38 is strongly expressed on lymphocyte precursors, weakly on peripheral B- and T-lymphocytes, but upregulated on activated lymphocytes and mature plasma cells. In B-cell follicles, CD38 is present on germinal center, but not mantle zone B-

cells (Fernandez et al, 1998). It is noteworthy that it is expressed at stages when cell-to cell interactions are important, such as in the cases of immature precursors and germinal center cells (Konopleva et al, 2000). CD38 has become a valuable tool in identifying different stages in lymphocyte development. It should also be noted that the expression of CD38 is very different in B-cell development in mice (Campana et al, 2000).

Most cases of pediatric T-and B-cell acute lymphocytic leukaemias express CD38 which does not have a prognostic implication in this condition (Koehler et al, 1993). The majority of acute myeloid leukaemias are CD38-positive; M7 subtypes were shown to be negative (Helleberg et al, 1997). CD38 is upregulated in AML M3 blast cells during all-trans retinoic acid (ATRA) treatment; the following interaction between CD38 and its ligand, CD31 on the lung endothelial cells, has an instrumental role in the ATRA-syndrome (Mehta K, 2000).

FUNCTIONS

A comprehensive overview of the multitude of the reported CD38 functions is beyond the scope of this introduction. Therefore, only functional aspects relevant to this Thesis are discussed.

Effects of CD38 ligation and its role as a signal transducer

Many studies have used ligation of CD38 by mAbs to investigate the function of this protein. In normal human B-cells, such ligation results in suppression of lymphopoiesis in immature progenitors (Kumagai et al, 1995), apoptosis in germinal center B-cells (Zupo et al, 1994) and modulation of BCR responses (Lund et al, 1996).

In CLL cells, CD38 is also known to have important modulatory effect on BCR signalling in that only in CD38+ cells does BCR crosslinking influence cell survival/differentiation. Thus, in CD38+ CLL, IgD crosslinking has been reported to enhance cell survival and induce plasmocytoid differentiation, while IgM crosslinking induces apoptosis (Zupo et al, 2000).

The effects of CD38 ligation on B-cells (and other cell types) are not dependent on either the intracellular or transmembrane portions of the molecule, but do require the presence of a "co-receptor" for signalling - the BCR in case of B-cells

(Lund et al, 1996). Thus, CD38 signalling in B-cells is dependent on cross-talk with the BCR, and uses the effector mechanisms of the antigen receptor for signalling.

The enzymatic activities of CD38

CD38 is a bifunctional ectoenzyme, having both cyclase and hydrolase activities. In this respect it differs from the Aplysia enzyme, which has only cyclase activity (Lee HC, 2000).

The major reactions catalysed by CD38 are shown on Fig.2. The synthesis of cyclic-ADP-ribose (Fig.2/A) includes three consecutive steps: first, NAD+ binds to the active site of CD38 in a folded conformation positioned by two tryptophan residues (Trp^{125} and Trp^{189})(1); in the second, intermediate step, adenosine-diphosphoribosylated CD38 is formed and nicotinamide is released (2); in the third, cyclisation step, cyclic-ADP-ribose is produced and released from CD38: the site of cyclization is at the N1-position of the adenine ring (3). The exchange reaction of NAADP formation is illustrated on Fig.2/B. In contrast to cADPR, NAADP is a linear molecule. The hydrolase function of CD38 is illustrated on Fig.2/C. The enzyme is capable to hydrolase both cADPR and NAD+ into ADPR. Under physiological conditions, the hydrolase reaction is dominant with a cyclase/hydrolase ratio of 1:100.

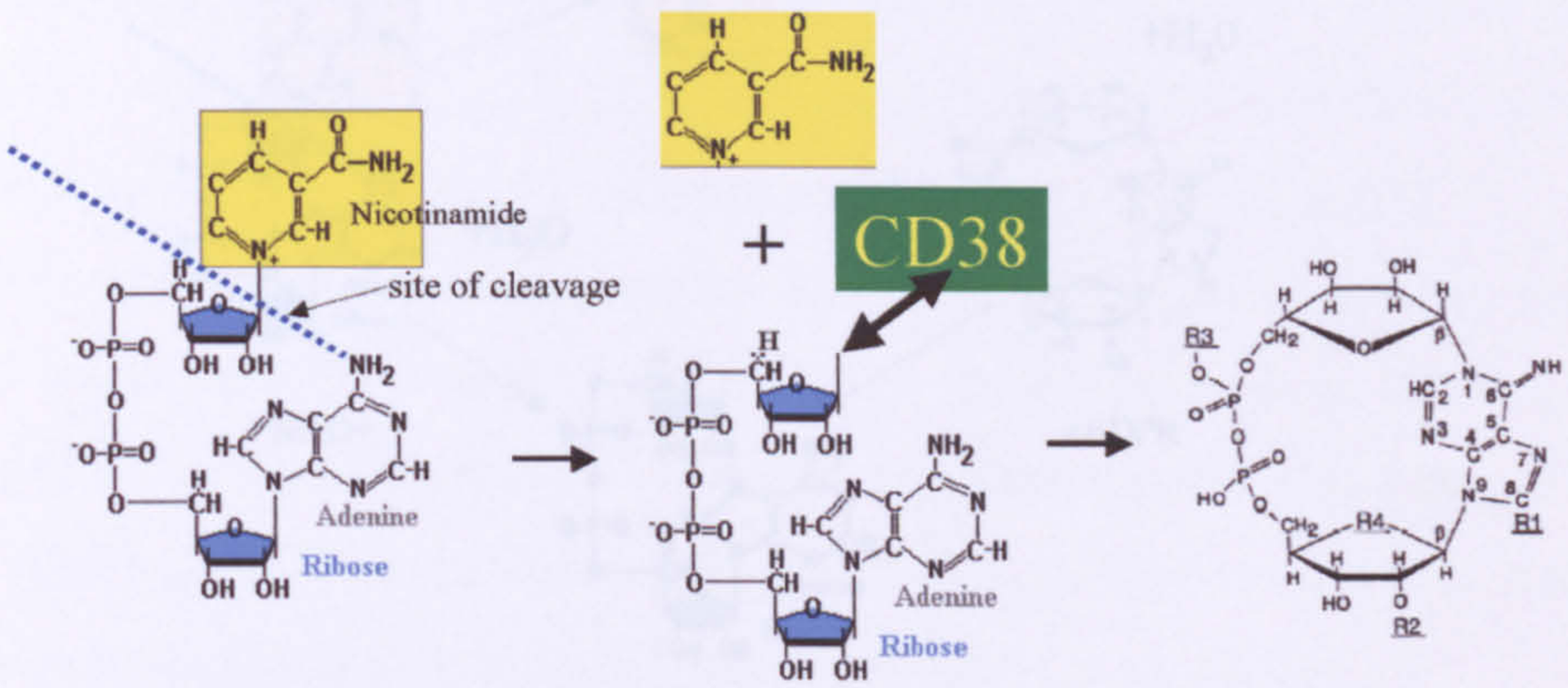


Fig. 2/A The cyclase function of CD38: synthesis of cADPR. Cyclic adenosine-diphosphoribose is formed from NAD+ via an intermediate product, adenosine diphosphoribosylated CD38. In the reaction nicotinamide is released.

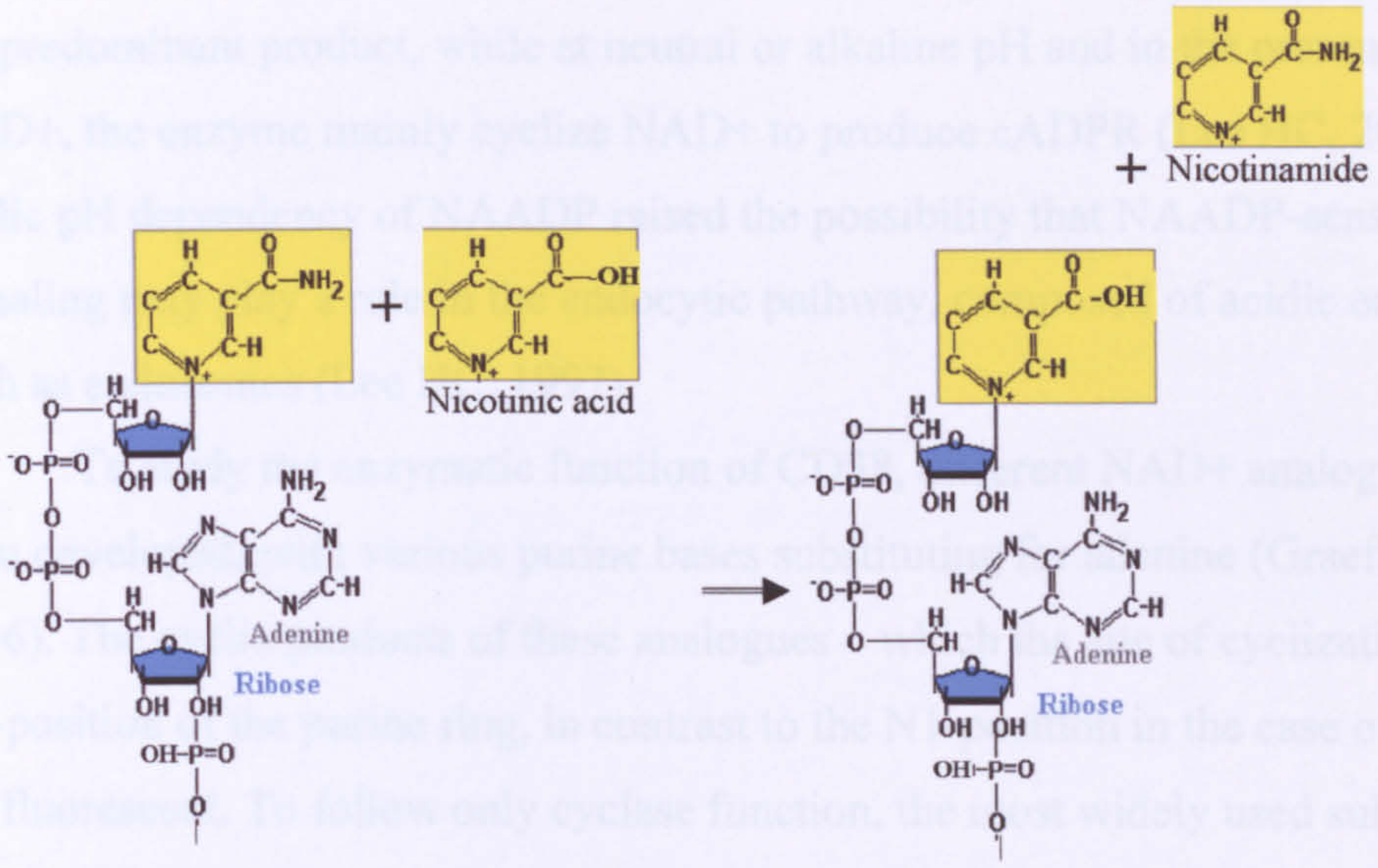


Fig. 2/B The cyclase function of CD38: synthesis of NAADP.

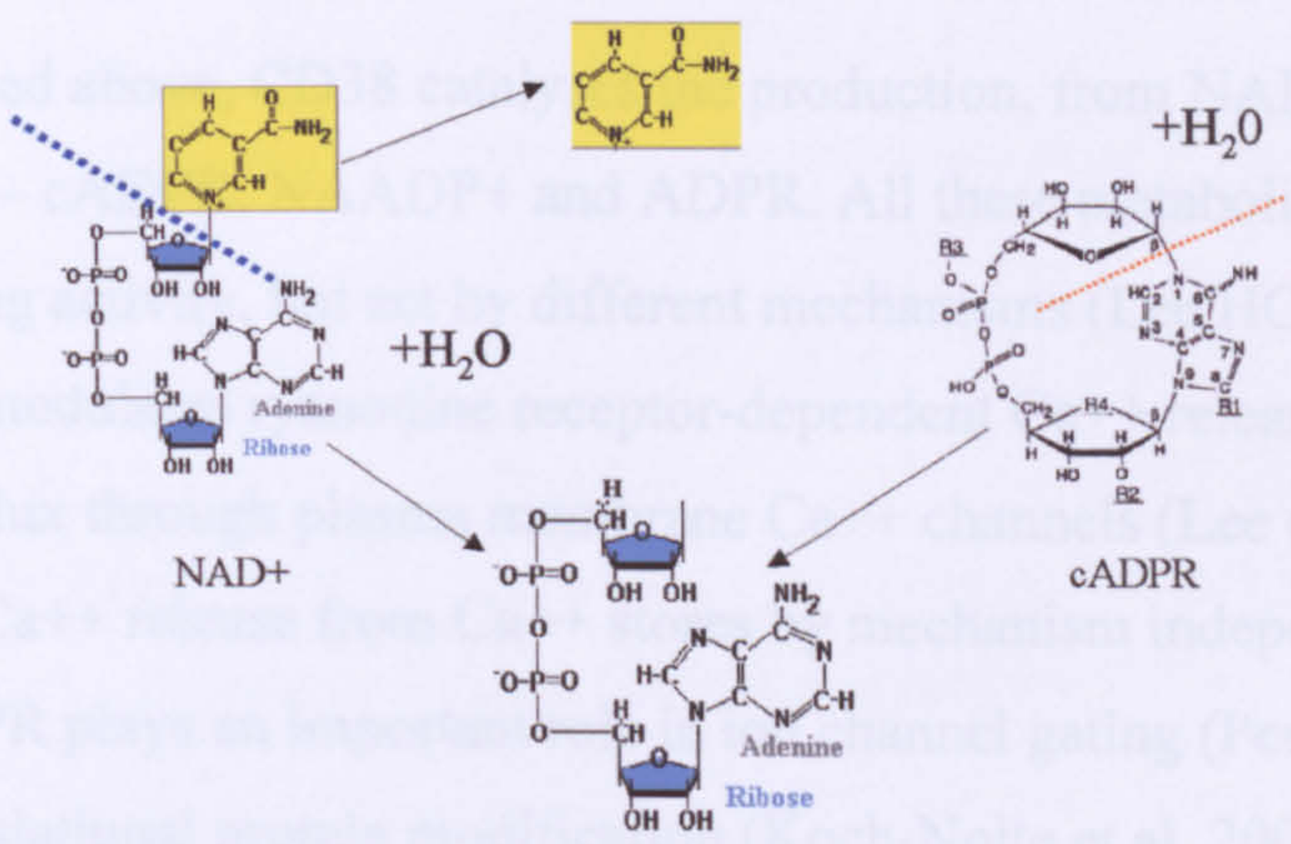


Fig. 2/C The hydrolase functions of CD38: both NAD+ and cADPR can be hydrolysed into ADPR.

Which catalytic path the enzyme takes is determined by pH and the availability of substrate. At acidic pH and in the presence of nicotinic acid, NAADP is the predominant product, while at neutral or alkaline pH and in the presence of NAD+, the enzyme mainly cyclize NAD+ to produce cADPR (Lee HC, 2001). The acidic pH dependency of NAADP raised the possibility that NAADP-sensitive Ca++ signaling may play a role in the endocytic pathway, composed of acidic organelles such as endosomes (Lee HC, 1997).

To study the enzymatic function of CD38, different NAD+ analogues have been developed, with various purine bases substituting for adenine (Graeff et al, 1996). The cyclic products of these analogues – which the site of cyclization at the N7-position of the purine ring, in contrast to the N1-position in the case of cADPR – are fluorescent. To follow only cyclase function, the most widely used substrate is GDPR (adenine substituted by guanine), since cGDPR is resistant to hydrolysis. For hydrolase function, $1,N^6$-etheno NAD is generally used – its hydrolytic products, cyclic etheno-ADP ribose and etheno-ADPR are fluorescent (Graeff et al, 1996). These are the two main substrates also used in this work; further details and results on CD38 enzymatic function in CLL cells are presented in Chapter 3.

Cyclic ADP-ribose and NAADP as calcium messengers

As outlined above, CD38 catalyses the production, from NAD+, of three main products – cADPR, NAADP+ and ADPR. All these metabolites have Ca++ mobilizing activity, but act by different mechanisms (Lee HC, 2001).
cADPR modulates ryanodine receptor-dependent Ca++ release and also regulates Ca++ influx through plasma membrane Ca++ channels (Lee et al, 2004). NAADP+ induces Ca++ release from Ca++ stores by mechanism independent of cADPR and IP3. ADPR plays an important role in ion channel gating (Perraud et al 2001) and post-translational protein modification (Koch-Nolte et al, 2001).

Both cADPR and NAADP have been shown to mediate Ca++ signaling in a wide variety of cells, with downstream functions including cell cycle regulation, insulin secretion, muscle contraction, neurotransmitter release and activation of gene transcription. Among others, cyclic ADP-ribose produced by CD38 has been shown to control neutrophil chemotaxis, thus having an important role in inflammation and

innate immunity (Partida-Sanchez, 2001) and to regulate insulin secretion (Okamoto et al, 2000).

Role in cell cycle

CD38 and cADPR have extensive links with cell proferation. This matter will be discussed in detail in Chapter 4; it is considered here only briefly. CD38 has been shown to be preferentially expressed on proliferating normal human B-cells (Golay et al, 1994) and on cycling haematopoietic stem cells (Holm et al, 1999). Cyclic-ADPR has been reported to have a long-term stimulative effect on human haematopoietic progenitors (Zocchi et al, 2001). CD38 transfection in HeLa cells results in increased cytosolic Ca++ concentrations and shortened doubling time (Zocchi et al, 1998). These associations, knowing the adverse prognostic significance of CD38 in CLL, led us to study the relationship between cell proliferation and CD38 in CLL (Chapter 4).

Cell adhesion and motility

In 1994, the Malavasi group reported that CD38 has weak adhesion properties and might mediate adhesion between CD38+ lymphocytes and endothelial cells (Dianzani et al, 1994). In 1996, a putative ligand for CD38 was identified as PECAM-1 (CD31), a molecule known to be involved in adhesion and extravasation and expressed on monocytes, granulocytes, platelets, a subpopulation of lymphocytes and endothelial cells (Deaglio et al, 2000).

The functional significance of CD38-CD31 interactions in CLL is still unclear. Since CD31 is involved in the phase of extravasation in which leukocytes migrate through endothelial cells (Fernandez et al, 1998), it is possible that CD38 – CD31 interactions may be involved in the transmigration of CLL lymphocytes.

CD38 is also important in leukocyte motility. Thus, neutrophils from CD38-/- knockout mice display defective motility to FMLP (Partida-Sanchez et al, 2001). It is therefore possible, that CD38 contributes to certain types of induced CLL-cell movements.

Regarding CD38 knockout mice, it is known that they develop normally and possess all lymphoid subsets apart from CD5+ B1-cells, which are reduced in the peritoneum. Their lymphocytes show normal in vitro activation assays, but in vivo responses particularly involving humoral immunity are defective. The mechanism of

these defective in vivo responses remains uncertain, but it has very recently been suggested that the defective motility of antigen-presenting cells may be involved (Partida-Sanchez et al, 2004). Thus, Partida-Sanchez et al showed that dendritic cell trafficking in response to a range of chemokines is defective in CD38 -/- animals.

CHAPTER 1

CD38 AS A PROGNOSTIC FACTOR IN CLL

INTRODUCTION

When the work for this thesis was begun (June 2000), there was much interest in whether or not surface CD38 positivity can be used as a surrogate for IgVH mutation in CLL.

The two back-to-back Blood papers in 1999 from Damle et al and Naylor et al, showing that the extent of IgVH mutation varies in CLL and that the levels of mutation correlate inversely with outcome, had generated much attention. One of these two studies (Damle et al, 1999) also looked at CD38 expression in relation to IgVH mutation and found that cases with >30% CD38-positive cells were nearly always unmutated and had a worse prognosis than cases with <30% CD38-positive cells and mutated IgVH genes. On the basis of these findings, Damle et al suggested that CD38 expression could serve as a surrogate for IgVH mutation as well as a prognostic marker, since its measurement is more straightforward than that of IgVH mutation.

Damle et al examined whether CD38 can be a surrogate for IgVH mutation because it was known that, together with surface IgD, expression of this molecule identifies different stages of mature B-cell development. For example, IgD+/CD38- tonsillar B-cells were known to be naïve and IgVH non-hypermutated, while IgD-/CD38+ cells were known to be mutated and of germinal-center origin (Naylor et al, 1999).

In April 2000, Hamblin et al also looked at the prognostic value of CD38 expression and its correlation with IgVH mutation (Hamblin et al, 2000b). These authors confirmed Damle's observation that CD38 expression confers an adverse prognosis, but found no correlation between CD38 expression and IgVH mutation. In response, Damle et al reported on an updated series of CLL cases and confirmed both that CD38 expression is an adverse prognostic marker and that there is a strong correlation between CD38 expression and IgVH mutation (Damle et al, 2000). As a

possible explanation for these inconsistent findings, differences in the procedures used for CD38 measurement were invoked by Damle et al.

Because of the different results obtained by the two groups, the aim of the work described in this Chapter was to examine the relationship between CD38 expression, IgVH mutation and survival in our cohort of CLL patients.

1.2. METHODS

1.2.1.CLL cells

Peripheral blood samples were obtained with informed consent and with the approval of the Liverpool Research Ethics Committee at a median interval of 48 months after diagnosis. Mononuclear cells were prepared by centrifugation over Lymphoprep (Gibco, Paisley, United Kingdom) and cryopreserved in liquid nitrogen. Samples were thawed and washed twice in RPMI containing 1% BSA before processing for FACS analysis.

40 CLL patients (27 men, 13 women) were included with known clinical history. The mean age was 65 (men 61.5, women 72.3) years, and the mean follow-up time 5.4 (1-23) years.

1.2.2. Measurement of surface CD38 on CLL cells by flow cytometry

1-2 x 10^6 CLL cells were resuspended in RPMI/BSA and incubated for 15 minutes with the anti-CD38 HB-7-PE and anti-CD19-FITC antibodies (both in 1:10 dilution) at room temperature in the dark. Both antibodies were purchased from Becton Dickinson. After incubation, cells were washed twice in PBS containing 1% BSA, then resuspended in PBS and analysed on a Becton Dickinson FACSscan flow cytometer (San Jose, CA). In each case, 10 000 events were acquired; data were analysed by the CellQuest software. Cases were considered CD38-positive if the number of CD38+/CD19+ cells was greater than 30% of all CD19+ cells.

1.2.3. IgVH mutation analysis: see Appendix (p.118)

1.2.4. Statistical analysis

Survival analysis was performed by the method of Kaplan and Meier, and significance by the log-rank test. Comparison of CD38 expression before/after freezing was calculated by the paired-samples T-test. For all analyses, the SPSS software was used.

1.3. RESULTS

1.3.1. Effect of cell preparation on CD38 expression

Since differences in cell processing can affect expression of surface molecules, this could potentially have an impact on CD38 expression of CLL cells. To examine this possibility, CD38 expression was compared in CLL cells from fresh whole blood and after freezing/thawing of Ficoll-separated cells. As shown in Fig.1.1, there was no significant difference in CD38 expression in both types of cell preparation. Therefore, in subsequent studies, frozen/thawed cells were routinely used.

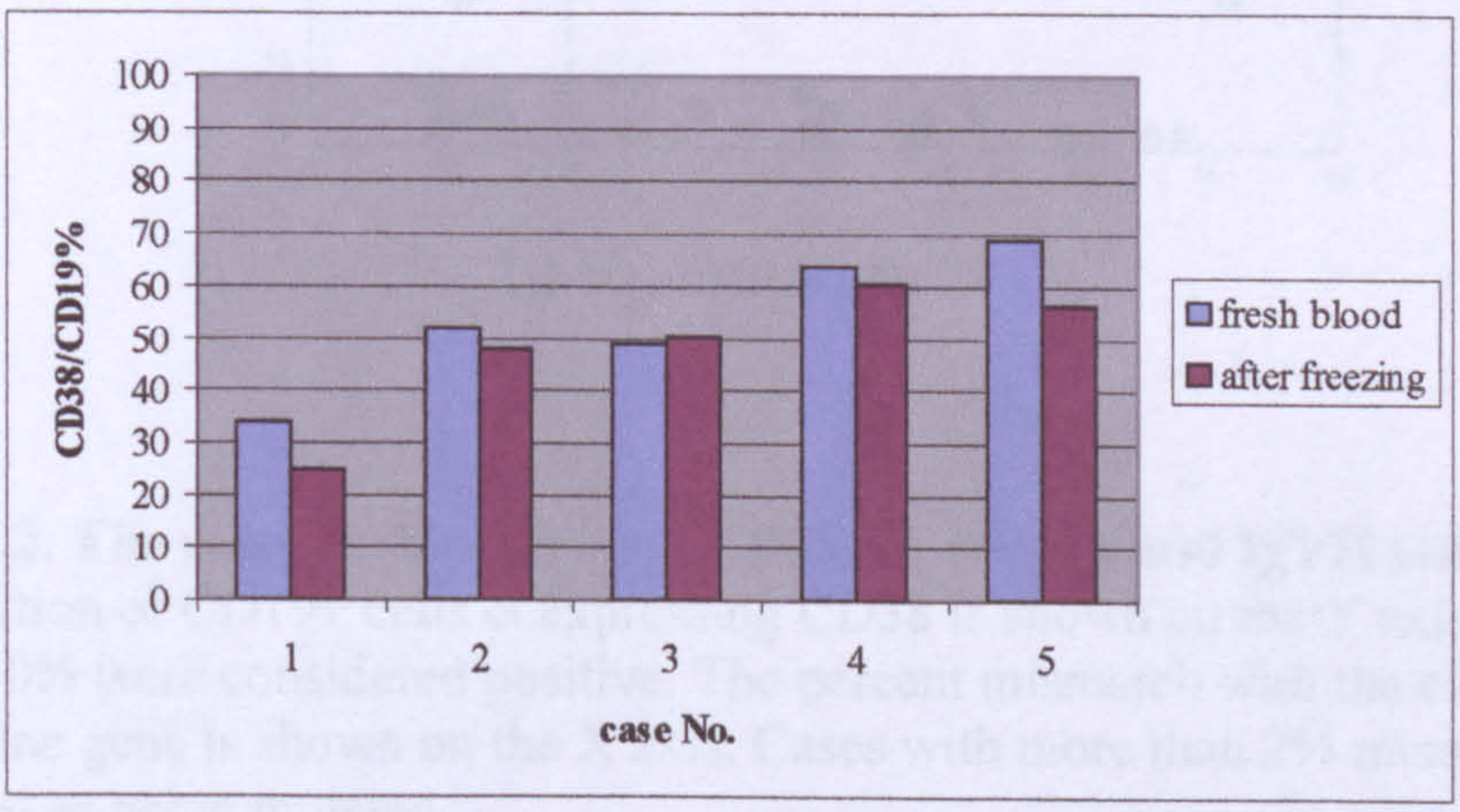


Fig.1.1 The effect of cryopreservation on CD38 expression.
CD38 expression was determined in 5 sCD38+ CLL cases comparing samples before/after freezing and thawing. No significant effect on CD38 expression was found.

1.3.2. Relationship between CD38 expression and IgVH mutation

Using ≥2% difference from germline as a cutoff for somatic hypermutation, 24/40 (60%) cases were classified as unmutated and 16/40 (40%) as mutated, with a male/female ratio of 2.4 in the unmutated and 1.6 in the mutated subgroups. CD38 was expressed in 2 (12.5%) of the 16 mutated cases and in 11 (46%) of the 24 unmutated cases. Among the 13 CD38+ cases, 2 (15%) were mutated and 11 (85%) unmutated. Twenty-seven cases were CD38-, of which 13 (48%) were unmutated and 14 (52%) mutated. These results are shown in Figure 1.2.

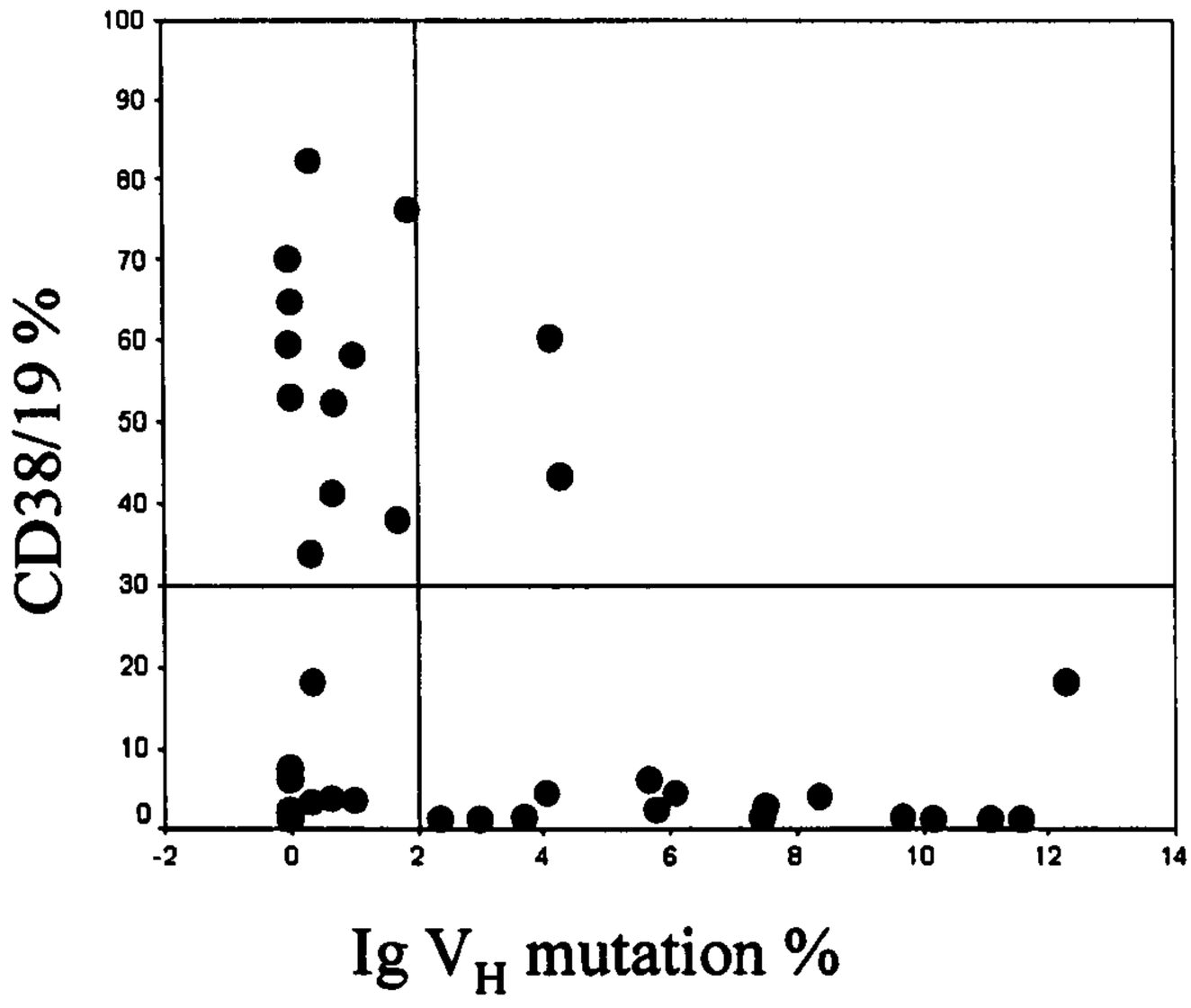


Fig. 1.2. The relationship between CD38 expression and IgVH mutation. The proportion of CD19+ cells coexpressing CD38 is shown on the Y axis. Values greater than 30% were considered positive. The percent mismatch with the closest IgVH germline gene is shown on the X axis. Cases with more than 2% mismatch were defined as being mutated.

1.3.3. CD38 expression and IgVH gene mutation as prognostic indicators

Survival analysis of mutated versus unmutated cases (Fig.1.3/A) showed that the absence of IgVH gene somatic mutation is associated with a worse disease outcome (median survival 9 years, versus not reached; $p<.05$). Similarly, CD38+ patients had a shorter median survival than the CD38- group (5 years versus 13 years; $p<.001$) (Fig.1.3/B).

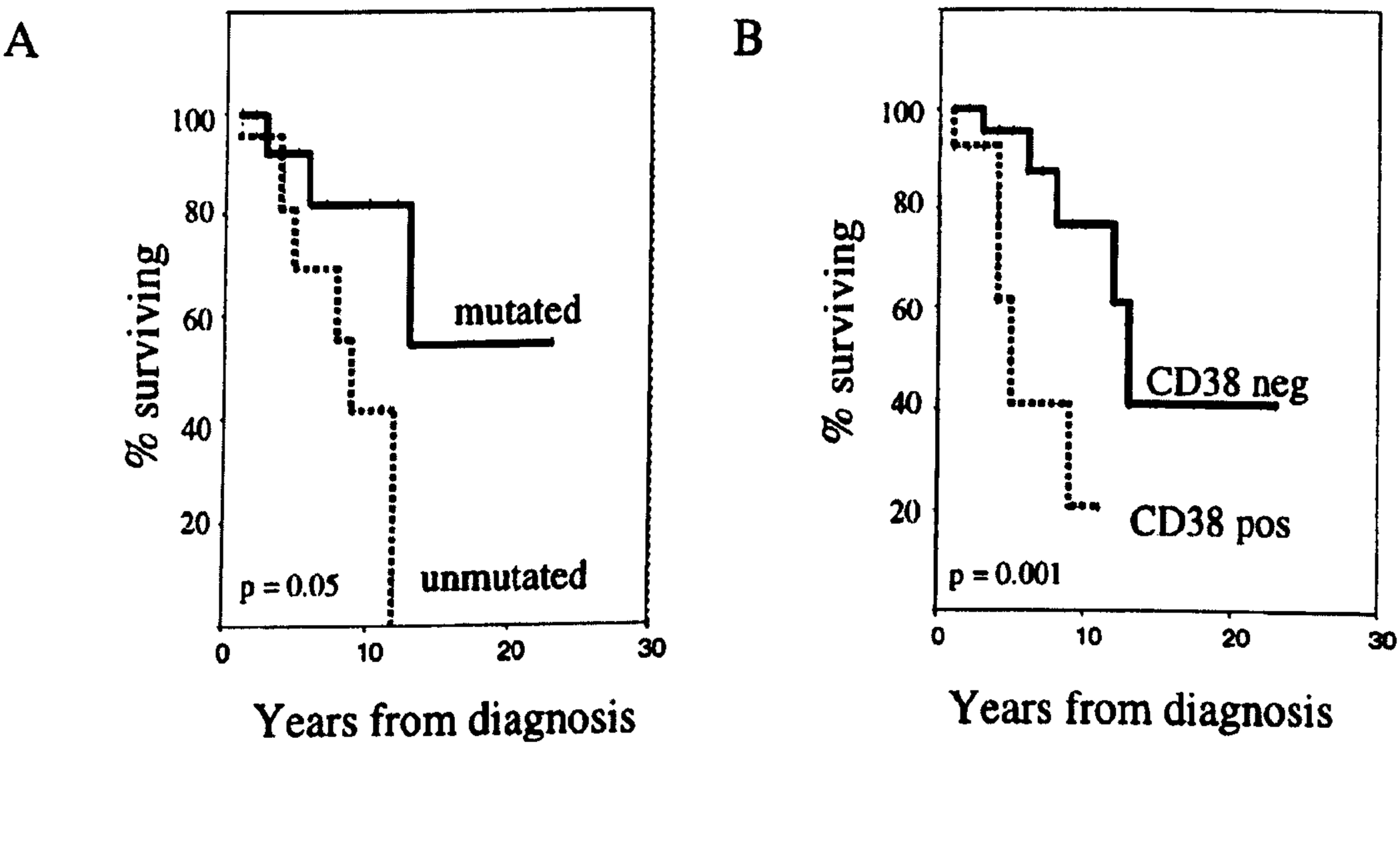


Fig. 1.3. CD38 expression and IgVH gene mutational status as prognostic factors in CLL. The figure shows Kaplan-Meier survival curves comparing cases with mutated vs unmutated IgVH genes (A) and CD38+ versus CD38– cases (B). Deaths not attributable to CLL were censored.

1.3.4. Distribution of CD38 expression in Liverpool cohort of CLL patients

When the above work was performed, the two previous studies on the relevance of CD38 expression for disease prognosis had used a 30% cutoff in defining cases as CD38-positive (Damle et al, 1999; Hamblin et al, 2000). To make the present work comparable with these published studies, the same cutoff was used. Subsequently, other studies have used a number of different cutoff values to define CD38 positivity. It therefore seemed of interest to see how CD38 positivity is distributed in our cohort of patients.

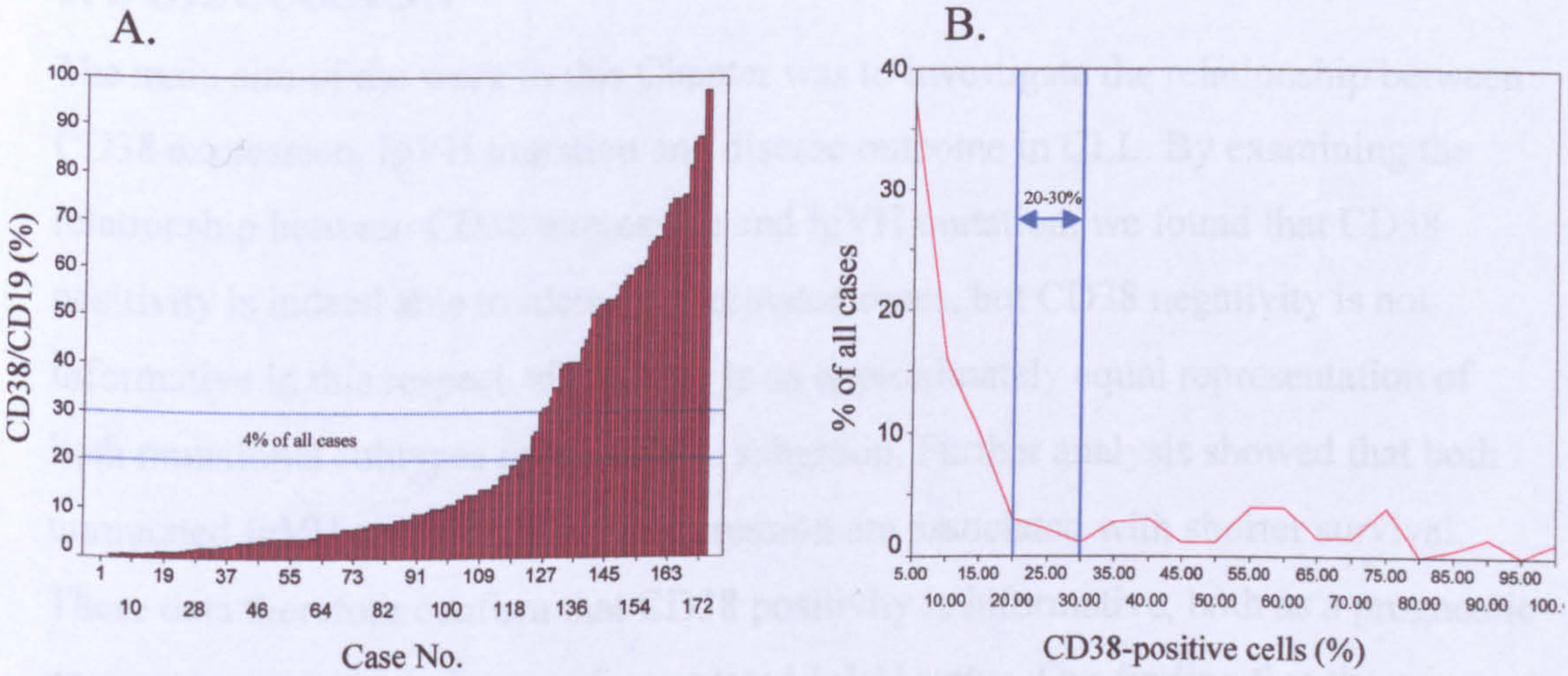


Fig.1.4. Distribution of CD38 values in our CLL patients. A. Distribution of CD38 values in a recently extended series of 174 CLL cases. **B**. Percentages of CD38+ cases in 5% increments in the number of CD38 positive cells. The cases in the first 3 increments (0-15% CD38+ cells) represent 63% of all cases, whereas in the next 3 intervals (15-30% CD38+ cells) this number of cases sharply drops to 6.3% and remains relatively constant in all subsequent increments. The figure also shows that in our cohort of patients, 20% CD38-positive cells is the optimal cutoff separating the two populations. However, since only 3% of all cases fall between 20% and 30%, 30% could be used as well as it was originally proposed (Damle et al, 1999) and used in the present work. Nevertheless, on the basis of Fig.B., a cutoff of 20% was used in the follow-up of our original communication (Lin et al, 2002).

Fig.1.4 shows that there are indeed two subgroups, one with relatively low CD38 values and one with a distinctly higher level of expression. This figure also shows that placing the cutoffs at either 20% or 30% would alter the positive/negative classification of only small number of cases.

1.4. DISCUSSION

The main aim of the work in this Chapter was to investigate the relationship between CD38 expression, IgVH mutation and disease outcome in CLL. By examining the relationship between CD38 expression and IgVH mutation, we found that CD38 positivity is indeed able to identify unmutated cases, but CD38 negativity is not informative in this respect, since there is an approximately equal representation of both mutational subtypes in the CD38- subgroup. Further analysis showed that both unmutated IgVH genes and CD38 expression are associated with shorter survival. These data therefore confirm that CD38 positivity is informative, both as a prognostic parameter and as an indicator of unmutated IgVH status. Our finding that there is a predominance of male gender in the unmutated subgroup is in keeping with those of Damle et al (1999).

Since the work in this Chapter was completed and reported (Matrai et al, 2001), many other investigators have addressed the issue of CD38 expression in CLL, providing a cumulative patient number of over 3000 by the year of 2004 (d'Arena et al, 2001; del Poeta et al, 2001; Heintel et al, 2001; Ibrahim et al, 2001; Jelinek et al, 2001; Morabito et al, 2001; Thornton et al, 2002; Domingo-Domenech et al, 2002; Durig et al, 2002; Hamblin et al, 2002; Kroeber et al, 2002; Lin et al, 2002; Ghia et al, 2003). All these studies uniformly found that CD38 is indeed able to identify progressive cases, even at an early stage of the disease. Moreover, CD38 expression closely correlated with other prognostic factors, such as bad-prognosis karyotypes (Chevallier et al, 2002; Dewald et al, 2003), high β2-microglobulin levels (del Poeta et al; Ibrahim et al, 2001), bulky lymphadenopathy (del Poeta et al; Ibrahim et al, 2001), atypical morphology (d'Arena et al, 2001; Chevallier et al, 2002) and stage (Hamblin et al, 2002). All these data, in conjunction with the fact that CD38 can be measured readily by FACS, make CD38 a valuable tool in the routine diagnostic workup of CLL.

Both 1999 studies that stimulated the present work used 2% IgVH divergence from germline to distinguish between mutated and unmutated CLL (Damle et al, 1999; Hamblin et al, 2000). Therefore, the same cutoff was used in the present work. However, other cutoff values have also been suggested (Kroeber et al, 2002) and

subsequent work from this Department has suggested that 5% might be a biologically more meaningful cutoff (Lin et al, 2002). Furthermore, a more recent study has shown that any deviation from germline probably represents somatic mutation (Davis et al, 2003). There is therefore still controversy about what level of hypermutation should be taken as an optimal cutoff for defining "mutated" and "unmutated" CLL. However, when CD38 expression is corrrelated with hypermutation defined on the basis of 2%, 4% or 5% cutoffs, similar results were obtained. Thus, all these reports are concordant in that unmutated IgVH genes are associated with CD38 expression and mutated genes with CD38 negativity in CLL. However, a not insignificant proportion (10-28%) of all cases give discordant results (Hamblin et al, 2002; Kroeber et al, 2002).

In all clinical and biological studies on CD38, an important point of debate has been how to define a cell population as "positive" or "negative". The most frequently used approach has been to regard a case as CD38-positive if the percentage of CD38-expressing CLL cells is above a certain cutoff. In this study, we used the 30% cutoff to ensure comparability with previous studies. However, there is still no consensus regarding the optimal cutoff for definition of CD38 positivity and other values have been employed, such as 20% or 5% (Ibrahim et al, 2001; Thornton et al, 2002). Therefore, we examined the distribution of CD38 positivity in our cohort of patients (Fig.1.4.) and found that up to two-thirds (67.2%) of the cases had less than 20% CD38-positive cells and only 4% were between 20% and 30%. In the remaining cases (28.1%), CD38 positivity steeply increased from case to case to almost 100%. The present data therefore support the notion that there are two distinct subgroups of CLL with regard to CD38 expression and indicate that in our patients, 20% is probably the best cutoff. However, since very few cases fall between 20% and 30%, it makes little difference which of these cutoffs are chosen.

In order to refine subgroup division, others used various statistical measures to find the optimal cutoff value for CD38 positivity. Hamblin et al found 30% using the Youden index (Hamblin et al, 2002), while Kroeber et al showed that 7% is better than 30% using maximally selected log-rank statistics (Kroeber et al, 2002).

As a departure from this confusing multitude of percentage cutoffs, Ghia et al proposed that the single most important fact having an impact on clinical behaviour is the presence or absence of a CD38-positive population within the clone, irrespectively of its size (Ghia et al, 2003). By this approach, three populations can be outlined - one purely positive, one purely negative and a bimodal group showing both a positive and

a negative subpopulation. It is worth noting that cases classified as positive or negative by this method would fall mostly into the same category using the 20% or 30% cutoffs. Bimodal cases, however, (which constituted 27% of all patients in the mentioned study) would be labelled differently by other cutoffs.

It has also been suggested by two reports that quantitative assessment of CD38 molecules on the surface of CLL cells might be superior to standard cutoffs (Mainou-Fowler et al, 2002; Hsi et al, 2003). Mainou-Fowler et al found that in stage A patients and in those younger than 60 years, antibody-binding capacity (ABC) was superior to the 30% cutoff in terms of predictive power. Using an arbitrary ABC cutoff of 250, 34% of cases were classified as "positive" – a value very much in line with the conventional 30% cutoff. These authors also demonstrated that ABC scores closely correlate with CD38 percentages. Hsi et al – using another quantitation system and a different ABC cutoff (100) – classified 70% of their patients as positive. Since these two investigators used different reagents to quantitate CD38 expression, direct comparison is not possible. However, its worth noting that the 70% given in the latter study as the proportion of CLL cases rated as "positive" seems a very high value given that in most series, around 30-40% of patients are CD38-positive (using the 30% cutoff). This questions the validity of their chosen cutoff. Probably partly for these considerations, and partly because the method is more difficult to apply, quantitative CD38 measurement has not been widely adopted.

Despite all these differences in approaches to measurement of CD38 positivity, all groups agree that positivity is related to bad prognosis. As regards the relationship between CD38 positivity and IgVH mutation, changes in cutoffs in defining positivity of both of these parameters will clearly affect the closeness of this relationship. Nevertheless, there is a general agreement – supported by the present study – that CD38+ clones are mostly unmutated, although the existence of a significant number of discordant results indicate that the two prognostic parameters are to some extent independent.

In all the above studies, including the present investigation, a single antibody (HB-7) was used. However, a report from the Malavasi group indicates that it probably matters which antibody is used to define CD38 positivity (Morabito et al, 2001). Thus, in their study, an antibody known as IB4 was used, and no correlation was found between CD38 expression and prognosis using the 30% cutoff. Only when a score system was derived combining CD38 percentage and fluorescence intensity

was a relationship found between CD38 expression and prognosis. It seems quite possible that the IB4 antibody detects a different form of CD38 from that reactive with HB-7. It seems appropriate therefore, to consider the choice of antibody as an important factor which might influence results, something to be reckoned with in interpreting clinical studies of CD38 in CLL. Since most investigators used HB-7 for the clinical surveys, it also seems important to determine the potential and limitations of this mAb to detect different forms of CD38 and to relate its immunoreactivity to other anti-CD38 antibodies. This concept will be developed further in Chapters 2 and 3.

CHAPTER 2

BIOCHEMICAL AND IMMUNOCYTOCHEMICAL STUDIES OF CD38 IN CLL

2.1. INTRODUCTION

In all the clinical studies discussed in detail in the previous chapter, CD38 expression of CLL cases was determined solely by FACS measurement of surface expression of the molecule (sCD38). However, in the last 5 years, much data have accumulated showing that, in both human and non-human tissues, CD38 is also present intracellularly - in the endoplasmatic reticulum, nuclear envelope, mitochondria and cytosol (Adebanjo et al, 1999; Sun et al, 2002). It has also been established that CD38 has several different molecular forms which have been detected under normal and pathological conditions (Funaro et al, 2000).

The standard transcript of the CD38 gene is a 45 kD type II transmembrane ectoenzyme consisting of 300 amino acids, with short cytoplasmic and transmembrane portions and a long ectocellular domain; this latter part harbours the active site of the molecule (Lee HC, 2000). Different molecular forms are generated partly as a result of the propensity of the monomeric protein to form dimers and tetramers with MWs of 90 and 190 kD respectively (Bruzzone et al, 1998). The ADP ribosyl cyclase activity of the molecule increases with the degree of multimerisation (Umar et al, 1996). The high MW forms of the molecule are progressively stabilised by the formation of intermolecular disulphides and ultimately by transpeptide links formed by transglutaminase. Other molecular forms of 39 & 78 kD have also been identified mainly in cell supernatants and are presumably produced by proteolytic shedding of different CD38 molecules (Funaro et al, 1996; Mallone et al, 1998). Given these data, it seemed important to study intracellular CD38 in CLL to determine what molecular forms of CD38 are present in sCD38+ vs CD38- cases and to establish their topology.

2.2. METHODS

2.2.1. Definition of CD38 positivity

As indicated in Chapter 1, most clinical studies of CD38 have employed surface staining with the HB-7 mAb and have chosen a cut-off of 20-30% CD38+/CD19+ cells in identifying a given case as CD38 positive or negative.

The work described in the present chapter shows that very different results are obtained with different mAbs or when cells are permeabilised before staining (see later). Nevertheless, the standard method of classifying a clone as CD38 positive or negative is retained in this chapter. Thus, a clone is designated as $sCD38^+$ when ≥30% cells possess surface reactivity with HB-7 and as $sCD38^-$ when <30% cells show surface positivity with this antibody.

Because CLL cells possess only small amounts of CD38, in most instances, cases with a high percentage of $sCD38^+$ cells (>40%) were used in studies of $sCD38^+$ clones. Also, since $sCD38^-$ clones can contain significant numbers of sCD38+ cells, clones with a low percentage of $CD38^+$ cells (<5%) were employed when studying $sCD38^-$ clones.

2.2.2. CLL cells

CLL cells were isolated from peripheral blood by Ficoll-Hypaque density gradient centrifugation and stored in liquid nitrogen before use. Frozen cells were thawed at 37C and slowly reconstituted in RPMI containing 1% BSA. To minimize contamination with non-CLL cells, cases with high WBC counts were chosen, resulting in >90% CD19+ cell percentages.

In certain experiments, further purifications were carried out by immunodepletion with magnetic beads. T-cells and monocytes were removed with beads coated with anti-CD3 and anti-CD14 antibodies. Surface CD38+ cells were removed by exposing the mononuclear cells to HB-7 mAb and then to beads coated with goat anti-mouse antibody.

2.2.3. Antibodies

The following mouse monoclonal anti-human antibodies were used in the experiments presented in this chapter: Ab-4 (NeoMarkers); C1-22 (BD Transduction); Ab-2 (Neomarkers); sc-7325 (Santa Cruz Biotechnology); C1586 (Sigma); pure and PE-conjugated HB-7 (Becton Dickinson); AT-1 (NeoMarkers). The polyclonal goat antihuman CD38 C-19 was purchased from Santa Cruz Biotechnology. Second-layer antibodies for Western Blotting were: goat anti-mouse Ig (BD Transduction); donkey anti-goat Ig (Santa Cruz). The second-layer antibody used in permeabilised FACS and confocal microscopic experiments was the goat anti-mouse-FITC (Becton Dickinson). For the APAAP experiments, the rabbit anti-mouse immunoglobulin and the APAAP immunocomplex were purchased from DAKO.

All antibodies used are known to be directed against the ectocellular domain of human CD38, containing the carboxy terminus. With the exception of C1-22, the exact location of the reactive epitope is not known; in the case of C1-22, the immunogenic peptide comprised of amino acids 171-292 of the ectocellular part of CD38. The various antibodies used, together with their specificities are listed in Table 2.1.

2.2.4. Cell lysates

In order to preserve CD38 multimeric structures, in most of the experiments the mild detergent CHAPS was used for the lysis of CLL and PCL cells (Umar et al, 1996). Briefly, cryopreserved CLL and PCL cells were thawed in RPMI/BSA, washed in PBS, then lysed in ice-cold PBS (pH 7.4) with 3% CHAPS and proteinase inhibitors (1mM phenylmethylsulfonyl fluoride/PMSF/, 1μg/mL aprotinin, 1μg/ml leupeptin, 5 mM EDTA and 1mM sodium orthovanadate) for 30 minutes on ice. The volume of lysis buffer was adjusted according to the number of cells lysed and varied depending on the type of experiments but, in the most instances, 10μl lysis buffer was used for 10^6 cells. Nuclei and insoluble cellular material were removed by centrifugation at 14000 rpm for 20 mins at 4°C. If needed for later use, lysates were frozen at –20°C.

antibody	clone	Manufacturer	nature	immunogen	epitope detected
Ab-4	38C04	NeoMarkers	mouse monoclonal	CEM T cell line	NK
CI-22	22	BD Transduction	mouse monoclonal	human CD38 peptide	aa.171-292 (ectocellular)
Ab-2	AT2	NeoMarkers	mouse monoclonal	CEM T cell line	NK
sc-7325	AT1	Santa Cruz	mouse monoclonal	NK	NK
C1586	HI157	Sigma	mouse monoclonal	CEM T cell line	NK
HB-7	HB7	BD	mouse monoclonal	BJAB cell line	ectocellular
AT1	AT1	Ancell	mouse monoclonal	CEM T cell line	NK
C-19	sc-7047	Santa Cruz	goat polyclonal	human CD38	peptide at carboxy term.

Reported activities[1]						
antibody	WB	IP	IC	FACS	IH	Own results
Ab-4	yes	yes	NK	yes	yes (F)	All CLL cells +ve by FACS; all -ve in frozen sections
CI-22	yes	NK	NK	NK	yes (F)	Strongest reactivity with CD38 monomer (WB)
Ab-2	yes	yes	NK	NK	yes	Unreactive in WBs
sc-7325	yes	NK	NK	yes	yes(F+P)	Unreactive with 45 kD monomer (WB)
C1586	NK	NK	NK	yes	NK	Unreactive in WBs
HB-7	NK	NK	NK	yes	NK	Used for CLL subclassification
AT1	yes	yes	NK	yes	NK	Inactive in WBs, but active in IPs
C-19	yes	NK	NK	yes	yes (F)	Reactive with 45 kD monomer (WB)

Table 2.1. Anti-CD38 antibodies used in this work and their reported activities.

[1]As reported in the product information. WB = Western blotting; IP = immunoprecipitation; IC = APAAP immunocytochemistry; IH = immunohistology in frozen (F), or paraffin-embedded (P) sections. NK = not known.

In some of the experiments performed early in the course of this work, a harsher procedure for cell lysis was also employed. Cells were thawed as above, then lysed in boiling Laemmli's sample buffer (DSSB) containing 4% SDS, β-mercaptoethanol and proteinase inhibitors as above. Gentle sonication was used to dissolve insoluble material.

2.2.5. Western blotting

Lysates prepared in DSSB were loaded directly on the gel, while those lysed in CHAPS were mixed with DSSB (in a 1:1 ratio) before loading. Along with the samples, prestained (Invitrogen Ltd) and, in some experiments, unstained molecular weight markers (Sigma-Aldrich) were applied. Samples were run on SDS-PAGE using a 10% separating gel and a 5% stacking gel in a BioRad minigel system. After electrophoresis, proteins were transferred to a nitrocellulose membrane (Immobilon-P; Millipore). All equipment used in these experiments was from BioRad. Membranes were blocked in a blocking buffer (5% non-fat milk in TRIS-buffered saline consisting of 10mM TRIS, 150 mM NaC1/pH 7.4 with 0.1% Tween (TBST) for 1 hour at room temperature. Membranes were incubated overnight at 4°C with the different anti-CD38 antibodies dissolved in blocking buffer at 1μg/ml concentration, then rinsed twice and washed 2 x 15 minutes in TBST. Incubation with a peroxidase-conjugated goat anti-mouse (Transduction Laboratories) (1:6000) or donkey anti-goat (Santa Cruz Biotechnology) (1:15000) secondary antibody was carried out for 1 hour at room temperature. After two further washings, the membranes were incubated in the ECL reagent (enhanced chemiluminescence system, Amersham International PLC) for 1 min. at room temperature, then wrapped in foil and exposed to a chemoluminescence sensitive film. Antibody binding was indicated by the blackening of the film.

2.2.6. Immunoprecipitation

CHAPS lysates of CLL and PCL cells were used for immunoprecipitation experiments. Protein-G beads were washed twice in 3% CHAPS, then coupled to the immunoprecipitating antibody by incubating for 1 hour at 4°C on a sample rotator. In most experiments, 2μg of the immunoprecipitating antibody were used per sample. To preclear the samples, cell lysates were incubated with protein-G beads for 3 hours at 4°C with continuous rotation; non-specifically bound proteins were discarded with the beads after centrifugation. Immunoprecipitation was performed by incubating the precleared cell lysates with the anti-CD38 antibodies coupled to protein-G overnight on a mixer. Immunoprecipitates were washed twice in CHAPS, dissolved in 30 μl DSSB and boiled for 5 mins at 95°C, than either submitted to electrophoresis immediately or were frozen at –20°C for later use.

2.2.7. APAAP immunohistochemistry

Cytospin preparations of 10^5 CLL and PCL cells were made, air dried, fixed and permeabilised in acetone for 5 mins. After washing with TBS-HC1 (pH 7.4), 50µl anti-human CD38 mouse mAbs (Ab-4, Ab-2, HB-7, C1-22, sc-7325) were added (1:10) in 1% human AB serum and incubated in a humidified chamber for 30mins. All incubations were performed at room temperature. After washing the slides 3 times with TBS, 50µl of rabbit-anti-mouse immunoglobulin (1:25) (DAKO) AB serum was added for 30 minutes. After 3 washes with TBS, 50µl APAAP immunocomplex (1:20) (DAKO) in AB serum were added and the slides incubated for a further 30 minutes. After rinsing, the previous two steps were repeated. Slides were washed 3 times and chromogenic substrate (naphthol AS-MX /3-hydroxy- 2-naphtoic acid 2,4-dimethylanilide/ phosphate plus Fast Red TR salt) added for 30 minutes. The reaction was stopped by adding TBS, then one drop of Harris haematoxylin was applied and after 3 minutes incubation, cytospins were washed in tap water for 5 minutes. Slides were mounted in aqueous mounting medium under coverslips.

2.2.8. Detection of surface and intracellular CD38 in CLL cells by flow cytometry

2.2.8.1. Surface staining

2×10^6 CLL cells were resuspended in RPMI/BSA and incubated for 30 minutes at 4C with the following unconjugated mouse mAbs: Cl-22, AT1, HB-7, Ab-4 and Ab-2 (all in 1:10 dilution). Cells were washed twice in PBS/BSA and incubated for 30 minutes at 4C in the dark with goat anti-mouse-FITC (1:15). After two final washings in PBS/BSA, cells were resuspended in PBS and analysed.

2.2.8.2. Intracellular staining

Cell permeabilisation and intracellular staining was performed according to the protocol described by Jacob et al (1991). Briefly, 1-2 x 10^6 CLL cells were incubated for 30 mins at 4°C in 50µl Hanks balanced salt solution with 0.3% saponin and 2% fetal calf serum (FCS), containing the different mouse anti-human CD38 antibodies (1:10). Cells were washed twice in PBS containing 1% BSA and 0.1% saponin and then incubated for 30 mins at 4°C in 50µl 0.3% saponin/Hanks solution/FCS containing the second-layer goat anti-mouse-FITC antibody (1:15). After two washes in PBS/BSA/saponin, cells were analysed on a FACScan flow cytometer. For each case studied, 10,000 cells were processed; data were analysed by the CellQuest software.

2.2.9. Detection of surface and intracellular CD38 by confocal microscopy in CLL cells before and after permeabilisation

Permeabilised and non-permeabilised cells were stained for CD38 exactly as for surface and intracellular FACS. After incubation with the FITC-conjugated goat anti-mouse second-layer antibody, cells were washed twice in PBS/BSA/saponin, cytospin preparations made, air dried, mounted and analysed on a Bio-Rad MicroRadiance confocal laser scanning microscope. Fluorescein was excited by an argon laser at 488nm and emission signals were collected with an HQ 515/30nm emission filter set. Optical sectioning was performed at 1µm increments. Images were acquired, stored and processed by the LaserSharp 2000 software.

2.2.10. Purification of CLL samples from T-cells, monocytes and CD38+ CLL cells by immunomagnetic beads

All equipment used for this procedure was purchased from Miltenyi Biotec. Before applying samples, columns were rinsed through with buffer. CLL cells (10^8/sample) were thawed, washed 1x in PBS and resuspended in 800 µl degassed buffer (PBS with 2 mM EDTA and 0.5% BSA). 200 µl anti-CD14 and anti-CD3 immunomagnetic beads (MACS)

were added, mixed and incubated for 15 minutes at 10C. Also, CLL cells were incubated with HB-7 mAb for 15 minutes at RT. After two washings with buffer, cells were then incubated with goat anti-mouse IgG microbeads. All three labelled fractions were then washed twice, resuspended in 500 µl buffer and run through on MACS LS+ separation columns. The negative, CD3, CD14 and CD38-purified fractions were collected and processed further for cell lysis and Western blotting and mRNA determinations.

2.3. RESULTS

2.3.1. Western blotting of whole-cell lysates

The aim of this part of the work was to examine CD38 expression in CLL cells by Western blotting in order to determine what molecular forms are present in cells expressing different levels of CD38 on their surface.

2.3.1.1. Comparison of anti-CD38 antibodies in detection of CD38 in CLL and PCL cell lysates

In preliminary experiments, six antibodies (Ab-4, C-19, Cl-22, Ab-2, AT-1 and C1586) were tested for their ability to detect CD38 in CHAPS lysates of sCD38+ CLL cells after SDS-PAGE under reducing conditions. Strongly sCD38+ plasma-cell leukaemia (PCL) cells were used as a positive control.

Three of these antibodies (Ab-4, C-19 and Cl-22) detected the 45 kD form of CD38 in both CLL and PCL cell lysates (Fig.2.1). This 45 kD band was much more prominent in the plasma cells, indicating that this cell type contains considerably more CD38 monomer than do CLL cells. In addition, a number of other reactive bands were seen. A band of ~60 kD was observed with both Ab-4 and C1-22. Also, high MW bands were detected with both of these antibodies, but these were of different sizes (~205 kD with Ab-4 and ~172 kD with C1-22). Lastly, a low MW band of ~27 kD was present in the Ab-4 and C-19 blots.

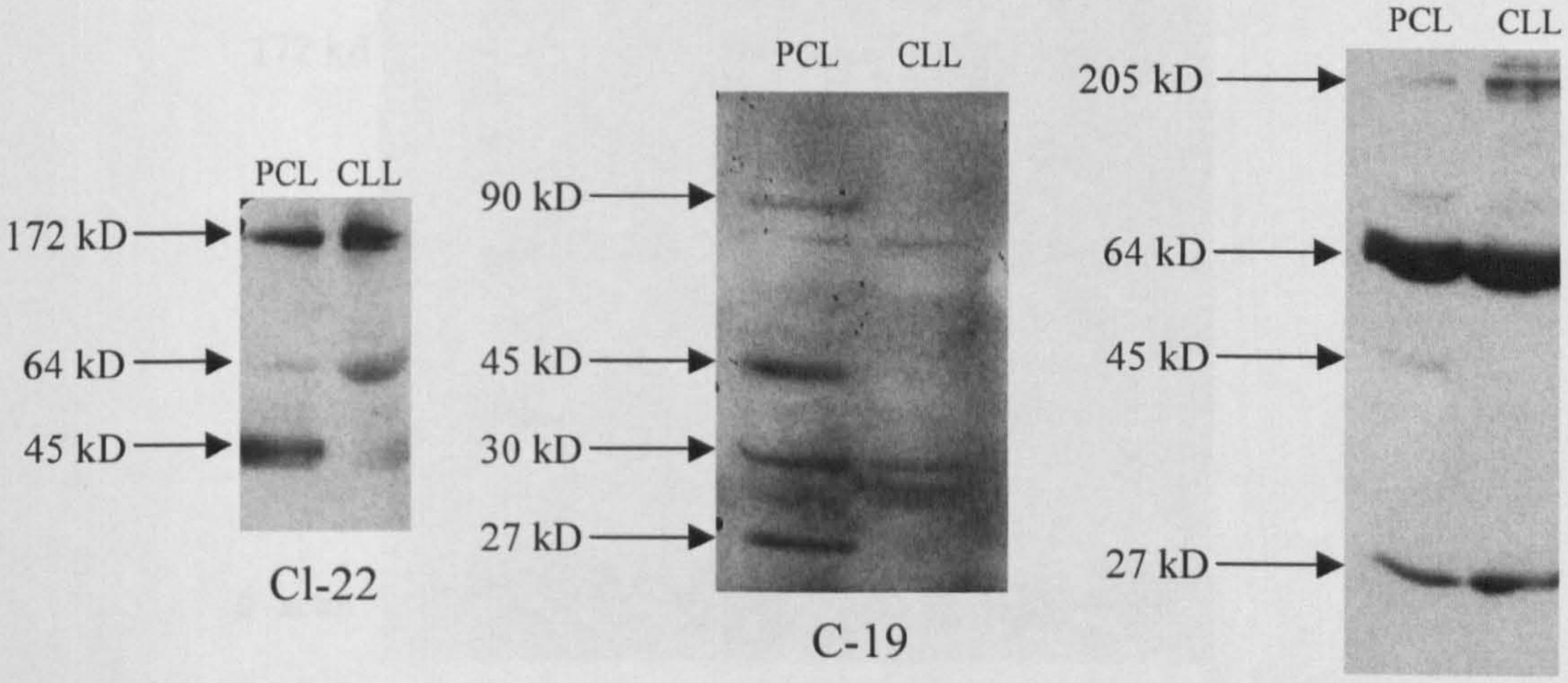


Fig.2.1. Antibodies detecting the 45 kD form of CD38.
Cell lysates of sCD38+ CLL and plasma-cell leukaemia (PCL) cells ($2x10^6$ per lane) were extracted with 3% CHAPS. After SDS-PAGE under reducing conditions and transfer to a nitrocellulose membrane, the separated proteins were exposed to the indicated anti-CD38 antibodies (1μg/ml). Bound antibody was detected by HRP-conjugated goat anti-mouse antibody or, in the case of the goat polyclonal antibody C-19, with HRP-conjugated donkey anti-goat Ig. Secondary antibody was then detected by the ECL reagent.

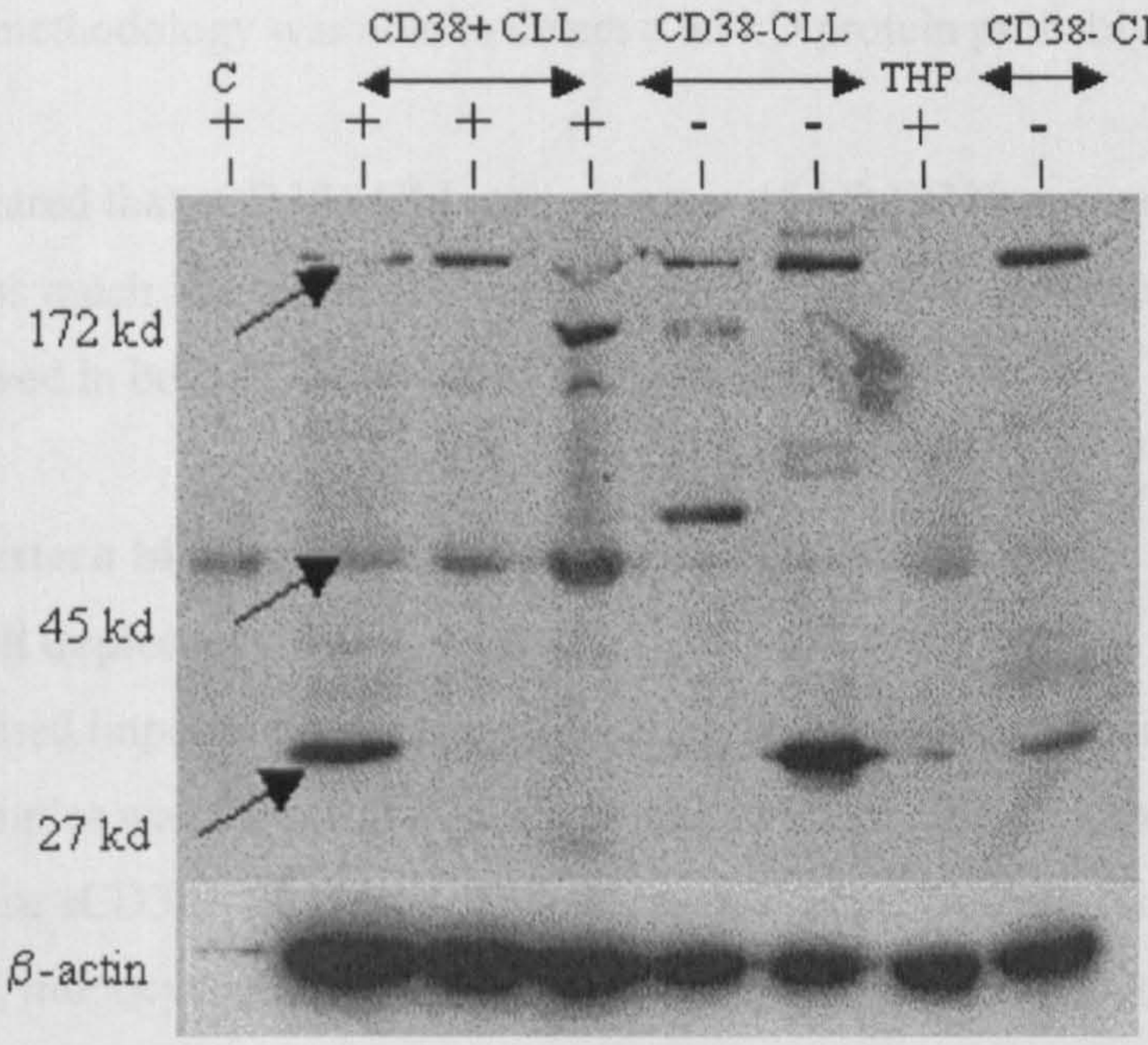


Fig. 2.2 Western blotting of sCD38+ and sCD38- CLL cell lysates with Cl-22 anti-CD38 antibody. Western blotting was performed as an Fig.2.1 except that double-strength sample buffer (containing 4% SDS) was used for extraction. Arrows mark the three main immunoreactive bands detected at 45, 27 and 172 kD. EB-1 (Burkitt) cells (C) and THP cells (THP) served as positive controls.The picture shown is a representative example of blots performed with more than 20 cases of sCD38+ and sCD38- CLL cases. The membrane was re-probed for β-actin to control for protein loading.

2.3.1.2. Western blotting of sCD38+ vs sCD38- CLL cell lysates

Because C1-22 gave the strongest 45 kD band in the positive plasma-cell leukaemia control (Fig.2.1), this mAb was subsequently used to probe sCD38+ versus sCD38- CLL clones (Fig.2.2). A 45 kD band was readily detected in sCD38+ CLL clones and was also weakly present in sCD38- CLL cells. Of the other bands strongly reactive with C1-22, the 172 kD protein was present in all CLL samples, regardless of their surface expression of CD38. A 27 kD band was detected in both sCD38+ and sCD38- clones, but only in some cases. Various other bands were also inconsistently present: the ~60 kD seen in CHAPS lysates was usually not observed in Cl-22 blots of lysates extracted in DSSB.

It was therefore concluded at this stage that, with at least 3 of the antibodies tested, the methodology was able to detect a 45 kD protein probably representing CD38 monomers.

It also appeared that sCD38+ CLL cells contain 45 kD CD38 monomer, while sCD38- cells possess much less molecular form. The other putative molecular forms of CD38 were observed in both sCD38+ and sCD38- clones.

2.3.1.3. Western blotting of CLL-cell lysates before/after T-cell, monocyte and sCD38+ cell depletion

Next it seemed important to determine whether or not the weak positivity observed in sCD38- samples was the result of the presence of either small numbers of T-cells, monocytes or sCD38+ CLL cells. To address this issue, Western blotting was repeated after T-cell, monocyte and sCD38+ cell depletion (by antibody-coated magnetic beads, see Methods). After this purification, the 45 kD band became barely detectable (Fig.2.3).

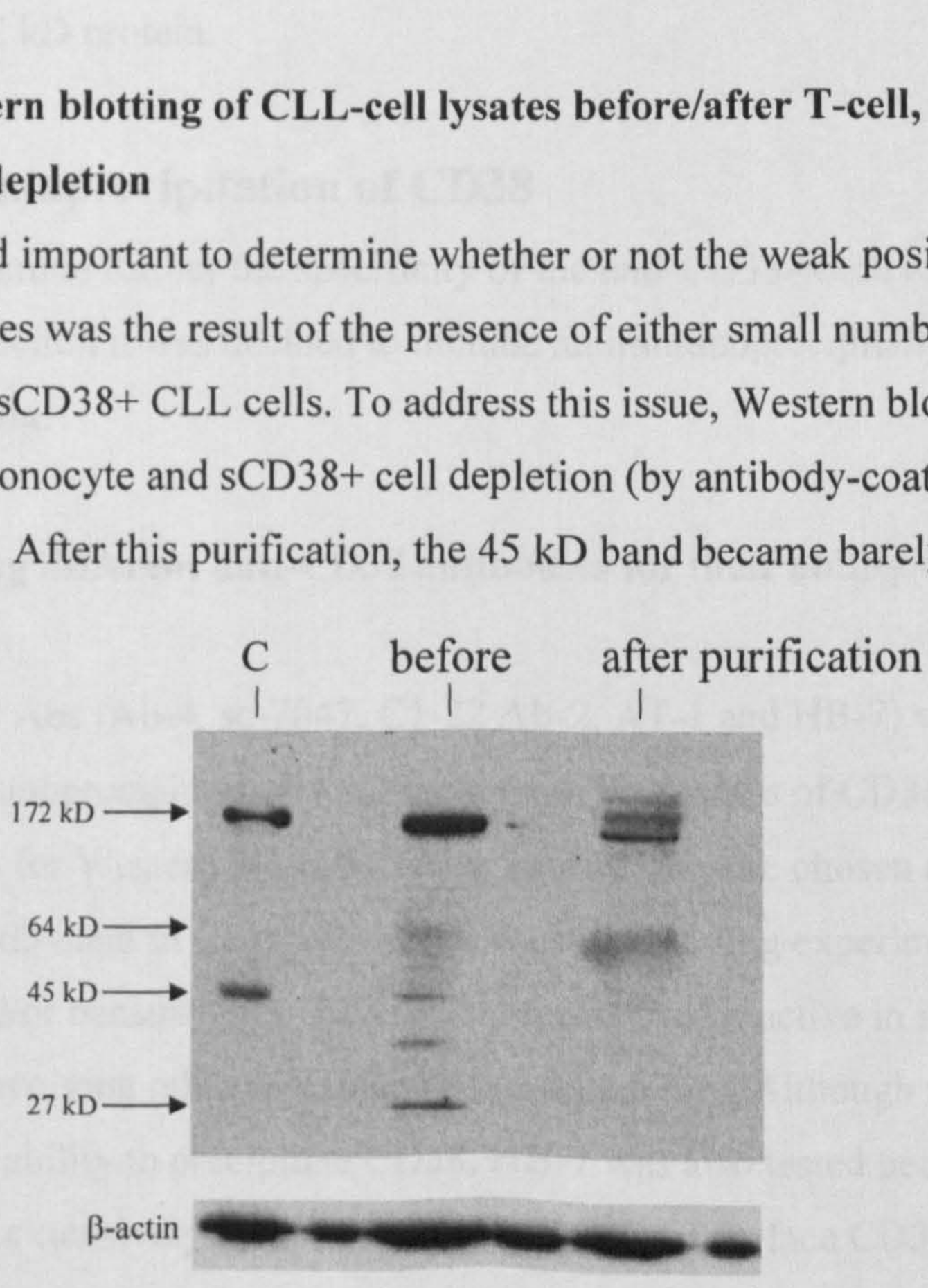


Fig.2.3. Western blotting for CD38 before/after T-cell, monocyte and sCD38+ cell depletion in a sCD38-negative CLL clone. Western blotting with C1-22 was performed as in Fig.2.1 using CHAPS for cell lysis. T-cells and monocytes were removed with CD3- and CD14 - coated magnetic beads, while sCD38+ cells were removed by exposure to HB-7 antibody, followed by magnetic beads coated with goat anti-mouse antibody. Before depletion, the expression levels of sCD38+ cells were ~5% and after depletion ~1%. There was a 10-fold reduction in monocytes and T-cells (from 1% in 0.1%). The control was the commercially provided lysate of EB-1 cells (C). The EB-1 control usually contained either a single 45 kD band or both 45 and 172 kD proteins.

The 172 kD band, although reduced in intensity, was still strongly present in cells of the highly purified sCD38-negative clone.

It was therefore concluded that the weak 45 kD band observed in sCD38- CLL cells is mainly derived from T cells or monocytes or from the few membrane CD38+ cells present in the peparation. It was also concluded that sCD38- CLL cells do indeed contain a ~172 kD protein.

2.3.2. Immunoprecipitation of CD38

In order to examine further the specificity of the anti-CD38-reactive bands described in the previous section it was decided to include an immunoprecipitation step prior to Western blotting.

2.3.2.1. Testing different anti-CD38 antibodies for their ability to immunoprecipitate CD38

Six anti-CD38 Abs (Ab-4, sc-7047, C1-22 Ab-2, AT-1 and HB-7) were tested for their ability to immunoprecipitate a 45 kD band from the lysates of CD38+ PCL cells, using the Cl-22 Mab for Western blotting. These antibodies were chosen either because they detected a 45 kD band in CLL cells in the Western blotting experiments (Ab-4, sc-7047 and Cl-22) and/or because they have been reported to be active in immunoprecipitation experiments involving other cell types (Ab-2 and AT-1). Although no data were available concerning its ability to precipitate CD38, HB-7 was also tested because this is the antibody most extensively used for FACS detection of surface CD38 in CLL. 3% CHAPS was employed for preparation of the lysates because others have previously used this detergent successfully in different studies of immunoprecipitated CD38 in other cell types (Umar et al, 1996).

Three of these mAbs (Ab-4, AT-1 and Cl-22) clearly precipitated the 45 kD CD38 monomer from PCL cells. Results with Ab-4 and AT-1 are shown on Fig.2.4. Ab-4 and AT-1 also readily precipitated a high MW protein detected as a band of ~172 kD.

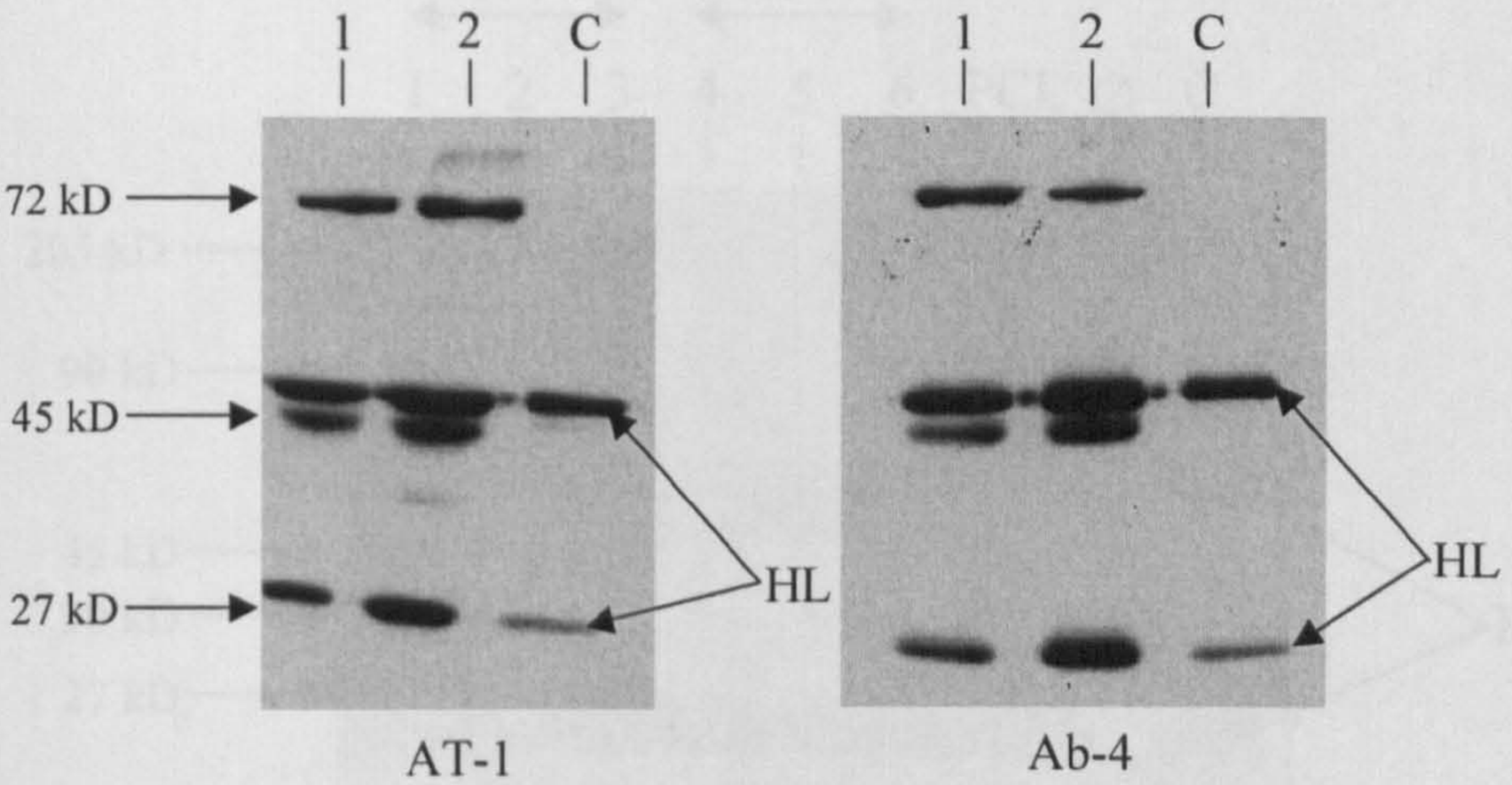


Fig.2.4. Immunoprecipitation of CD38 from plasma cell leukaemia cells. Plasma cell leukaemia cells (10^7 per lane) were lysed in 3%CHAPS and CD38 immunoprecipitated with AT-1 and Ab-4 antibodies. The antibodies were used in concentrations of either 0.5 μg/sample (1) or 1 μg/sample (2). Immunoprecipitates were processed by Western blotting and probed with Cl-22. The control lane (C) contained only lysis buffer and the IP antibodies. Both antibodies immunoprecipitated the 45 kD CD38 monomer and a HMW band at ~172 kD. More CD38 was immunoprecipitated with the higher (2) antibody concentrations. Arrows indicate heavy and light chains of mouse immunoglobulin (HL).

2.3.2.2. Immunoprecipitation of CD38 from CLL-cell lysates

Having identified conditions for immunoprecipitating candidate CD38 from PCL cells, the same methodology was used to compare sCD38+ versus sCD38- CLL cells employing Ab-4 for immunoprecipitation. As shown in Fig 2.5., immunoprecipitates from sCD38+ cells contained the 45 kD CD38 monomer, while those from sCD38- CLL cells did not.

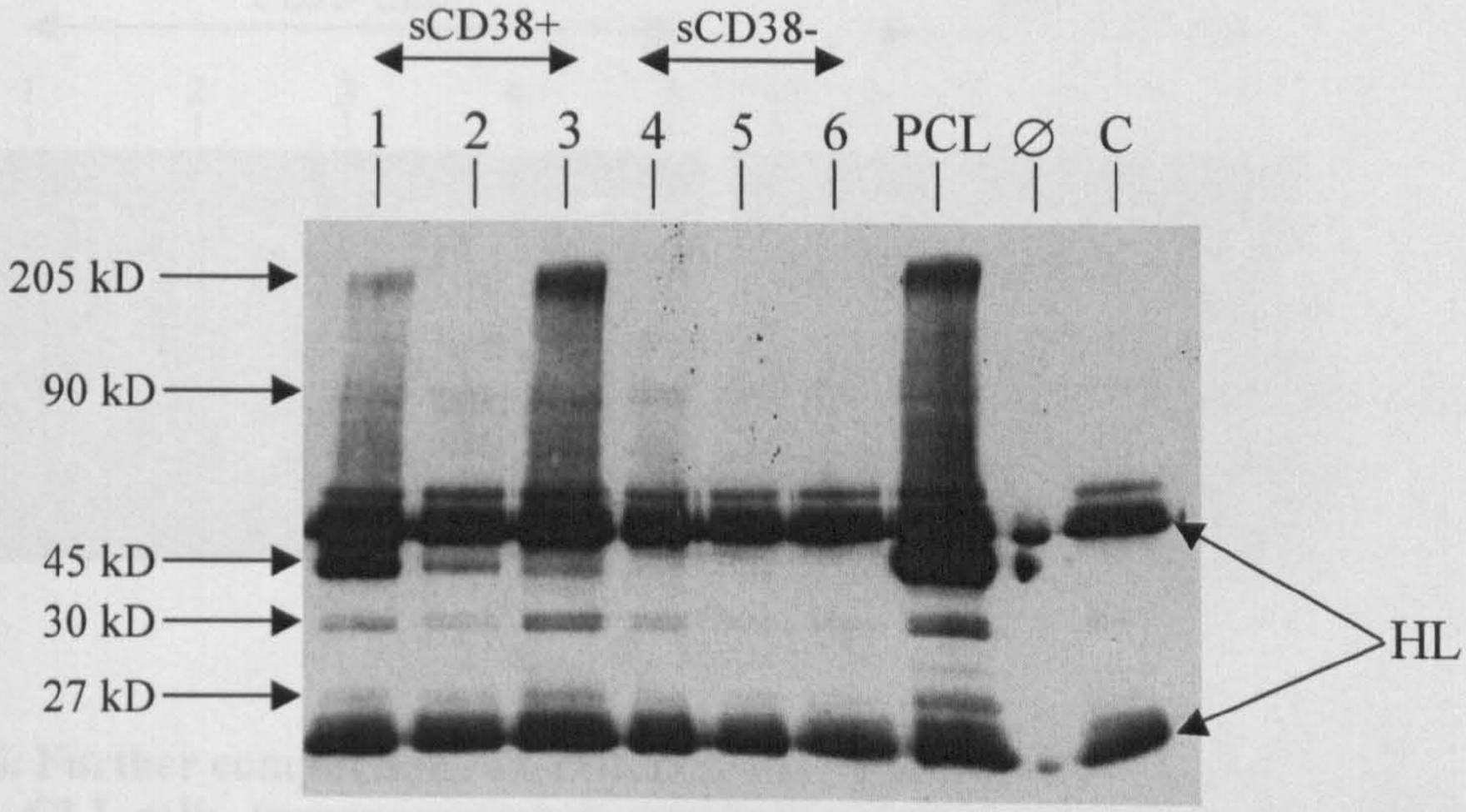


Fig.2.5. Immunoprecipitation of CD38 from CD38+ vs CD38- CLL cells. Ab-4 (2 μg/sample) was used for immunoprecipitation of CD38 from CHAPS lysates of sCD38+ (cases 1-3) and sCD38- (cases 4-6) CLL cells. 10^8 cells were used per sample. Immunoprecipitates were Western blotted and probed with Cl-22. PCL cells were used as positive control (PCL) and antibody in lysis buffer as a negative control (C). The 45 kD CD38 monomer was present only in sCD38+ cell lysates. A HMW band at ~205 kD was also immunoprecipitated in two of the sCD38+ cases. Arrows mark the heavy and light chain of the mouse IP antibody. ∅ signifies empty lane.

In addition, a ~205 kD protein was variably present, especially in the CD38+ cases. With very long exposure, HMW bands of 172 kD were apparent in both CD38 subgroups (not shown).

In order to avoid the simultaneous detection of both human CD38 and mouse Ig heavy and light chains, the same procedure was repeated using the C-19 goat antihuman antibody for Western blotting together with a donkey anti-goat second-layer antibody. Fig.2.6. shows that all sCD38+ cases tested (n=5) contained the 45 kD CD38 monomer, while all sCD38- ones (n=5) lacked this protein. This experiment did not detect the HMW band which is in keeping with the lack of reactivity of C-19 with HMW forms of CD38 in previous Western blotting experiments (Fig.2.1.)

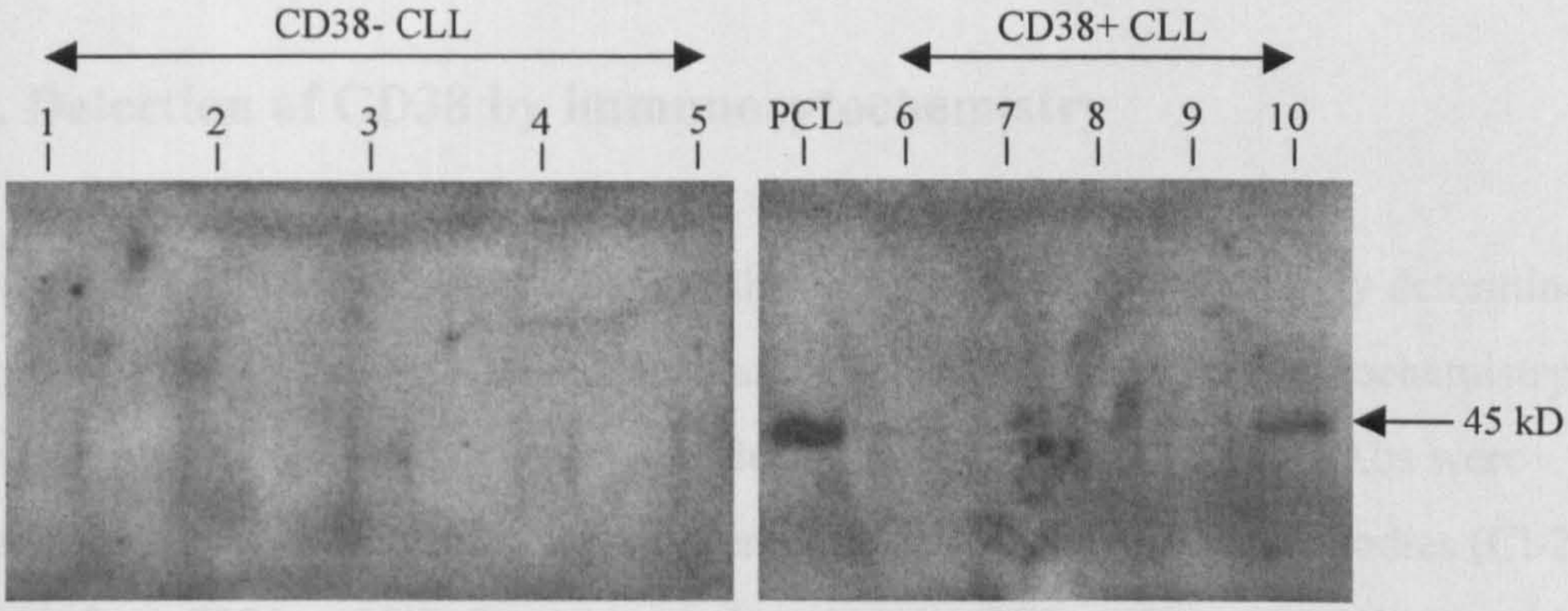


Fig. 2.6. Further comparison of CD38 immunoprecipitates from sCD38+ and sCD38- CLL cells. Immunoprecipitation with Ab-4 was performed exactly the same way as in the previous figure except that for the Western blotting, the C-19 polyclonal goat anti-human CD38 antibody was used which was then detected by a donkey anti-goat-HRP conjugate. 5 sCD38+ and 5 sCD38- CLL cases were applied; PCL cells served as a positive control. The 45 kD band was present in all 5 sCD38+ CLL cases but not in the 5 sCD38- ones. The dark background is probably caused by the reactivity of the second layer antibody with the protein content of the blocking buffer. Empty lanes were left between samples on the blot with sCD38- cases.

These results therefore confirmed the earlier Western blotting data that CD38+ CLL cells possess the 45 kD monomeric form of CD38, while sCD38- ones largely lack this form. In addition, this immunoprecipitation work confirmed the earlier conclusion, based on Western blotting, that both sCD38+ and sCD38- CLL cells express HMW forms of CD38.

2.3.3. Detection of CD38 by immunocytochemistry

In order to support the biochemical data of the previous section and directly determine the topography of CD38, cells were also examined by APAAP immunocytochemistry, FACS analysis and confocal microscopy. Because different anti-CD38 mAbs were shown in the previous sections to have different reactivities, 5 mouse antibodies (Cl-22, Ab-4, Ab-2, sc-7325 and HB-7) were tested, and again, PCL cells were used as positive controls. Three of the antibodies (Ab-4, sc-7325 and Cl-22) were shown in the previous section to detect/capture the 45 kD protein. In addition, Ab-2 and HB-7 were employed because the former antibody has been reported to be suitable for immunocytochemistry (Neomarkers: cat.number: MS-439- P1), while the latter has been used in most previous FACS studies of CLL.

Fig. 2.7 shows PCL cells stained with the 5 anti-CD38 antibodies using acetone fixation (this allows access of antibody to intracellular proteins). Four of the antibodies (Ab-4, Ab-2, sc-7325 and HB-7) produced similar staining, while reactivity with Cl-22 was weaker. For all 5 antibodies, there was considerable cell-to-cell variation in staining, but cells with plasma cell morphology were much more reactive than were most of the smaller cells of lymphoid morphology.

Fig. 2.8 shows sCD38+ CLL cells stained in the same way and with the same antibodies used in Fig 2.7. The CLL cells stained with all 5 antibodies, but reactivity was markedly weaker than that of PCL cells. With all 5 antibodies, occasional small, intensely reactive cells were observed; their nature is unclear but they may represent contaminating T-lymphocytes serving as an internal positive control.

Fig. 2.9 shows sCD38- cells stained for CD38 with the same 5 antibodies, cells showing variable reactivity; the strongest staining was observed with Ab-4. The positivity often did not extend to the plasma membrane and sometimes appeared granular.

It was therefore concluded that, following permeabilisation with acetone, CD38 is detectable in sCD38- CLL cells.

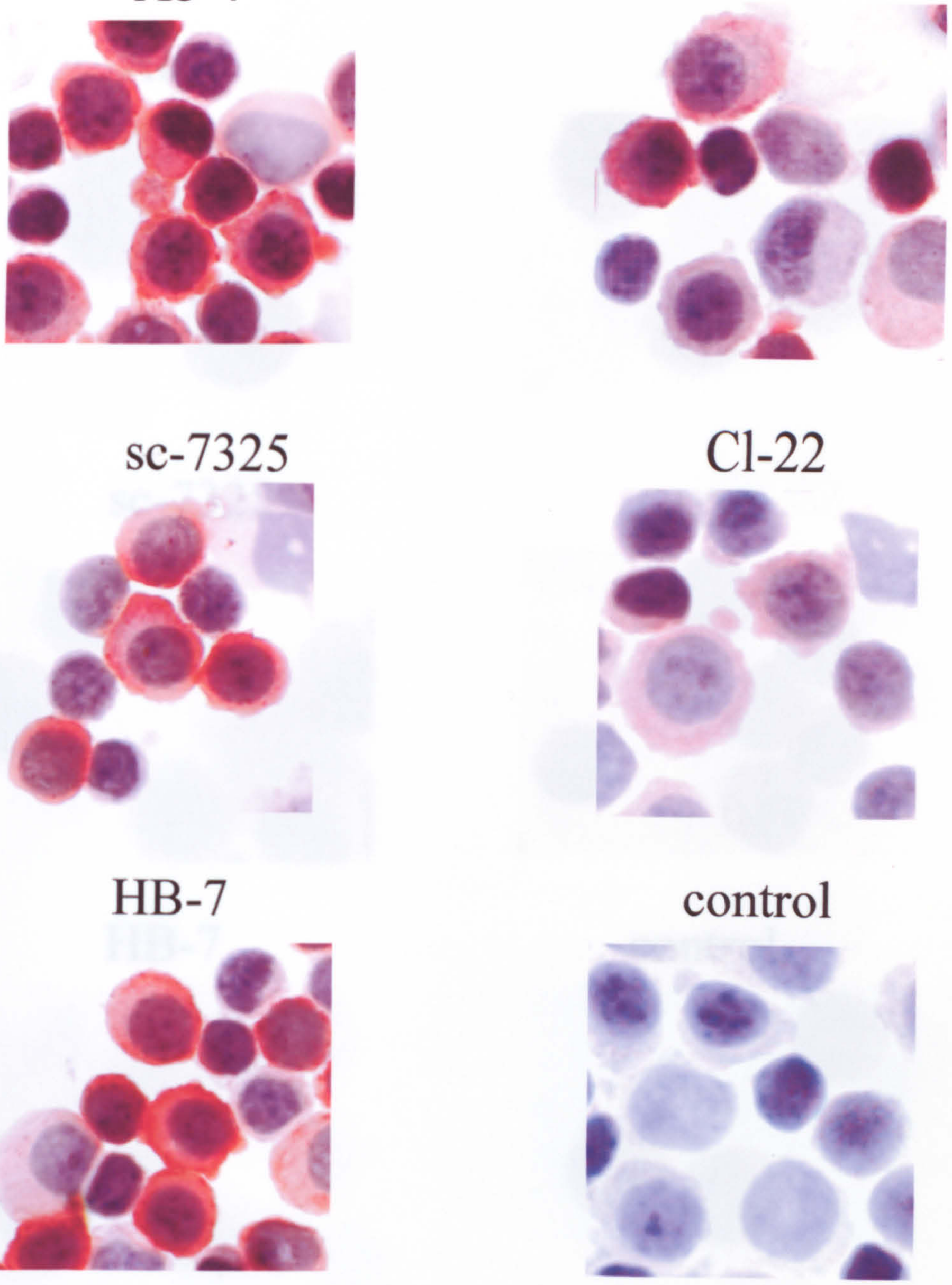


Fig. 2.7. Immunocytochemical detection of CD38 in plasma cell leukaemia cells with different antibodies. Cells were fixed and permeabilized in acetone and stained with the indicated mAbs. Staining was then detected by the APAAP method.

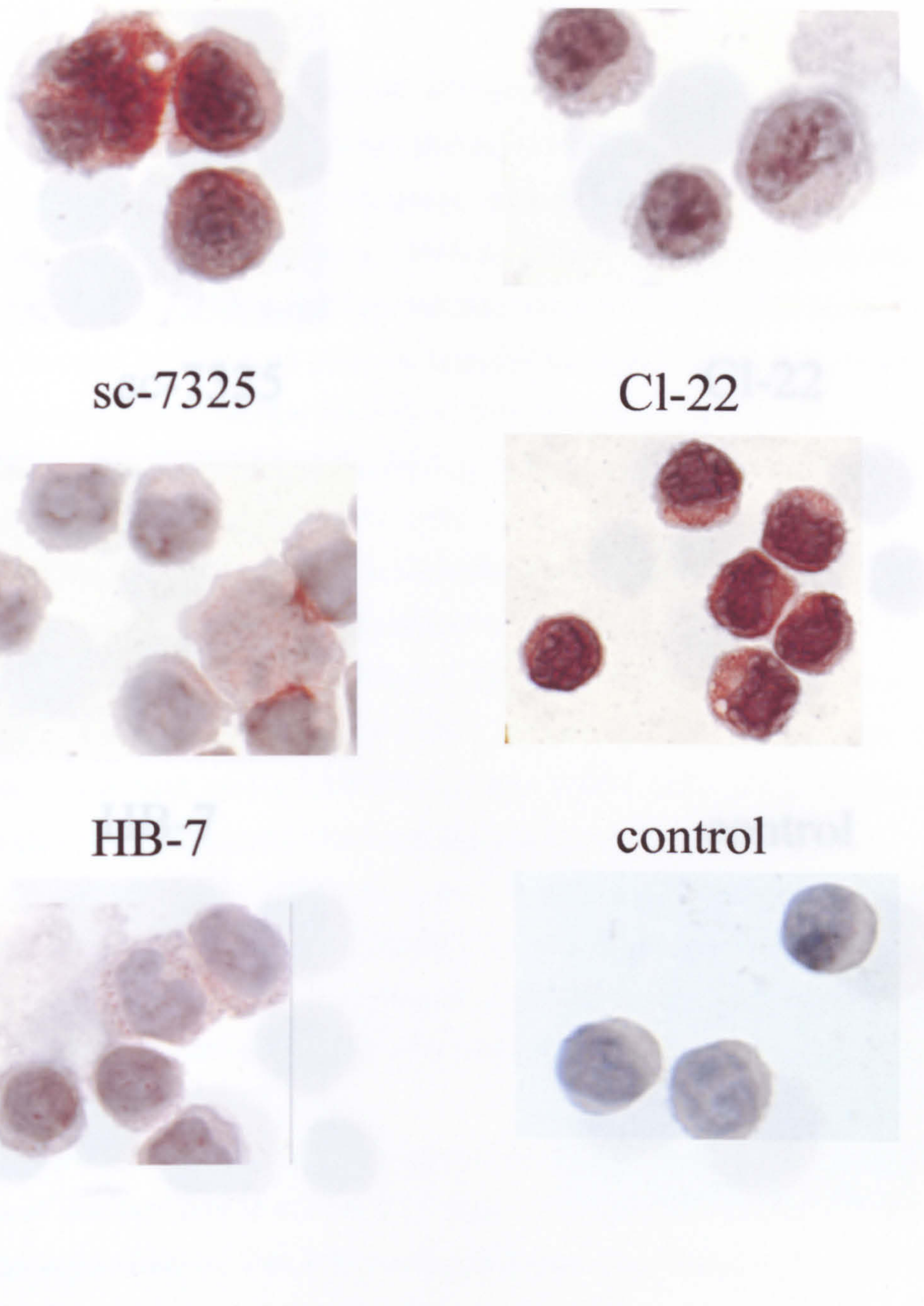


Fig. 2.8. Immunocytochemical detection of CD38 in sCD38-positive CLL cells. Cells were prepared exactly as in Fig.2.7. In this case, CD19+/CD38+ cells (with HB-7) constituted 95% of all mononuclear cells. Similar results were obtained in 2 further sCD38-positive cases.

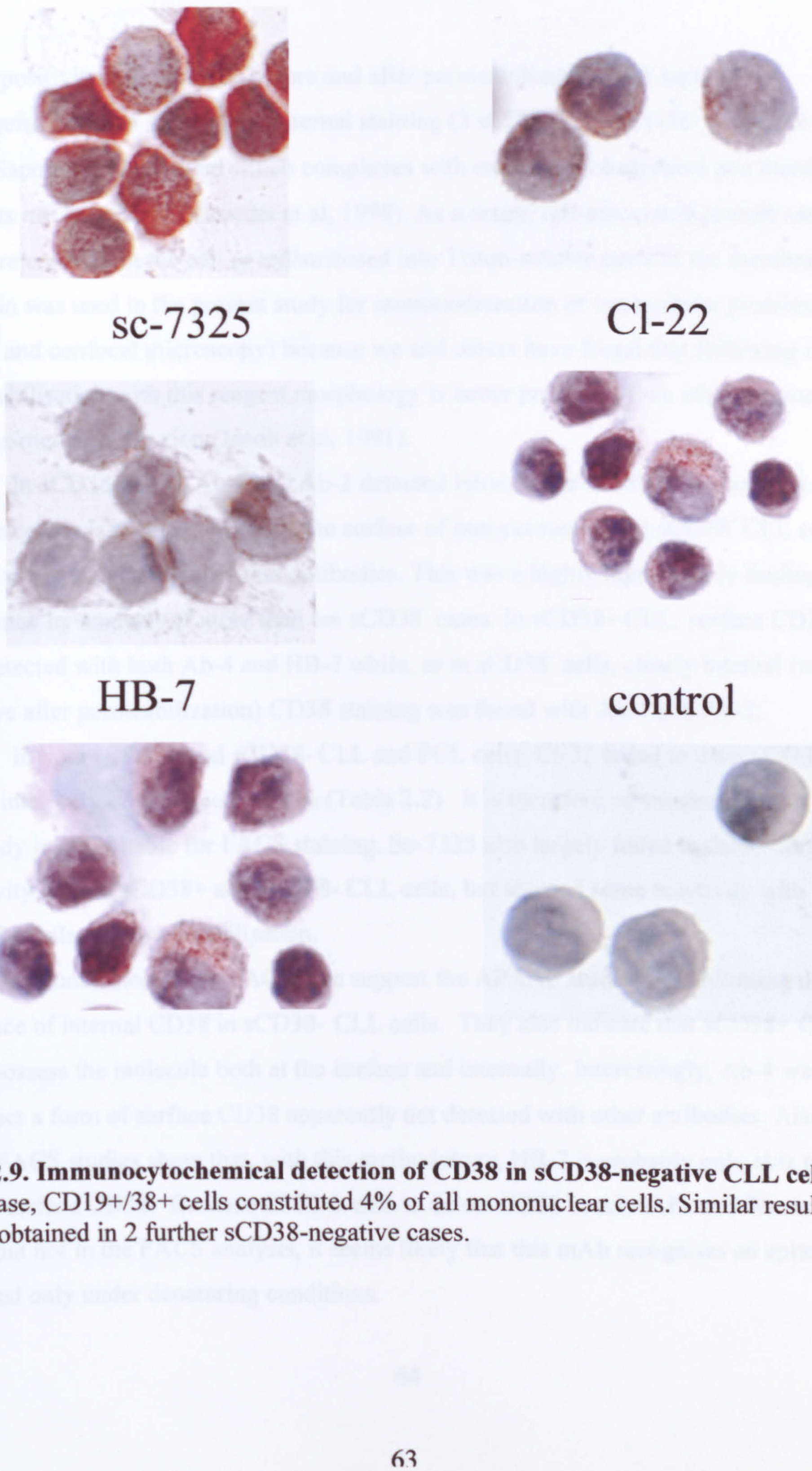


Fig. 2.9. Immunocytochemical detection of CD38 in sCD38-negative CLL cells. In this case, CD19+/38+ cells constituted 4% of all mononuclear cells. Similar results were obtained in 2 further sCD38-negative cases.

2.3.4. FACS analysis

CD38 positivity was analysed before and after permeabilisation with saponin to distinguish between surface and internal staining (3 sCD38+ and sCD38- cases, see Table 2.2). Saponin is a detergent which complexes with membrane cholesterol and thereby disrupts raft structure (Schroeder et al, 1998). As a result, raft-associated protein can be either released from the cell or redistributed into Triton-soluble parts of the membrane. Saponin was used in the present study for immunodetection of intracellular proteins (by FACS and confocal microscopy) because we and others have found that following cell permeabilisation with this reagent morphology is better preserved than after acetone or acetone/methanol fixation (Jacob et al, 1991).

In sCD38 - CLL, Ab-4 and Ab-2 detected intracellular CD38. Unexpectedly, Ab-4 also detected clear-cut reactivity at the surface of non-permeabilised sCD38$^-$ CLL cells which were unreactive with other antibodies. This was a highly reproducible finding confirmed by analysis of more than ten sCD38$^-$ cases. In sCD38+ CLL, surface CD38 was detected with both Ab-4 and HB-7 while, as in sCD38$^-$ cells, clearly internal (more positive after permeabilization) CD38 staining was found with Ab-4 and Ab-2.

In both sCD38+ and sCD38- CLL and PCL cells, Cl-22 failed to detect CD38 either internally or at the cell surface (Table 2.2). It is therefore concluded that this antibody is not suitable for FACS staining. Sc-7325 also largely failed to detect surface positivity in both sCD38+ and sCD38- CLL cells, but showed some reactivity with sCD38+ cells after permeabilisation.

In conclusion, these FACS data support the APAAP studies in confirming the presence of internal CD38 in sCD38- CLL cells. They also indicate that sCD38+ CLL cells possess the molecule both at the surface and internally. Interestingly, Ab-4 was able to detect a form of surface CD38 apparently not detected with other antibodies. Also, these FACS studies show that, with this methodology, HB-7 is probably only able to detect surface CD38. Because Cl-22 is able to detect CD38 in APAAPs and Western blots but not in the FACS analysis, it seems likely that this mAb recognises an epitope exposed only under denaturing conditions.

	Antibody	sc-7325[d]				Ab-4			
	Permeabilization[b]	Before		After		Before		After	
	CD38 positivity[c]	%	RFI	%	RFI	%	RFI	%	RFI
sCD38- [(a)]	CLL 1	0.7	1	0.6	1.2	40.2	5.4	43.3	2.5
	CLL 2	0.3	1.2	0.2	1	55.8	6.5	98.7	8
	CLL 3	1.1	0.8	0.5	1	73.9	10.5	99.9	11.2
	All (n=3)	**0.7±0.4**	**1±0.2**	**0.4±0.2**	**1±0.2**	**56.6±16.9**	**9.5±2.7**	**80.6±32.3**	**7.2±4**
sCD38+	CLL 4	23.5	4.3	15.7	1.9	61.2	9.2	98.5	11.1
	CLL 5	1.7	1.9	31.7	2.8	43.4	5.8	99.7	18.3
	CLL 6	1	2.1	43	3.3	33.3	6.6	99.9	13.6
	All (n=3)	**8.7±12.7**	**2.8±1.3**	**30.1±13.7**	**2.7±0.7**	**45±14.1**	**7.2±1.8**	**99.4±0.8**	**14.3±3.7**
	PCL[e]	**92**	**52**	**91**		**96**	**82**	**99**	

	Antibody	Ab-2				Cl-22[d]			
	Permeabilization	Before		After		Before		After	
	CD38 positivity	%	RFI	%	RFI	%	RFI	%	RFI
sCD38-	CLL 1	1	1.1	0.1	1	0.3	1	0.4	1.2
	CLL 2	1.2	1.5	72.4	14.5	1	1.1	0.4	1.3
	CLL 3	1.5	1.5	99.2	5.8	0.3	0.9	6	2.6
	All	**1.2±0.2**	**1.4±0.3**	**57.2±51.2**	**3.3±2.4**	**0.5±0.4**	**1±0.1**	**2.3±3.2**	**1.7±0.8**
sCD38+	CLL 4	16.7	6.3	95.2	6.8	0.3	1.4	6.7	2.5
	CLL 5	2.7	2.5	99.6	8.8	3.6	1.6	7.2	2.2
	CLL 6	2.7	2.8	98.6	7.6	0.1	1	1.9	2.1
	All	**7.4±8**	**3.9±2.1**	**97.8±2.3**	**7.7±1**	**1.3±2**	**1.3±0.3**	**5.3±2.9**	**2.3±0.2**
	PCL[e]	**92**	**79**	**72**		**0.4**	**1**	**1.9**	

	Antibody	HB-7[d]			
	Permeabilization	Before		After	
	CD38 positivity	%	RFI	%	RFI
sCD38-	CLL 1	1.3	1.2	1	1.3
	CLL 2	0.8	1.3	0.3	1
	CLL 3	2.5	2	0.9	1.4
	All	**1.5±0.9**	**1.5±0.5**	**0.7±0.4**	**1.2±0.2**
sCD38+	CLL 4	62.8	5.3	56.5	3.7
	CLL 5	68	2.2	46	2.6
	CLL 6	80.5	3.6	29.9	2.3
	All	**70.4±9.1**	**3.7±1.6**	**44.1±15.3**	**2.9±0.7**
	PCL[e]	**90**	**90.5**	**70**	

Table 2.2. FACS analysis of sCD38- and sCD38+ CLL cells before/after saponin permeabilization. Cells were stained by an indirect technique using the primary antibody at 20 μg/ml (1:10) and the second-layer goat anti-mouse FITC at 17 μg/ml (1:15) concentration. (a) Surface CD38 reactivity was defined on the basis of reactivity with HB-7. (b) Cells were examined unpermeabilised and after permeabilisation with 0.3% saponin. (c) Reactivity was measured as both % positive cells and RFI as compared with the staining obtained with the class-specific control. RFI (relative fluorescence intensity) was calculated as the ratio of MFI (mean fluorescence intensity) of test cells per MFI of cells stained with the class-specific control. (d) Because sc-7325, Cl-22 and HB-7 produced too weak staining by APAAP, these antibodies were also employed at 60 μg/ml cc. Cells remained unreactive (n=3, data not shown). (e) Plasma cell leukaemia cells were used as a positive control.

2.3.5. Confocal microscopy

The same six cases (3 sCD38+ and 3 sCD38-) used earlier for both APAAP and FACS analysis were also examined by confocal microscopy using cells prepared in the same way as for FACS. PCL cells were used as positive controls.

In the sCD38- cases, only cells stained with Ab-4 were examined by confocal microscopy, since this was the only antibody to produce clear positivity (both surface and intracellular) in the FACS studies of sCD38- CLL cells. Surface CD38+ cells were stained with 4 of the 5 antibodies used for FACS (Ab-4, Ab-2, sc-7325, HB-7). Cl-22 was not studied because it was unreactive in the FACS analyses.

2.3.5.1. Surface CD38-negative CLL cells

In non-permeabilised cells, Ab-4 produced clear membrane staining located in multiple clusters (Fig.2.10). These confocal results therefore confirmed the FACS findings indicating that CLL cells classified as sCD38- on the basis of lack of surface staining with HB-7, show surface immunoreactivity with Ab-4.

After saponin permeabilisation, reactivity was again observed with Ab-4 and the staining was mainly located within the cytoplasm: the positivity was of either a granular or diffuse pattern. The clustered membrane staining observed before permeabilisation was now no longer observed. This suggests that the surface clusters became dispersed after cell treatment with saponin, strongly indicating that clustered CD38 is localised in cholesterol- and sphingolipid-rich microdomains (lipid rafts).

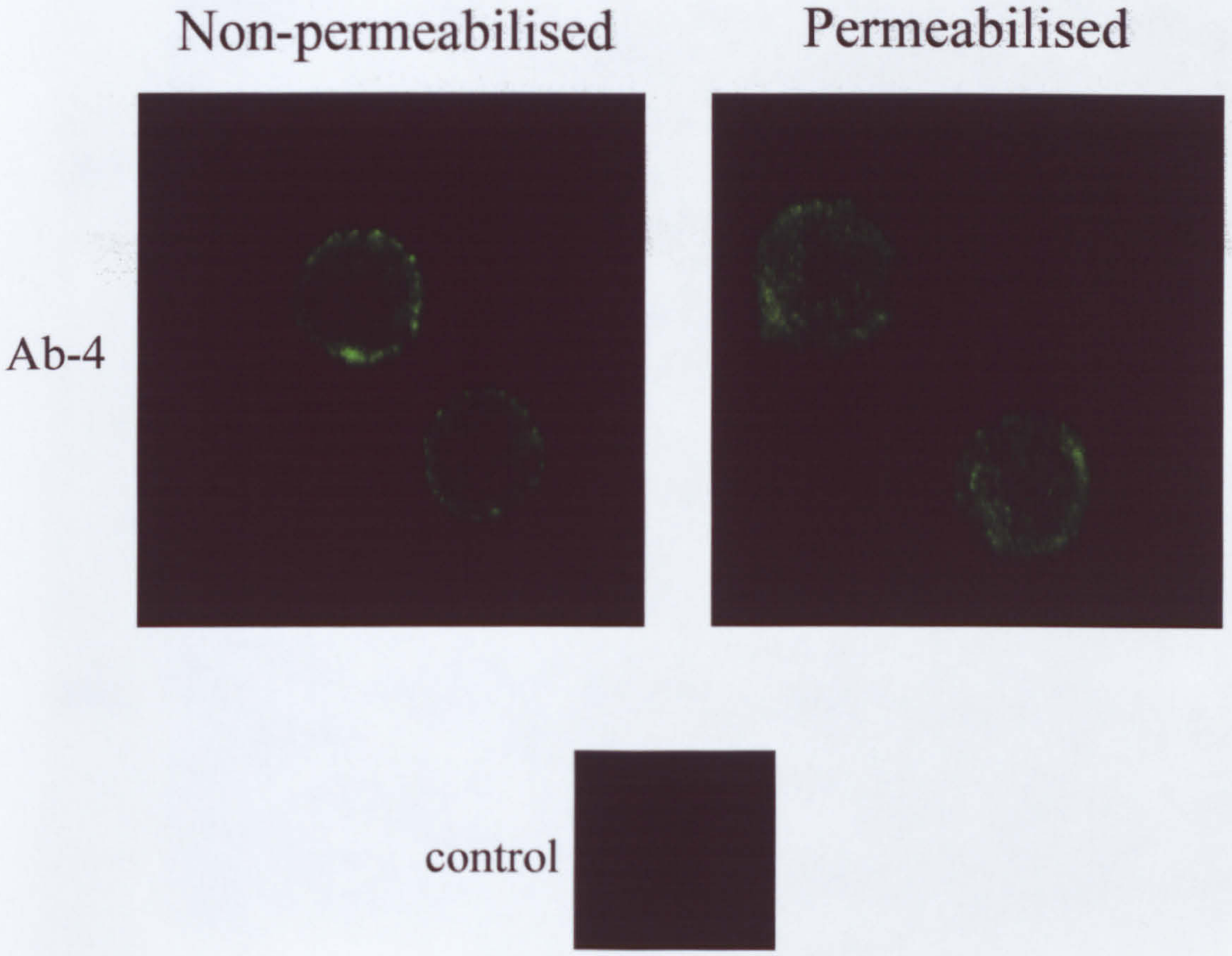


Fig.2.10. Confocal microscopic analysis of sCD38- CLL cells (as determined by HB-7 staining). Cells were prepared exactly as for FACS and stained with the Ab-4 antibody before/after permeabilization with saponin. Cytospin preparations were then examined by confocal microscopy. Representative confocal slices are shown (n =3). In this particular case, 3% of cells displayed CD38 positivity with HB-7.

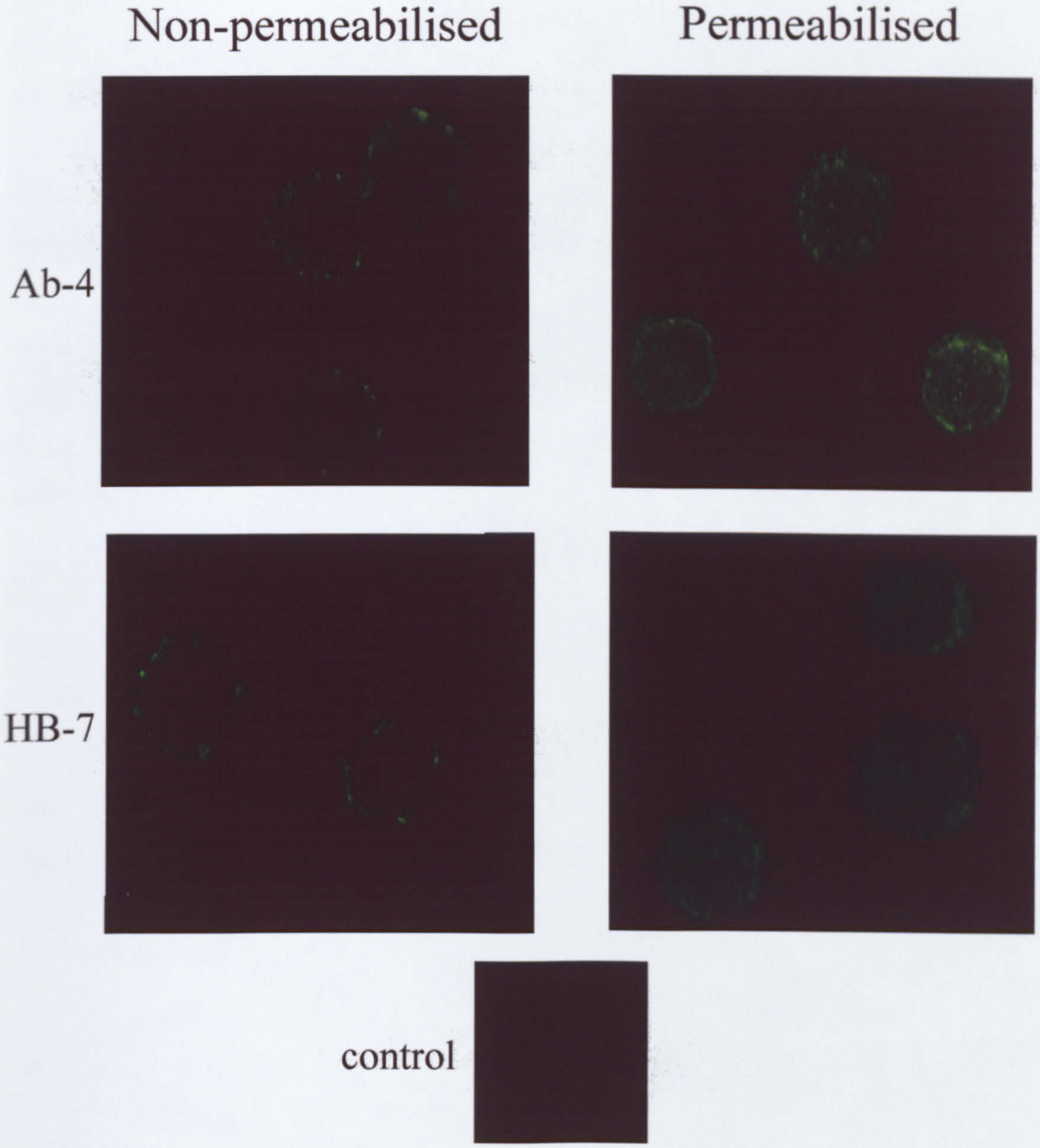


Fig.2.11. Confocal microscopic analysis of sCD38+ CLL cells. Cells were prepared and examined as for the previous figure and staining was performed with the Ab-4 and HB-7 antibodies. Representative confocal slices are once more shown. (n=3). In this case, 74% of the cells were CD38-positive with HB-7.

2.3.5.2. Surface CD38-positive CLL cells

Ab-4 staining was very similar to that observed in sCD38- cases. Before permeabilisation, Ab-4 produced clustered membrane staining. After permeabilisation, Ab-4 again resulted in fine granular cytoplasmic reactivity (Fig.2.11)

With Ab-2, non-permeabilised cells showed little or no reactivity, while permeabilised cells displayed cytoplasmic reactivity resembling that seen after Ab-4 staining (not shown).

With HB-7, the reactivity of non-permeabilised cells closely resembled that observed after Ab-4 staining; clustered membrane positivity was again seen (Fig.2.11.). After permeabilisation, very weak intracellular staining appeared. With sc-7325, there was no reactivity on non-permeabilised cells. After permeabilisation, very weak intracytoplasmic reactivity was detected in a proportion of the cells (not shown).

Taken together, these confocal studies support the earlier conclusion that sCD38-CLL cells contain intracellular CD38. Furthermore, the clustered positivity of surface staining on non-permeabilised cells suggests that sCD38, as in other cell types (Zubiaur et al, 2001) is likely to be located in membrane rafts.

2.3.6. Further Western blotting employing Ab-4 and HB-7

Having shown in the previous section that non-permeabilised sCD38- CLL cells are strongly reactive with Ab-4, it seemed of interest to perform Western blotting with this antibody and compare the results with those obtained with HB-7 – the antibody used throughout the world to classify CLL cells as CD38 positive or negative (this antibody was not used earlier in this Chapter for Western blotting because it is has not been recommended for this purpose in previous literature). Because CD38 contains several cysteines that form intra-and inter- molecular disulphide bonds, and in the hope of shedding further light on the various MW bands described earlier in this Chapter, CLL-cell lysates extracted in CHAPS were analysed by Western blotting after electrophoresis under non-reducing as well as reducing conditions (Fig.2.12).

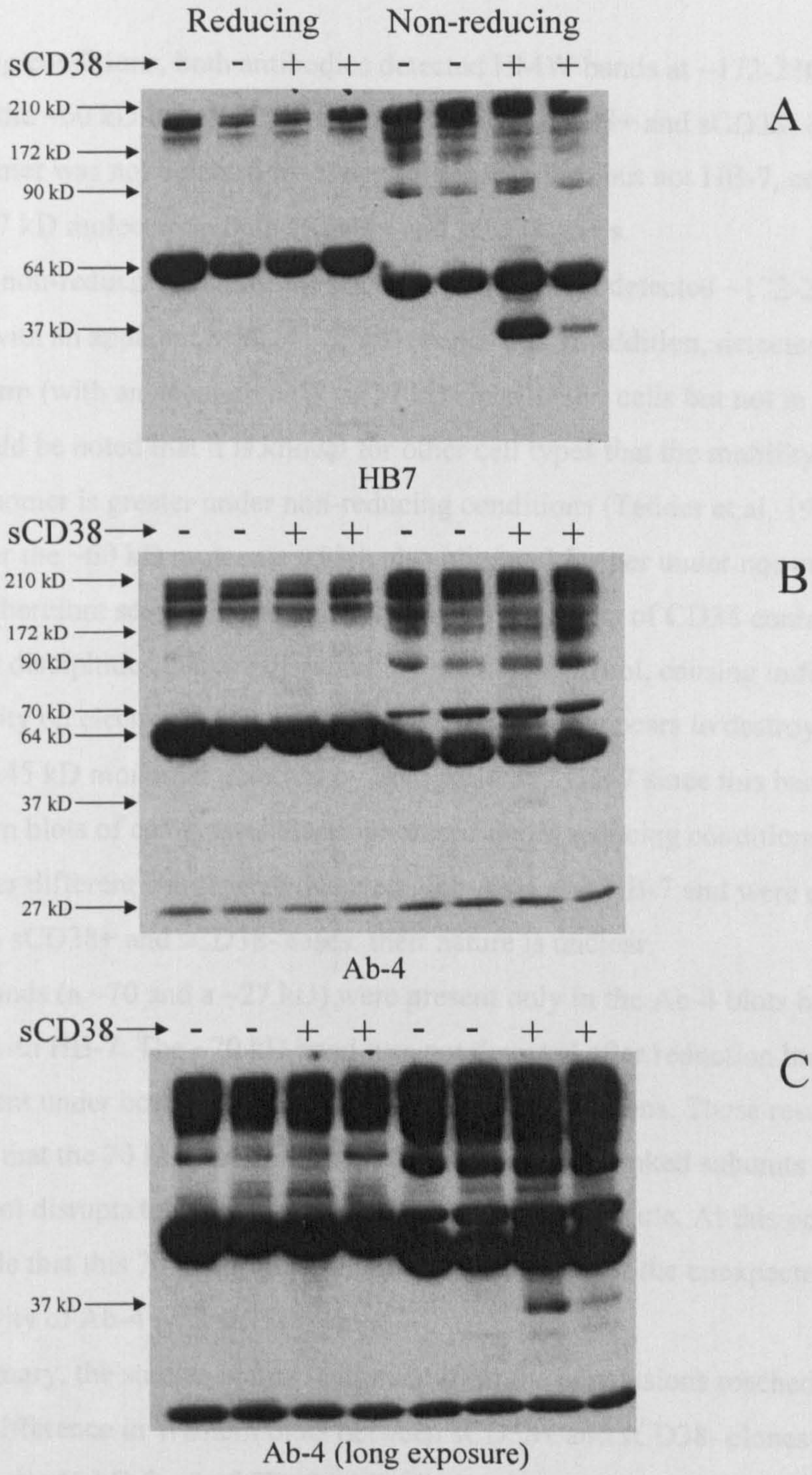


Fig 2.12. Western blotting of CLL cell lysates with HB-7 and Ab-4. sCD38- (n=2) and sCD38+ (n=2) CLL cells (2 x 10^6/lane) were extracted in 3% CHAPS and Western blotted with HB-7 (A) or Ab-4 (B) under reducing vs non-reducing conditions. With longer exposure, the 37 kD band becomes visible on the Ab-4 blot (C).

Under reducing conditions, both antibodies detected HMW bands at ~172-210 kD, together with the ~60 kD form of the molecule, in both sCD38+ and sCD38- clones; the ~45 kD monomer was not detected by either antibody. Ab-4, but not HB-7, consistently detected a ~ 27 kD molecule in both sCD38+ and sCD38- cells.

Under non-reducing conditions, both antibodies again detected ~172-210 and 60 kD (but now with an apparent MW of ~55 kD) bands but, in addition, detected the monomeric form (with an apparent MW of 37 kD) in sCD38+ cells but not in sCD38- clones. It should be noted that it is known for other cell types that the mobility of the 45 kD CD38 monomer is greater under non-reducing conditions (Tedder et al, 1984). The same is true for the ~60 kD molecule which also migrated further under non-reducing conditions. It therefore seems that both the 45 and 60 kD forms of CD38 contain intramolecular disulphides that are disrupted by mercaptoethanol, causing unfolding and reduced mobility on electrophoresis. Also, mercaptoethanol appears to destroy the epitope on the 45 kD monomer detected by both Ab-4 and HB-7 since this band was not seen in Western blots of cell lysates electrophoresed under reducing conditions. A number of other different bands were detected with Ab-4 and HB-7 and were equally present in both sCD38+ and sCD38- cases; their nature is unclear.

Two bands (a ~70 and a ~27 kD) were present only in the Ab-4 blots but not in those probed with HB-7. The ~70 kD band was not detected after reduction but the 27 kD band was present under both reducing and non-reducing conditions. These results indicate either that the 70 kD protein is composed of disulfide-linked subunits or that mercaptoethanol disrupts the Ab-4 reactive epitope of this molecule. At this point, it seemed possible that this 70 kD protein might be responsible for the unexpected immunoreactivity of Ab-4 with sCD38- cells.

In summary, the studies in this section confirm the conclusions reached earlier that the main difference in Western blots between sCD38+ and sCD38- clones is that the former possess the 45 kD form of CD38 while the latter contain little or none of this form. The studies in the present section also confirm the earlier conclusion that both CD38+ and CD38- CLL cells contain HMW forms of CD38 together with ~60 kD and 27 kD molecular forms. Both Ab-4 and HB-7 detected HMW forms of clearly higher

molecular weights (172-210 kD) than that detected by Cl-22 (172 kD). The ~27 kD molecule detected by Ab-4 was observed in Western blots with two other anti-CD38 antibodies (with C-19 and variably with C1-22) and presumably therefore represents a form of CD38.

2.3.7. Detection of CD38 mRNA in sCD38+ and sCD38- CLL cells

Initially, primers were designed to amplify gene fragments representing both full-length and the known splice variant of CD38. These primers were used in conventional RT-PCR experiments to screen unpurified cells from 35 CLL cases for CD38 transcription. To minimise the contribution of contaminating T cells and monocytes, only cases with high-count (>40 x 10^9/l) disease were employed.

RT-PCR products were submitted to agarose gel electrophoresis, transferred to a nylon membrane, and hybridised to CD38 and actin probes (Fig.2.13). A single CD38-positive band was detected in all cases and its electrophoretic motility was consistent with its representing the full-length transcript (the higher MW band in lane 12 is not of appropriate size for any known CD38 transcript). There was therefore no evidence of expression in CLL cells of the known CD38 splice variant (Nata et al, 1997). There was no obvious correlation between levels of CD38 message and surface protein expression (as measured by HB-7). It was also noted that the CD38 PCR product was not readily visualized on ethidium bromide-stained agarose gels, suggesting that the levels of CD38 mRNA are uniformly low in CLL cells.

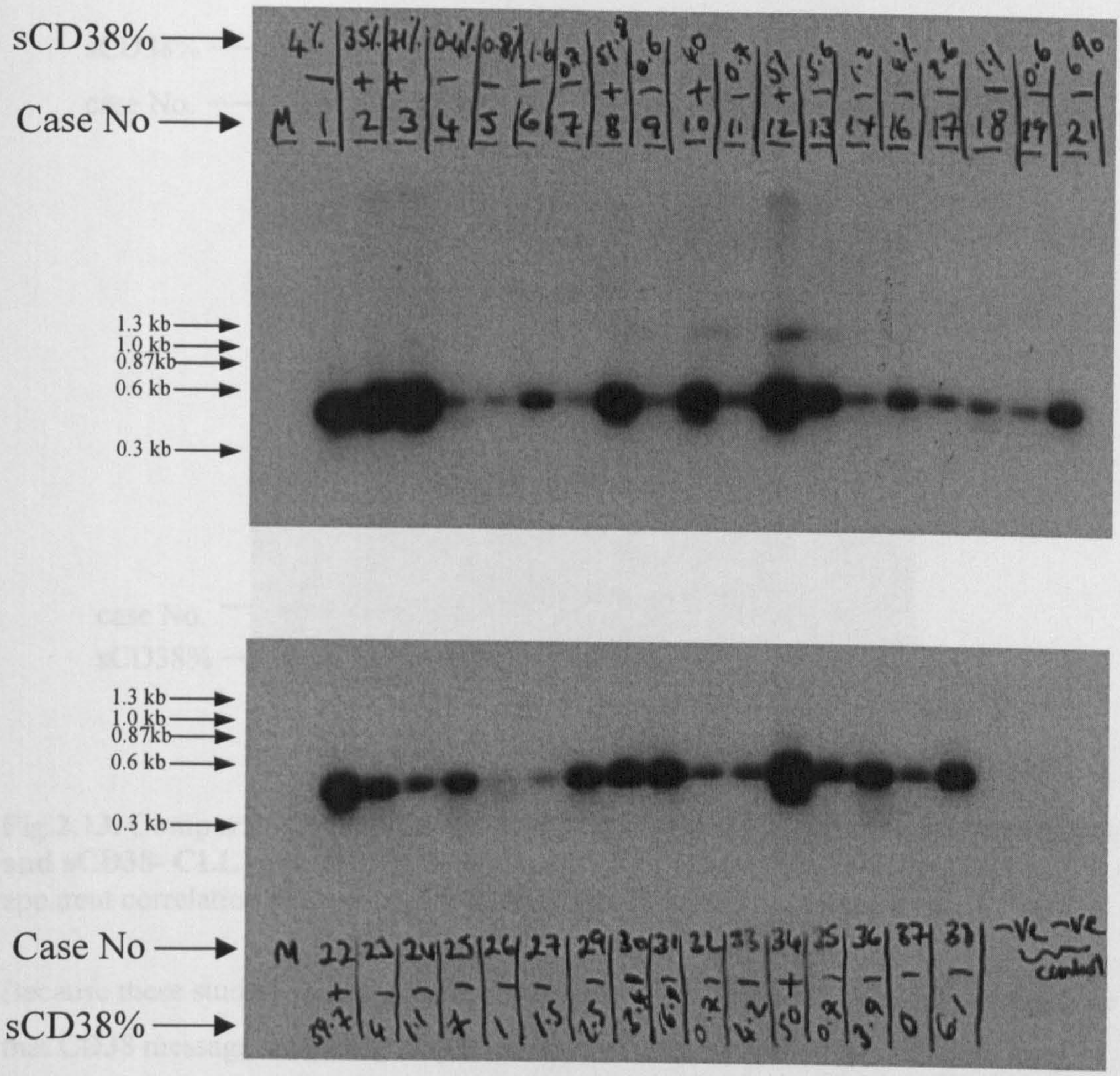


Fig.2.13/A. Detection of CD38 mRNA in sCD38+ and sCD38- CLL cells by RT-PCR. PCR products corresponding to the full-length CD38 mRNA (438 bp) is are detected in all 35 cases studied.

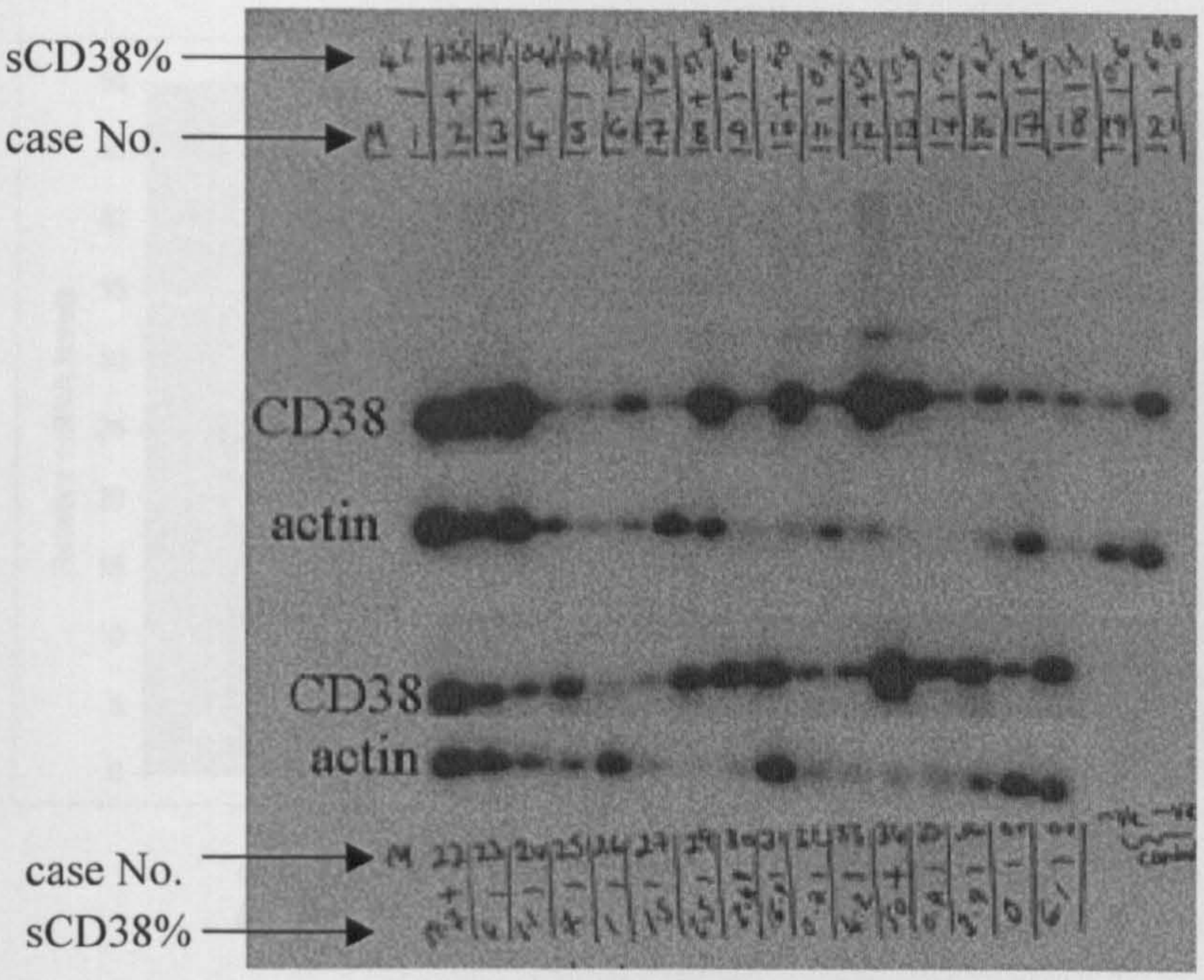


Fig.2.13. Comparison of cell surface CD38 (%) and mRNA expression in sCD38+ and sCD38- CLL cells. mRNA determination was done by RT-PCR. There is no apparent correlation between surface CD38 and the mRNA expression.

Because these studies were performed with unpurified CLL cells, it remained possible that CD38 message was being amplified from the small numbers of contaminating sCD38+ non-CLL cells present in the preparations. Also, these studies were performed (November 2000) before the Department acquired a quantitative real-time thermal cycler. Therefore, the studies were repeated using the new analyser and cells purified by removing T cells, monocytes and NK cells either by FACS sorting or by depletion with immunomagnetic beads.

For the FACS method, CLL mononuclear cells were depleted by staining with anti-CD3-PE, anti-CD14-FITC, and anti-CD16-FITC antibodies and removal of positive cells by sorting. sCD38+ and sCD38- clones had very similar amounts of CD38 message (Fig.2.14).

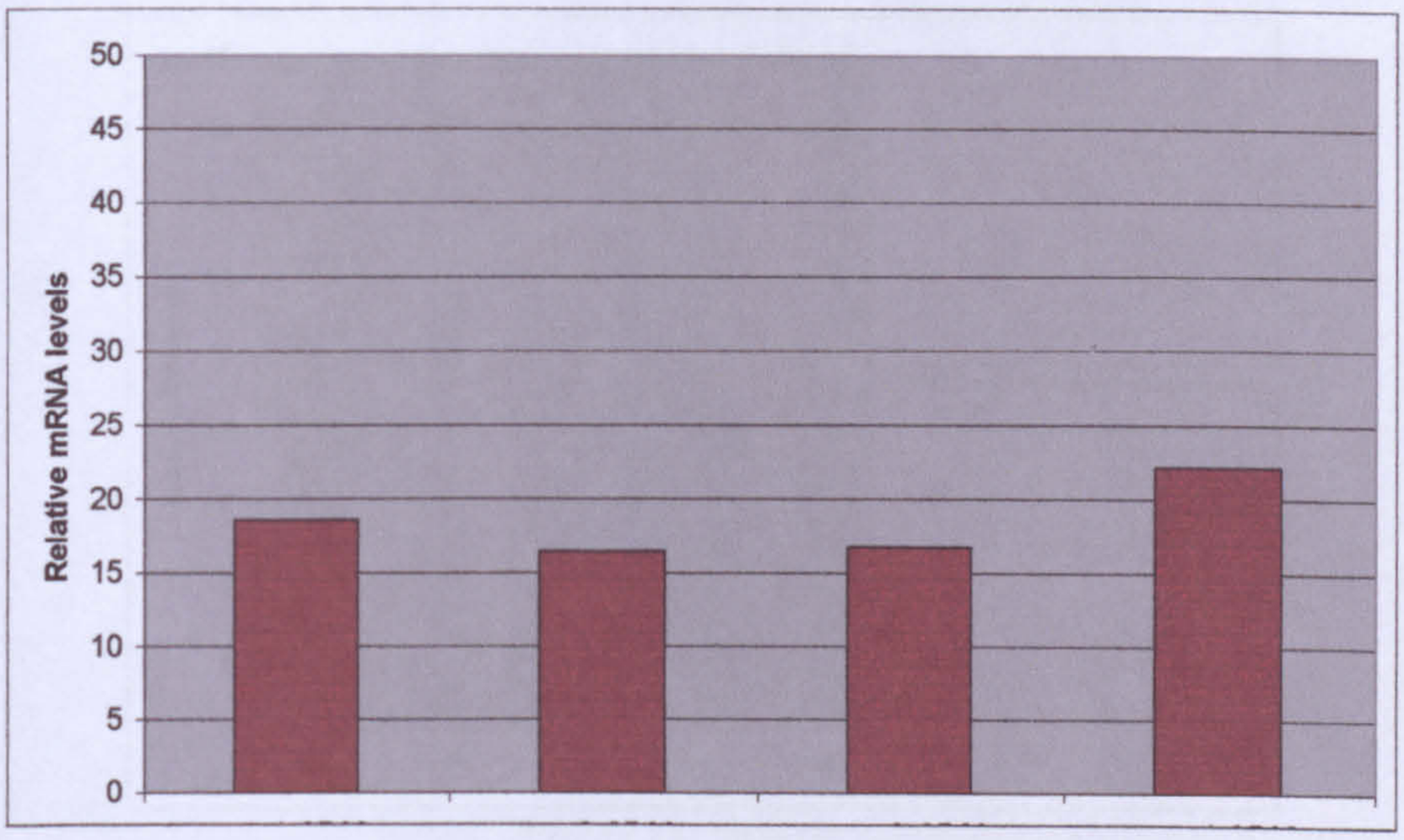
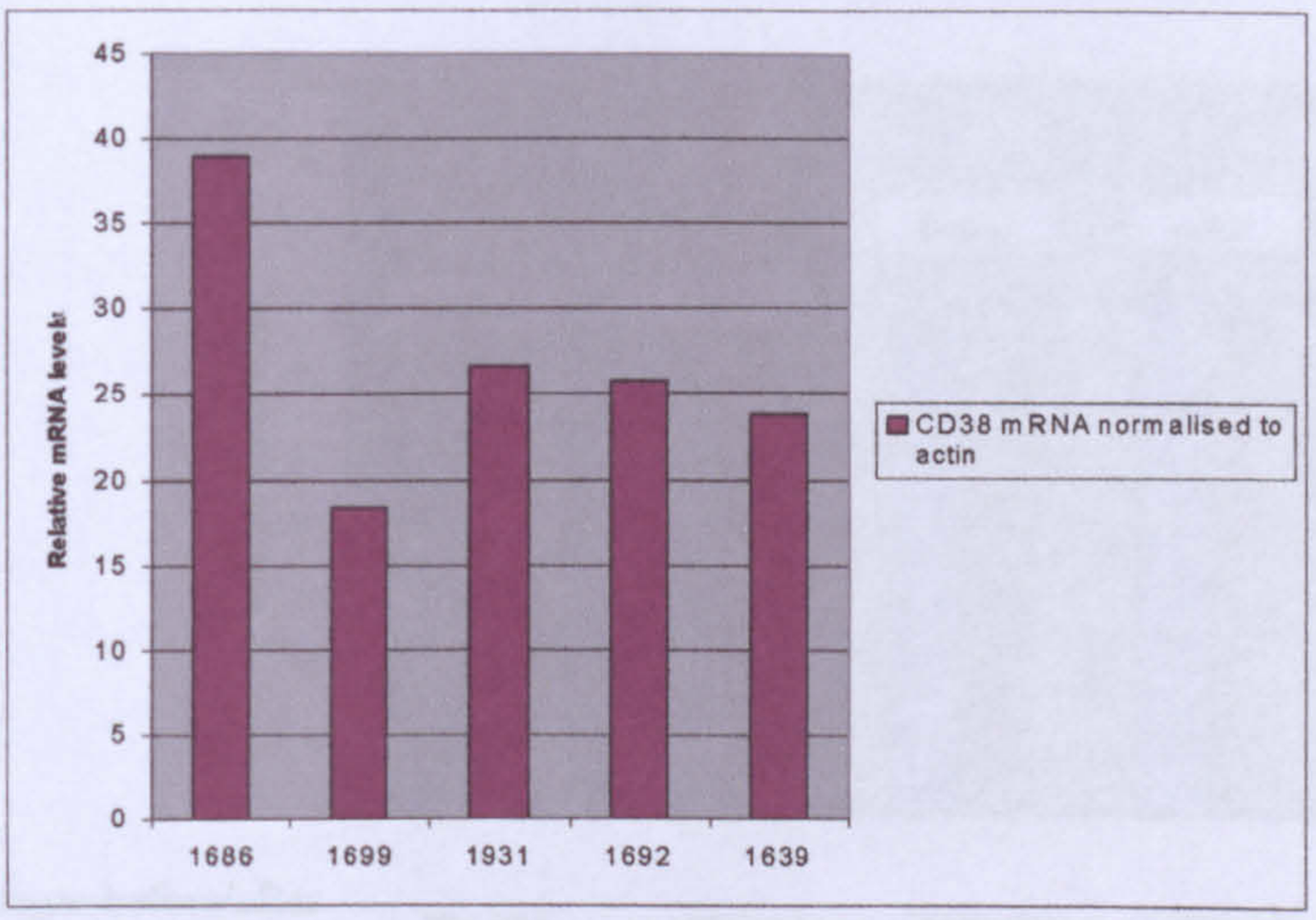


Fig. 2.14. CD38 mRNA levels in sCD38+ vs sCD38- clones depleted of T-cells, monocytes and NK cells by FACS sorting. Contaminating cells were removed by FACS depletion of cells reactive with CD3, CD14 and CD16. CD38 and actin message were amplified using a real-time thermal cycler. The levels of CD38 message were normalised to actin as described in the Methods. The results represent the means of duplicate measurements.

For the immunomagnetic-bead purifications, the same 3 antibodies were employed and the reactive cells removed with magnetic beads coated with goat anti-mouse Ig antibodies. In these experiments, sCD38- CLL clones contained CD38 message in amounts similar to those observed in sCD38+ clones (Fig.2.15)



Case number	1686	1699	1931	1692	1639
% surface CD38	60	82	81	0	0.6
Relative CD38 mRNA level	38.9	18.3	26.6	25.8	23.9
CD3+ T cells	2.6	2.7	2.7	1.8	2.5
CD14+ monocytes	0.06	0.06	0.03	0.22	0
CD16+ NK cells	1.6	2.1	0.74	1.25	1.1

Fig. 2.15. CD38 mRNA in sCD38+ vs sCD38- clones depleted of T cells, monocytes and NK cells using magnetic beads. CLL cells were purified as in Figure 2.14., except that immunoreactive contaminating cells were removed with magnetic beads. Levels of CD38 message were normalised to actin as in Figure 2.14. The mRNA results represent the means of duplicate experiments.

Finally, to exclude the possibility that the small number of sCD38+ CLL cells present in the sCD38- clones were affecting the PCR results, sCD38+ cells were removed from T-, monocyte- and NK-depleted CLL populations. CD38 message levels were then compared before and after this sCD38+ depletion step. Whether normalised against actin or L27, CD38 mRNA levels were very similar before and after depletion of sCD38+ cells (Fig 2.16).

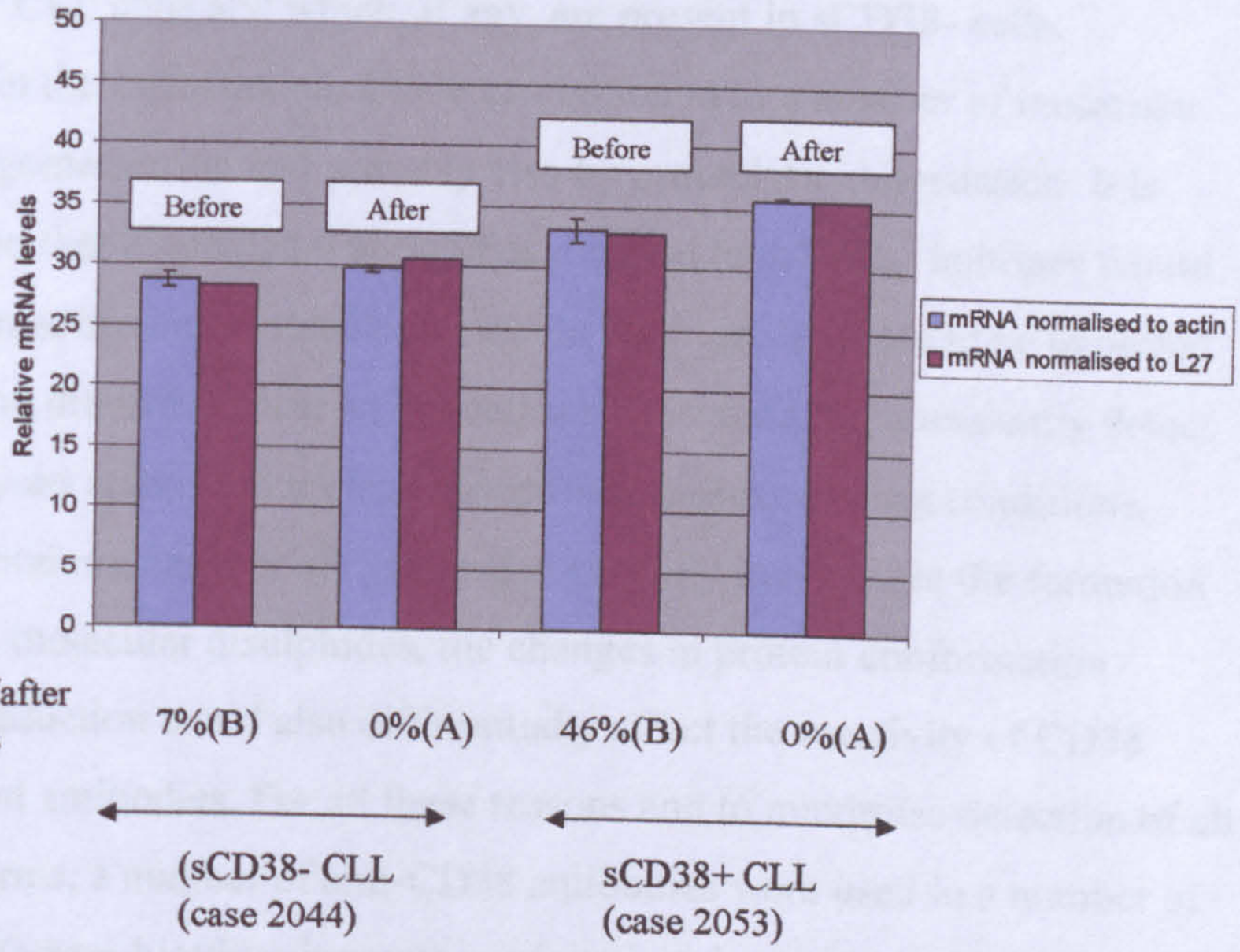


Fig. 2.16. CD38 mRNA levels before and after depletion of sCD38+ cells from CLL clones depleted of T cells, monocytes and NK cells. Surface CD38+ cells were removed with immunomagnetic beads from CLL cells purified as for Fig.2.15. One sCD38- (case 2044) and one sCD38+ (case 2053) CLL clone were studied. The CD38 message was normalised either against actin (two replicates in two experiments) or against L27 (two replicates in one experiment) as described in the Methods.

In all these real-time PCR experiments, it was noted that more amplification cycles were needed to detect fluorescence than in the positive control (PCL cells, not shown). These results therefore are consistent with the standard PCR findings, suggesting that CD38 message levels are uniformly low in CLL cells.

In conclusion, all 4 methods indicate that sCD38- CLL cells contain CD38 mRNA at levels comparable to those observed in sCD38+ clones. Furthermore, within both CD38 subgroups, sCD38+ and sCD38- cells have similar levels of message. In all cases, the levels of CD38 message are low.

2.4. DISCUSSION

The aim of the work in this Chapter was to determine which molecular forms of CD38 are present in sCD38+ CLL cells and which, if any, are present in sCD38- cells.

As mentioned in the Introduction, CD38 can be found in a number of molecular forms produced by oligomerisation and possibly also by proteolytic degradation. It is therefore to be expected that monoclonal antibodies directed to different epitopes would not equally well recognise distinct molecular forms. In addition, it is also to be expected that antibodies detecting the native form on the cell surface would not necessarily detect proteins in detergent-lysed cells or after electrophoresis under denaturing conditions. Lastly, since CD38 contains a number of cysteines potentially involved in the formation of both inter- and intra-molecular disulphides, the changes in protein conformation caused by disulphide reduction could also differentially affect the reactivity of CD38 molecules with different antibodies. For all these reasons and to maximise detection of all candidate molecular forms, a number of anti-CD38 antibodies were used in a number of different techniques (Western blotting; immunoprecipitation; immunocytochemistry, FACS and confocal microscopy of permeabilised and non-permeabilised cells).

Taken together, these approaches indicated for the first time that sCD38- as well as sCD38+ CLL cells possess immunoreactive CD38 proteins. Furthermore, both cell types were shown to express comparably low levels of CD38 message. However, the two cell types were shown to differ with regard to their content of 45 kD CD38 monomer. Thus, both Western blotting and immunoprecipitation experiments with different antibodies consistently demonstrated 45 kD CD38 in sCD38+ CLL and this band was largely absent from sCD38- cells. Since the presence of this 45 kD protein in Western blots corresponded to positivity detected with HB-7 in non-permeabilised cells by FACS analysis, this suggests that at least some of the 45 kD molecular form is expressed at the cell surface either as a monomer or as multimer(s). Although the sCD38- CLL cells (as defined by absence of surface reactivity with HB-7) largely lacked the 45 kD form of CD38, APAAP, FACS and confocal microscopic studies showed that these sCD38- cells possess immunoreactive CD38. The present biochemical studies indicate that this immunoreactivity is largely attributable to non-45 kD forms of CD38. Thus, both sCD38+ and sCD38- CLL cells were consistently shown to contain high-molecular

weight immunoreactive proteins. These bands were detected with a number of different antibodies in both Western blotting and immunoprecipitation experiments. Since the proteins were detected under both reducing and non-reducing conditions, they are likely to represent covalently linked multimers of CD38, perhaps produced by transglutamination (Umar et al, 1996).

This is the first time that the HMW multimeric forms of CD38 have been identified in CLL cells, and the findings are compatible with studies of other cell types showing that CD38 can associate into multimers as a result of intermolecular bonding by a number of different mechanisms (Bruzzone et al, 1998). The present study demonstrated a number of bands of MW between 172 and 210 kD. HB-7 and Ab-4 both reacted with these multiple bands, but Cl-22 detected only a 172 kD protein. Because Cl-22 is able to detect CD38 in APAAPs and Western blots but not in cells prepared for FACS analysis, it seems likely that this antibody reacts mainly with an epitope exposed only under denaturing conditions. It therefore seems probable that the 172 kD molecule is an altered form of CD38 perhaps produced by proteolytic cleavage of the 190 kD transglutaminase-crosslinked tetramer described in other cell types.

In addition to the 45 kD and >172 kD molecules, 2 further bands at ~60 kD and 27 kD were frequently observed in both sCD38+ and sCD38- CLL cells. The ~60 kD protein was best observed after the relatively gentle extraction achieved with CHAPS detergent and was demonstrable with 3 different anti-CD38 antibodies. The origin of this form is unclear but since it resists reduction, it may be derived from the transglutaminase-crosslinked tetrameric form.

The 27 kD band was also observed in both sCD38+ and sCD38- clones and was best detected with Ab-4. Its nature is also unclear, but it may represent a proteolytic fragment and it could also possibly represent a non-glycosylated form of CD38.

During the FACS experiments, it was unexpectedly observed that non-permeabilised sCD38- CLL cells (so classified on the basis of negative staining with HB-7) were consistently reactive with the Ab-4 mAb. Western blotting experiments indicated that both sCD38+ and sCD38- CLL cells contained bands of ~70 and 27 kD reactive after Ab-4 staining but not with HB-7. It therefore seemed possible at this stage that Ab-4 is detecting one or both of these bands on the surface of sCD38- CLL cells - this subject

is considered further in the next Chapter. Whatever the significance of these specific Ab-4 immunoreactive bands, the present findings indicate that it is the cell's possession of the 45 kD monomer, rather than CD38 per se, that is associated with the yet-to-be-defined functional property of sCD38+ CLL cells that confers an adverse prognosis. Since all CLL cells are positive with Ab-4, the form of CD38 detected by this antibody is probably irrelevant from a prognostic point of view, although it might have functional significance.

Further insight into surface-expressed CD38 was provided by confocal microscopy. Following staining with several antibodies, the positivity was detected in distinct clusters and these were dispersed after saponin treatment. This shows that, after staining (and possibly also before), surface CD38 is located in lipid rafts. Confocal microscopy also confirmed the intracellular presence of CD38 in both sCD38+ and sCD38- CLL cells. The staining pattern was finely granular or diffuse and therefore distinctly different from the surface staining.

Finally, the mRNA studies warrant a brief comment. First of all, the low levels of message in both CD38 subgroups are compatible with the fact that CD38 protein expression is also weak; these data are also in line with the literature (Zent et al, 2003). Furthermore, the fact that sCD38- cells are making comparable amounts of CD38 mRNA lends support to the idea that the immunoreactivity of these cells really is attributable to CD38. However, why the 45 kD form is only demonstrable in the sCD38+ clones, remains unclear.

In conclusion, the present studies, employing a range of techniques, showed that both sCD38+ and sCD38- CLL cells possess CD38 and that, with regard to the expression of this molecule, the two types differ in the surface expression of the 45 kD form of CD38 which, presumably can be present also as non-covalently associated dimers. Furthermore, the present studies indicate that various anti-CD38 antibodies react differently with different molecular forms of CD38 and with native vs denatured protein.

Although the present work shows that sCD38+ and sCD38- CLL cells contain different molecular forms of CD38, the precise location and function of these various molecules remains to be clarified. Their location and enzymatic function are considered further in the following Chapter.

CHAPTER 3

TOPOGRAPHY AND ENZYMATIC FUNCTIONS OF DIFFERENT MOLECULAR FORMS OF CD38

3.1. INTRODUCTION

In Chapter 2, it was established that both sCD38+ and sCD38- CLL clones express immunoreactive CD38, that both cell types contain various molecular forms of the molecule, and that CD38+ and CD38– cells differ mainly in their expression of the 45 kD form of CD38.

The purpose of the studies presented in this Chapter was to extend those in Chapter 2 with the aim of determining the cellular location and enzymatic function of the different molecular forms of CD38. To do this, a number of approaches were used. Firstly, CLL cells were submitted to subcellular fractionation and different molecular forms identified in the fractions by Western blotting. Secondly, to determine what molecular forms are expressed at the cell surface, lysates of surface radioiodinated cells were immunoprecipitated and analysed by SDS-PAGE and autoradiography. To assess the cyclase and hydrolase functions of CD38, a fluorimetric method has been established next, which was used first to compare the enzymatic activities of sCD38+ vs sCD38- CLL cell lysates. The same method was then applied to determine enzyme activity of only cell-surface bound CD38, by comparing activities before/after proteolytic removal of surface proteins. Finally, enzyme activities were determined in eluates extracted from different MW sections of SDS-gels.

Taken together, these studies showed, for the first time, that CD38 is expressed on the cell surface in a HMW form of ~205 kD in both sCD38+ and sCD38- CLL cells. Consistently resisting reduction, this form probably corresponds to transglutaminase-crosslinked tetramers, described in other cell types (Umar et al, 1996). The results also documented that in sCD38+ CLL cells, a proportion of cell-surface CD38 is expressed in the 45 kD monomeric form. That the bulk of the enzymatic activity in CLL cells is

attributable to these surface-located forms in both CD38 subgroups was shown by surface protein digestion experiments. The sCD38- cells largely lacked the 45 kD form on the surface, yet possessed both cyclase and hydrolase activities. Since other molecular forms of CD38 (27, 60, 172 kD) described earlier in Chapter 2 were not demonstrable at the cell surface, it seems that these are intracellular forms, and represent either biosynthetic intermediates or proteolytic fragments.

3.2. METHODS

3.2.1. Subcellular fractionation

Cryopreserved CLL and PCL cells were thawed and resuspended in 10mM TRIS-HCl buffer (pH 7.4) with 250 mM sucrose and 1mM PMSF. Cells were lysed in this buffer by three freeze-thaw cycles (Umar et al, 1996). Nuclei were separated by centrifugation (3000g for 10 min). The supernatant was centrifuged further by 18000g for 30 minutes; after separating the pellet (mitochondria, lysosomes, rough endoplasmic reticulum), the supernatant was collected and ultracentrifuged by 100,000g for 90 minutes, separating cellular membranes and ribosomes (pellet) and cytosolic proteins (supernatant). The fractions were solubilized in Laemmli's sample buffer, boiled at 95°C for 3 minutes and analysed by Western blotting with the C1-22 antibody.

3.2.2. Surface radioiodination of CLL cells

Iodobeads (Pierce) were washed twice in PBS (pH 8.0) and resuspended in 200 µl PBS. 1 mCi of Na [^{125}I] (Amersham) was added and incubated for 5 minutes at RT. 10^8 CLL cells, resuspended in 1 ml PBS pH 7.2 were added and incubated for 15 minutes at RT. Cells were then washed 3 times with PBS pH 7.2 and lysed in 3% CHAPS. CD38 has been immunoprecipitated with either Ab-4 or HB-7 as described in Chapter 2. Immunoprecipitates were run on SDS-PAGE under reducing or non-reducing conditions, transferred to a nylon membrane and radioactive bands visualized by autoradiography.

PCL cells were used as positive control and antibody only with lysis buffer as negative control.

3.2.3. Fluorimetric assays to measure cyclase/hydrolase activities of CD38

In these experiments, CHAPS lysates and immunoprecipitates of CLL and PCL cells were used. The fluorimetric method applied was based on previously published data (Graeff et al 1996). For CD38 cyclase activity, lysates were incubated with 1mM NGD^+ in the presence of 20 mM TRIS-HC1 (pH 7.0) at 37°C. For hydrolase activity, 0.2-0.8 mM εNAD was used as substrate at the same temperature and pH as in the cyclase reaction. All reagents were purchased from Sigma. Measurements were made at 300 nm excitation and 410nm absorption wavelengths in a SpectraMax Gemini spectrofluorometer. Data were analysed by the SoftMax Pro software and cyclase/hydrolase activities were expressed in relative millifluorescence units/min/10^8 cells.

3.2.4. Protease digestion of surface proteins in CLL cells with Streptomyces griseus protease

10^8 CLL cells were washed and resuspended in 0.1 M TRIS-HC1 pH 7.5, then slowly added to 5 ml pre-heated (37°C) Streptomyces griseus protease (Pronase, Sigma) working solution (0.5 mg/ml). Cells were incubated at 37°C with permanent rotation for 40 minutes, then centrifuged, washed twice in 0.1M TRIS-HCl and resuspended in PBS/BSA. Cells were lysed in 3% CHAPS and lysates assayed for hydrolase activities as described above.

3.2.5. Intragel assay for determining CD38 cyclase/hydrolase activities

The method applied for the intragel detection of CD38 was based on previous data (Bruzzone et al, 1998; Ziegler et al, 1997). Briefly, CHAPS lysates of CLL/PCL cells and

immunoprecipitates (Ab-4) were mixed with modified Laemmli's sample buffer (containing 1.5% SDS and no β-mercaptoethanol) and run in 10% polyacrylamide gel at 4°C. Prestained MW markers (Invitrogen) were also applied. For assessing cyclase and hydrolase activities of separated proteins, gels were incubated in 50 mM TRIS-HCl containing 3mM NHD^+ and 0.2 mM εNAD^+ respectively, for 20 minutes at 37°C. After incubation, fluorescence was visualised on a UV-transilluminator. Subsequently, gels were stained with Coomassie-blue to determine the MW of fluorescent bands.

3.2.6. Recovery of proteins from polyacrylamide gels

This has been performed according to the syringe maceration extraction (SME) method described earlier (Scheer et al, 2001). Briefly, whole-cell lysates and immunoprecipitates of CD38 were run in SDS-PAGE using 10% separating and 5% stacking gels. The running buffer contained 1.5% SDS and no β-mercaptoethanol. Pre-stained MW markers (Invitrogen) were also applied. After electrophoresis, the gel was sliced horizontally into 3 sections according to the expected sizes of the main CD38 molecular forms (monomers, dimers and tetramers: <64 kD; 64-116 kD; and >116 kD, respectively.). The gel slices were placed into a 5 cc.syringe without a needle, and forced through into a second syringe. The procedure was repeated 5 times, and the minced gel was collected in a 2 ml Eppendorf tube. 2 ml TRIS-HCl (pH 7) was added and mixtures vortexed for 2 minutes, than centrifuged at 12 000 g for 1 minute. The supernatant, containing the eluate was preserved and processed for cyclase and hydrolase assays.

3.2.7. Statistical analysis

Comparisons of cyclase and hydrolase activities in sCD38+ and sCD38- CLL cell lysates were performed by the independent-samples T-test. For all analyses, the SPSS software was used.

3.3. RESULTS

3.3.1. Distribution of different CD38 molecular forms in distinct subcellular fractions

Having identified different molecular forms of CD38, it seemed relevant to analyse the distribution of these proteins in distinct subcellular fractions of both sCD38+ and sCD38- CLL clones.

To do this, CLL cells were disrupted by freezing and thawing and separated by ultracentifrugation into nuclear, mitochondrial, membrane (consisting of both plasma and intracytoplasmic membranes including fragments of the endoplasmic reticulum) and cytosolic fractions as described in the Methods; similarly fractionated PCL cells were used as a positive control. The different cell fractions were then solubilised in DSSB, separated on SDS-PAGE, transferred and probed with C1-22. C1-22 was used for Western blotting because these studies were performed relatively early on during the thesis before Ab-4 and HB-7 had been investigated and at a stage when it was clear that C1-22 works satisfactorily in Western blots.

As shown in Fig 3.1, the distribution of CD38 molecular forms was very different in the four fractions.

In the nuclear fraction, little or no CD38 was detectable in either sCD38+ or sCD38- CLL clones. In contrast, large amounts of 45 kD monomer were detected in the nuclei of the PCL cells.

In the membrane fractions (consisting of both intracellular and surface membranes), the ~172 kD molecule was the most prominent band in both sCD38+ and sCD38- CLL cells.

Regarding the mitochondrial fraction, the ~172 kD molecule was demonstrable in both sCD38+ and sCD38- CLL cells, as well as in the PCs. The 45 kD monomer was readily detected in the mitochondrial fraction of the sCD38+ CLL cells, but was not present in the sCD38- clones.

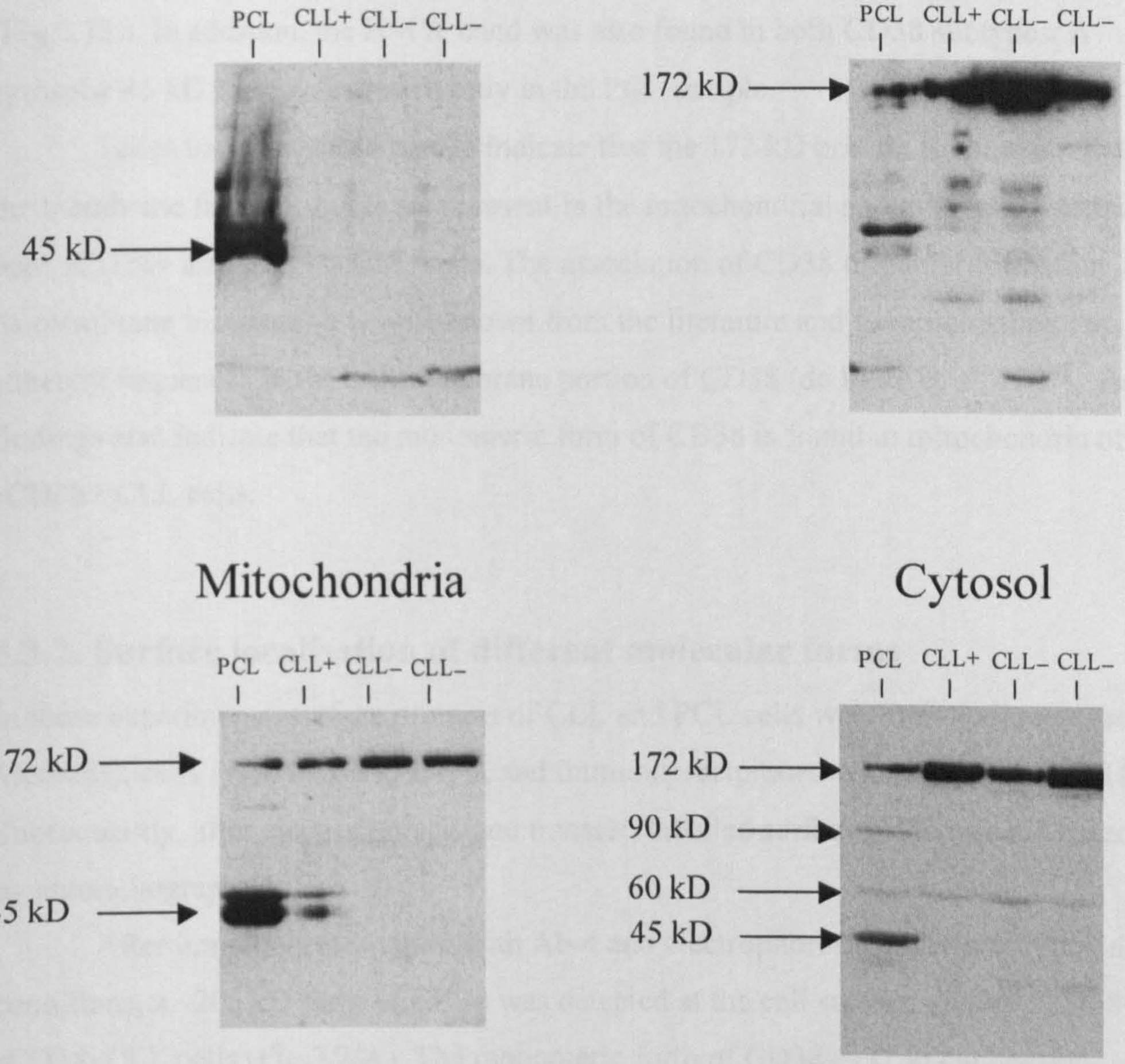


Fig 3.1. Western blotting for CD38 in different subcellular fractions of CLL cells. 10^{∞} CLL and PCL cells were disrupted by freeze/thaw cycles and fractionated by ultracentrifugation as described in the Methods. Surface CD38+ (CLL+) and surface CD38- (CLL-) CLL cases were used. Plasma-cell leukaemia cells (PCL) were the positive control. Different fractions were solubilised in DSSB, Western blotted and probed with Cl-22.

The cytosolic fraction differed from the other 3 fractions in containing a clear band at ~60 kD in both CD38 subsets as well as in PCL cells. It seems likely that this band corresponds to the band of similar MW described in CHAPS lysates in Chapter 2 (Fig.2.12.). In addition, the HMW band was also found in both CD38 subtypes. A cytosolic 45 kD band was present only in the PCL sample.

Taken together, these results indicate that the 172 kD protein is concentrated in the membrane fraction, but is also present in the mitochondrial and cytosolic fraction of both sCD38+ and sCD38- CLL cells. The association of CD38 oligomer formation and its membrane localisation is well-known from the literature and is explained by the adherent sequences in the transmembrane portion of CD38 (de Flora et al, 1997). The findings also indicate that the monomeric form of CD38 is found in mitochondria of sCD38+ CLL cells.

3.3.2. Surface localisation of different molecular forms

In these experiments, surface proteins of CLL and PCL cells were radioiodinated (see Methods), cells lysed in 3% CHAPS, and immunoprecipitated with either Ab-4 or HB-7. Subsequently, after electrophoresis and transfer, labelled surface proteins were detected by autoradiography.

After immunoprecipitation with Ab-4 and electrophoresis under non-reducing conditions, a ~205 kD form of CD38 was detected at the cell surface of both sCD38+ and sCD38- CLL cells (Fig.3.2/A). The monomeric form of CD38 (~37 kD under non-reducing conditions) was detected at the surface of sCD38+, but not sCD38-, CLL clones. After reduction, the high molecular weight band disappeared on both sCD38+ and sCD38- cells. On sCD38+ cells, the 45 kD monomeric form of CD38 became stronger and was accompanied by a new band of ~50 kD. In sCD38- CLL cells, as expected, no 45 kD molecule was detected after reduction but, as in sCD38+ cells, a ~50 kD molecule was again observed.

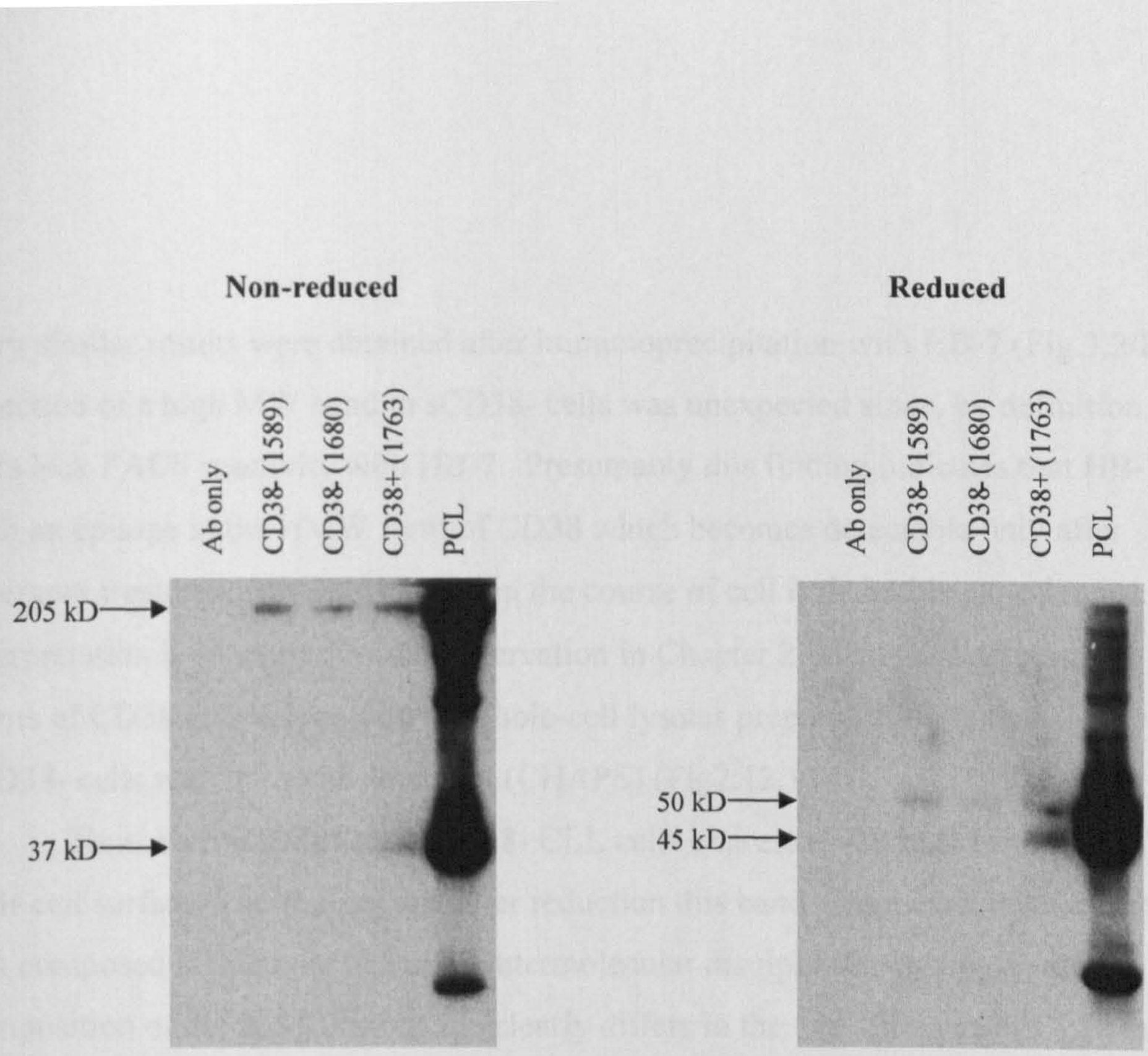


Fig.3.2/A. Immunoprecipitation of CD38 from surface radioiodinated CLL cells with Ab-4

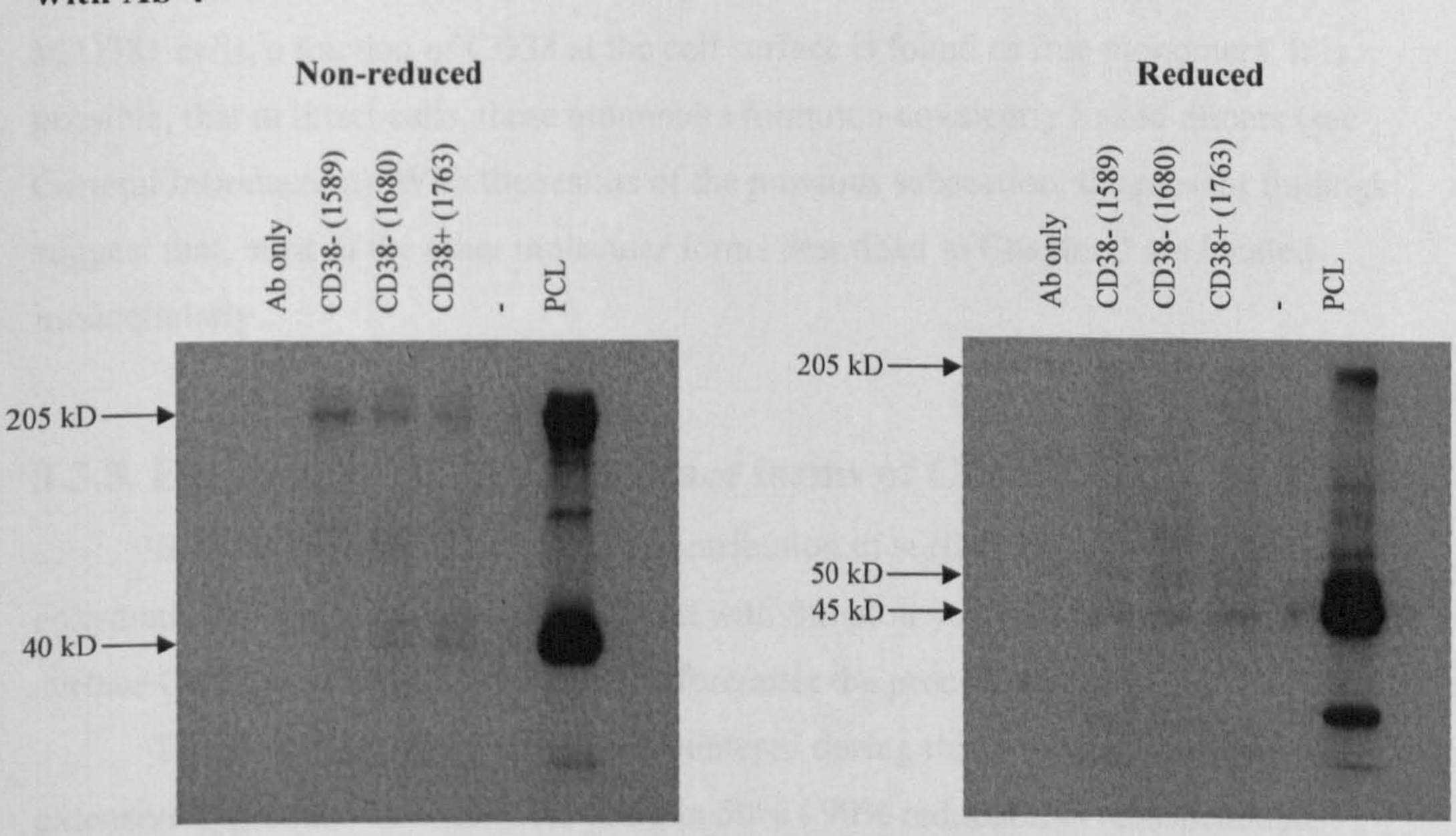


Fig.3.2/B. Immunoprecipitation of CD38 from surface radioiodinated CLL cells with HB-7

Very similar results were obtained after immunoprecipitation with HB-7 (Fig.3.2/B). The detection of a high MW band in sCD38- cells was unexpected since, by definition, these cells lack FACS reactivity with HB-7. Presumably this finding indicates that HB-7 reacts with an epitope in the HMW form of CD38 which becomes detectable only after detergent treatment (which was used in the course of cell lysis in this experiment). This interpretation is supported by the observation in Chapter 2, that HB-7 detects high MW forms of CD38 in Western blots of whole-cell lysates prepared from both sCD38+ and sCD38- cells with the same detergent (CHAPS) (Fig2.12.).

Thus, both sCD38+ and sCD38- CLL cells express a ~205 kD form of CD38 at their cell surface. The finding that after reduction this band disappears, indicates that it is composed of subunits linked by intermolecular disulphides. However, the composition of the 205 kD molecule clearly differs in the two subtypes of CLL: in sCD38+ cells, the 45 kD form is a major component of this HMW molecule while, in sCD38- cells, this protein has a different structure in which 45 kD monomers are not directly incorporated via disulphide linkages. These experiments also show that, in sCD38+ cells, a fraction of CD38 at the cell surface is found as free monomers. It is possible, that in intact cells, these monomers form non-covalently linked dimers (see General Introduction). With the results of the previous subsection, the present findings suggest that, most of the other molecular forms described in Chapter 2 are located intracellularly.

3.3.3. Enzymatic activities of surface forms of CD38

In order to examine the relative contribution of surface CD38 to the total enzymatic activity, CLL cells were treated with Streptomyces griseus protease to remove surface CD38 and activities compared before/after the procedure.

The major technical problem encountered during this protease treatment was extensive agglutination of cells resulting in 50% - 90% reduction in resuspendable lymphocytes. For this reason, the more sensitive hydrolase assay was used to compare the enzymatic activities before and after protease treatment. FACS analysis showed that

surface CD38 (assessed with Ab-4 and HB-7) had been completely removed without affecting the viability of the cells.

After protease treatment, the hydrolase activity of sCD38+ cells was markedly reduced (by ~95%), indicating that most of the enzyme activity is located at the cell surface of these cells (Fig.3.3). The hydrolase activity of sCD38- cell lysates was also markedly reduced (by ~90%) by protease treatment. These data indicate that in both sCD38+ and sCD38- CLL cells, the bulk of hydrolase activity is attributable to cell-surface-located CD38. Conversely, the fact that relatively little enzyme activity was detected in cell lysates after protease treatment indicates that the intracellular forms of CD38 have only low levels of functional enzyme.

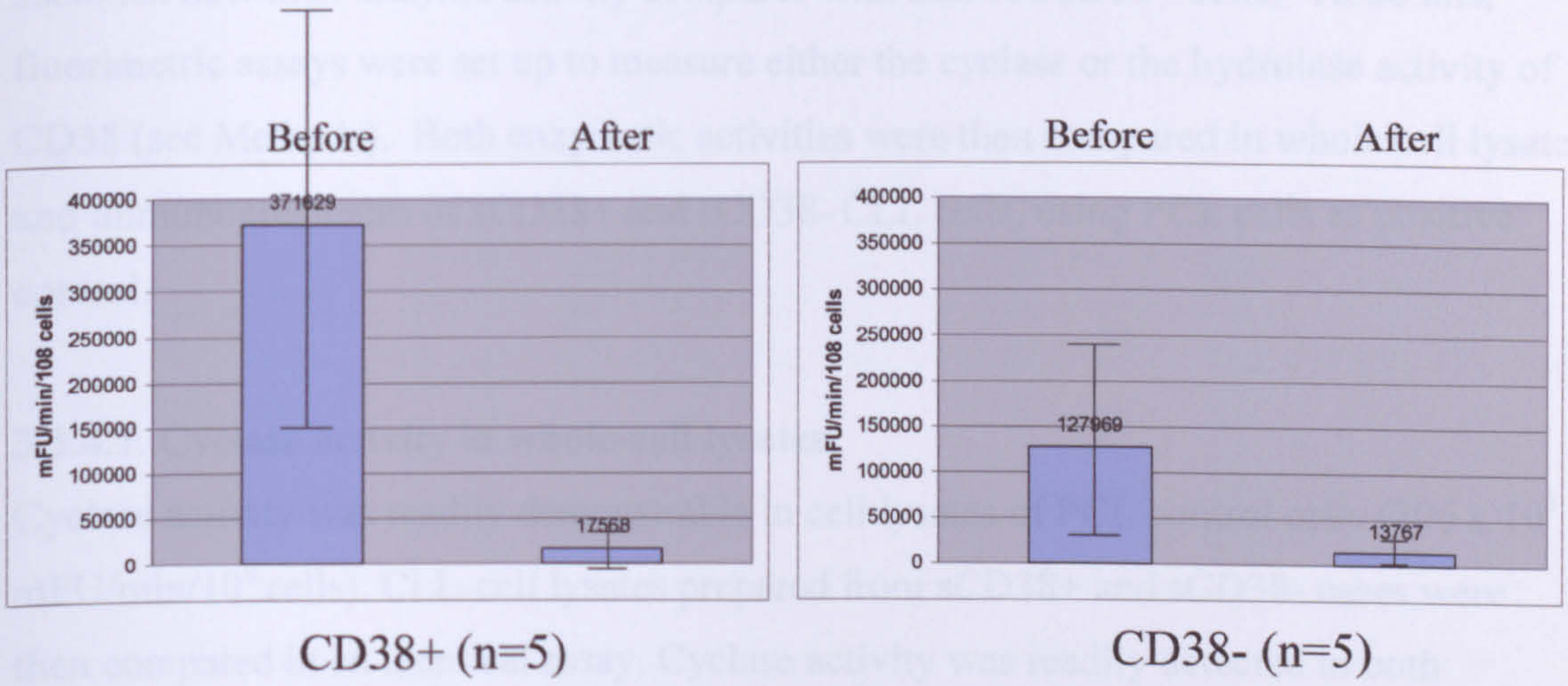


Fig.3.3. Hydrolase activities of sCD38+ and sCD38- CLL cell lysates before/after surface protein removal with Streptomyces griseus protease. CHAPS lysates of CLL cells were prepared before/after incubation of CLL cells with 0.5 mg/ml Pronase. Hydrolase activities were compared by the fluorimetric method, using εNAD as substrate.

Taken together with the surface radiolabeling experiments, the present findings indicate that in sCD38- cells the ~205 kD molecule has hydrolase activity. The results allow no firm conclusions about the relative contributions of the 205 and 45 kD forms to the overall surface enzymatic activity in sCD38+ cells. Also, it is tempting to suggest that the 45 kD molecule might be responsible for the greater enzymatic activity observed in sCD38+ cells.

3.3.4. CD38 enzyme activities in sCD38+ and sCD38- cells

Having shown in Chapter 2 that sCD38- CLL cells contain CD38, it seemed important to establish how their enzyme activity compares with that of sCD38+ cells. To do this, fluorimetric assays were set up to measure either the cyclase or the hydrolase activity of CD38 (see Methods). Both enzymatic activities were then compared in whole cell lysates and immunoprecipates of sCD38+ and sCD38- CLL cells, using PCL cells as positive control.

3.3.4.1. Cyclase activity in whole-cell lysates

Cyclase activity was readily demonstrable in cell lysates of PCL control cells (206 x 10^3 mFU/min/10^8 cells). CLL-cell lysates prepared from sCD38+ and sCD38- cases were then compared in an identical assay. Cyclase activity was readily detected in both sCD38+ and sCD38- CLL cell lysates (Fig.3.4); the activities were much lower than in PCL cells.

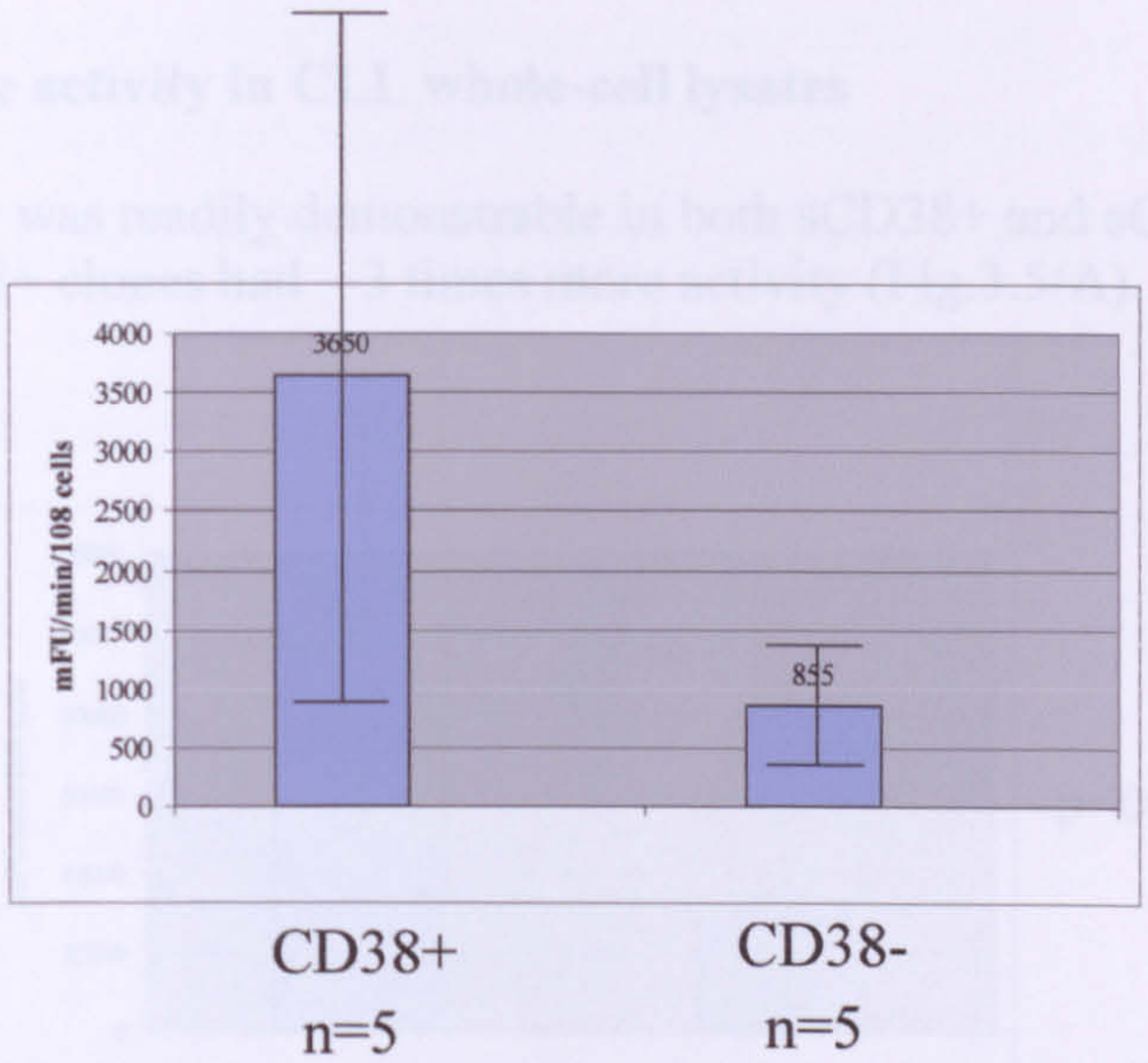


Fig.3.4 Comparison of CD38 cyclase activities in cell lysates of sCD38+ and sCD38- CLL cells. CD38 cyclase activities were measured in CHAPS lysates of sCD38+ and sCD38- CLL cells by a fluorimetric method using NGD^+ as substrate (see Methods). Data are expressed as arbitrary units/min/10^8 cells. Lysates correspond to 10^8 CLL cells per sample; 5 cases were studied in each subgroup. Columns represent the mean and error bars the SD values.

In sCD38- CLL cells the cyclase activity was around 25% of that found in the sCD38+ cells, and it seems unlikely therefore that the activity could be attributed solely to the small numbers of sCD38+ cells (<5% CD38%) present in the samples. The finding that sCD38- clones possess cyclase activity supports the biochemical and immunocytochemical data presented earlier indicating that these cells do express CD38.

3.3.4.2. Hydrolase activity in CLL whole-cell lysates

Hydrolase activity was readily demonstrable in both sCD38+ and sCD38- CLL cell lysates, but sCD38+ clones had ~ 3 times more activity (Fig.3.5/A).

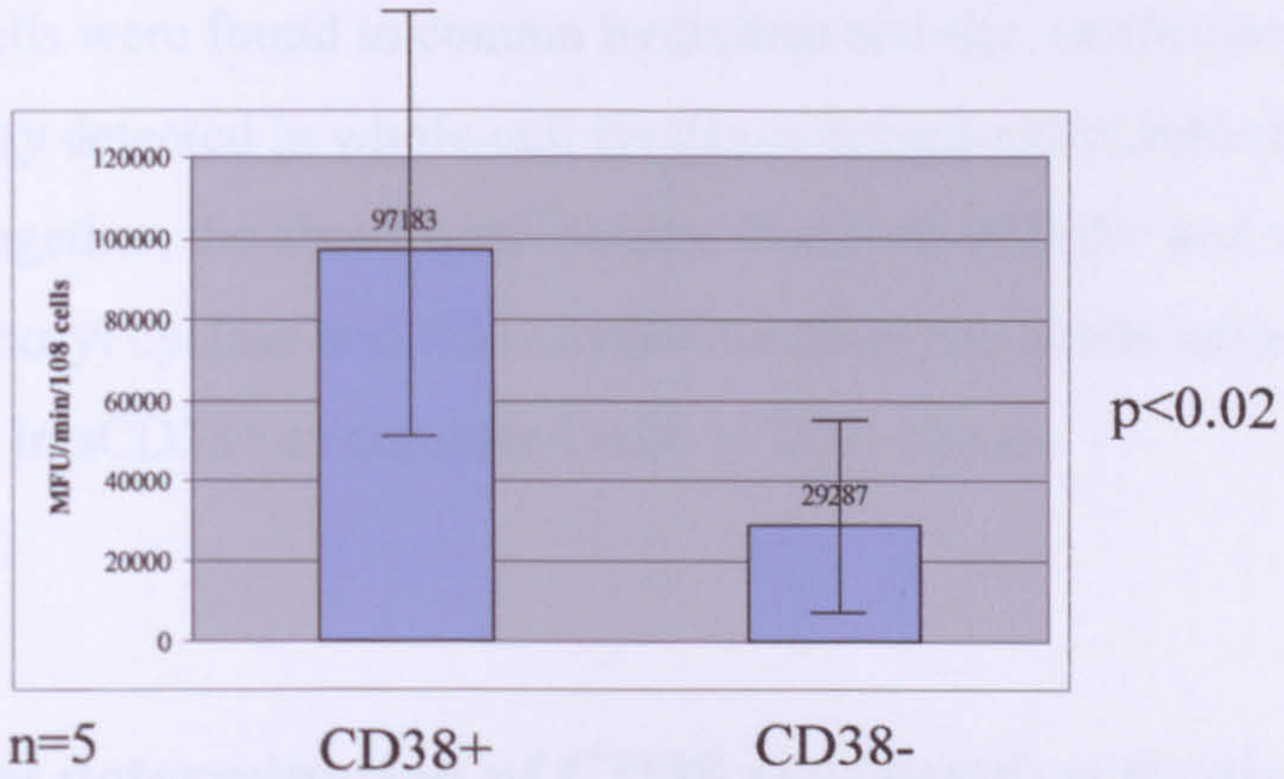


Fig.3.5/A Comparison of CD38 hydrolase activities in cell lysates of sCD38+ and sCD38- CLL cells. CD38 hydrolase activities were measured in CHAPS lysates of sCD38+ and sCD38- CLL cells using εNAD$^+$ as substrate. Data are expressed as arbitrary fluorescence units/min/10^8 cells. 5 cases were studied in each subgroup.

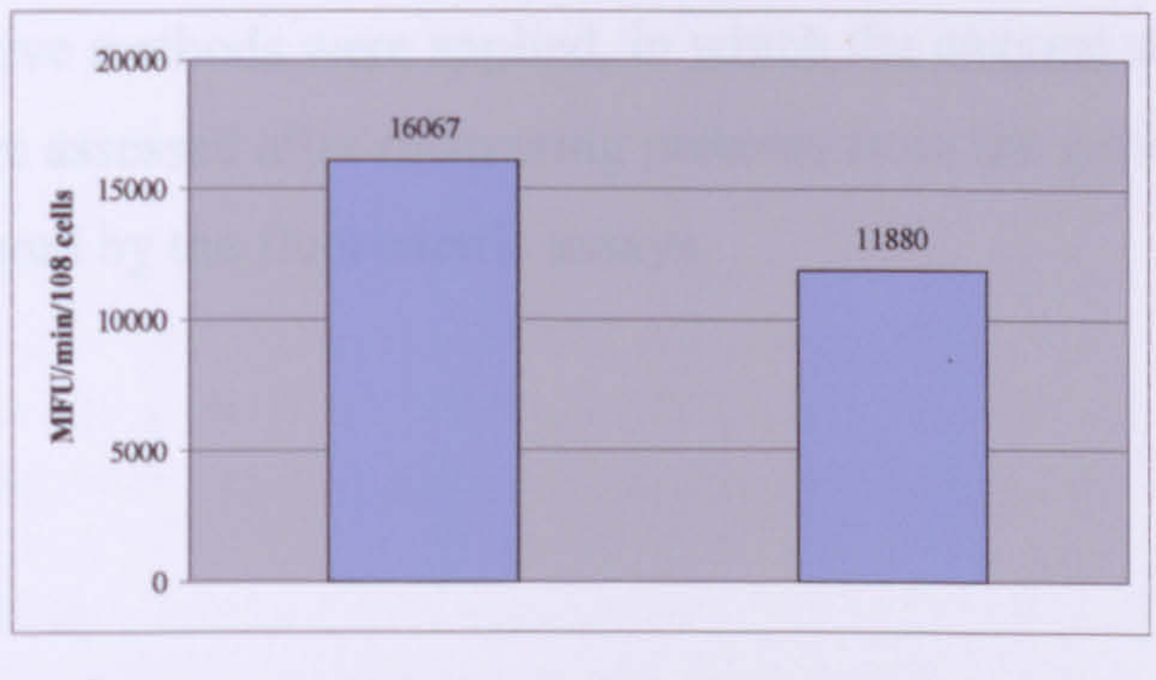


Fig.3.5/B Comparison of CD38 hydrolase activities in immunoprecipitates from sCD38+ and sCD38- CLL cells. CD38 hydrolase activities were measured in immunoprecipitates of sCD38+ and sCD38- CLL cells using εNAD$^+$ as substrate. Immunoprecipitation was performed in CHAPS lysates of CLL cells with the Ab-4 mAb, using 10^8 cells per case. 2 cases were studied in each subgroup.

Since CD38 is not the only NAD-hydrolase in mammalian cells, to provide an assurance that the hydrolase activity demonstrated in the CLL cell lysates is indeed mainly attributable to CD38, cell lysates were immunoprecipitated with Ab-4 and assayed for hydrolase activity. As shown in Fig.3.5/B, immunoprecipitates of both sCD38+ and sCD38- CLL-cells were found to contain hydrolase activity, confirming that the NAD-hydrolase activity detected in whole-cell lysates is indeed attributable to CD38.

Taken together, the above data indicate that both sCD38+ and sCD38- CLL clones possess ADP ribosyl cyclase and NAD hydrolase activities attributable to CD38 and that both are greater in sCD38+ as compared with sCD38- clones.

3.3.5. Intragel determination of CD38 enzymatic activities

In order to visualize directly the activity of different MW forms of CD38, an intragel assay was set up (see Methods). This method successfully demonstrated the hydrolase activity of the 37 kD CD38 monomer in immunoprecipitates of PCL cells (Fig.3.6.). However, it was not able to show enzyme activities in CLL-cell immunoprecipitates in further experiments (not shown). It was therefore concluded that this method is not sufficiently sensitive to detect the low levels of enzyme activity present in CLL cells. Thus, alternative methods were applied, in which the enzyme activities in different areas of the gel were assessed after recovering proteins from the gels and activities of the eluates measured by the fluorimetric assays.

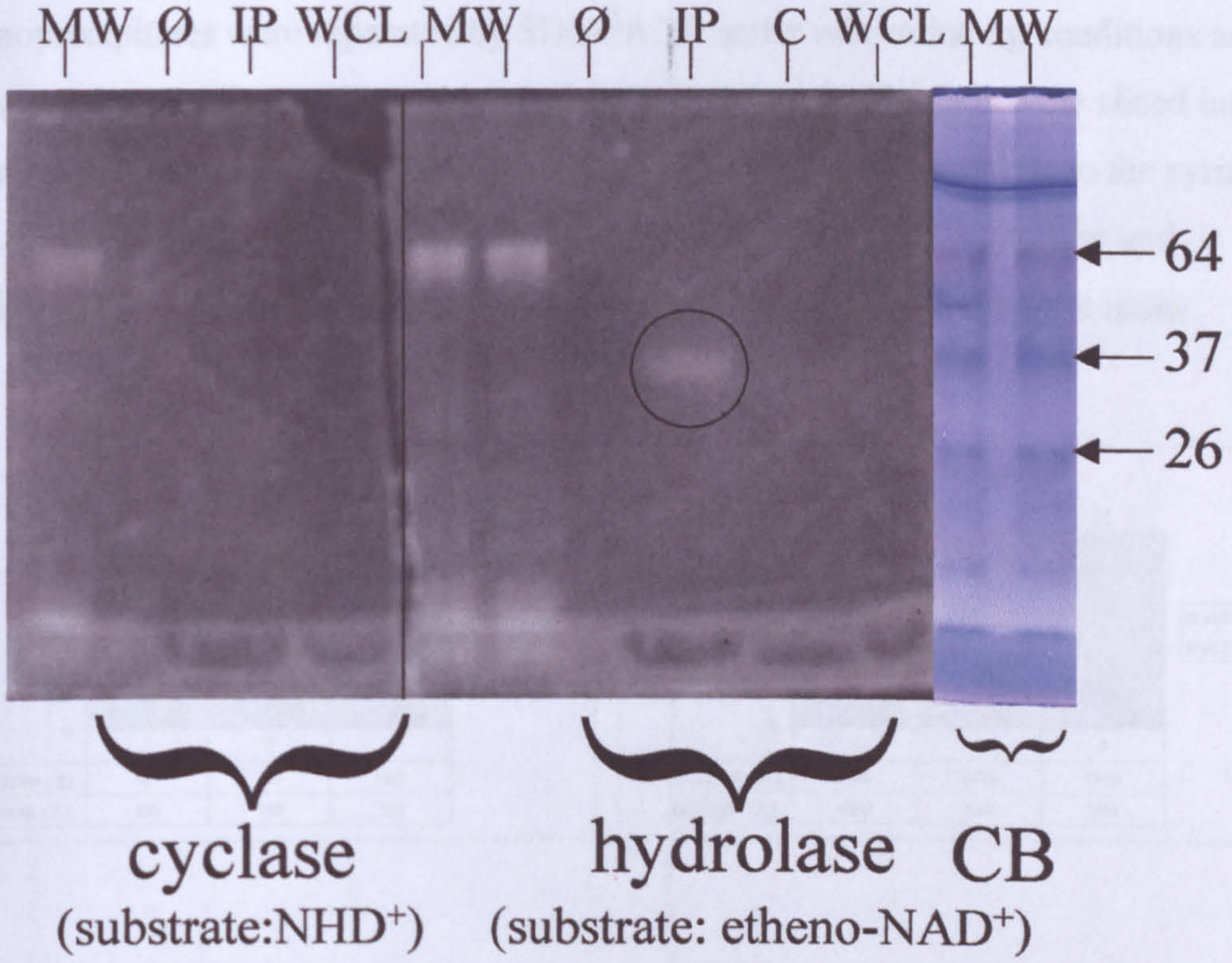
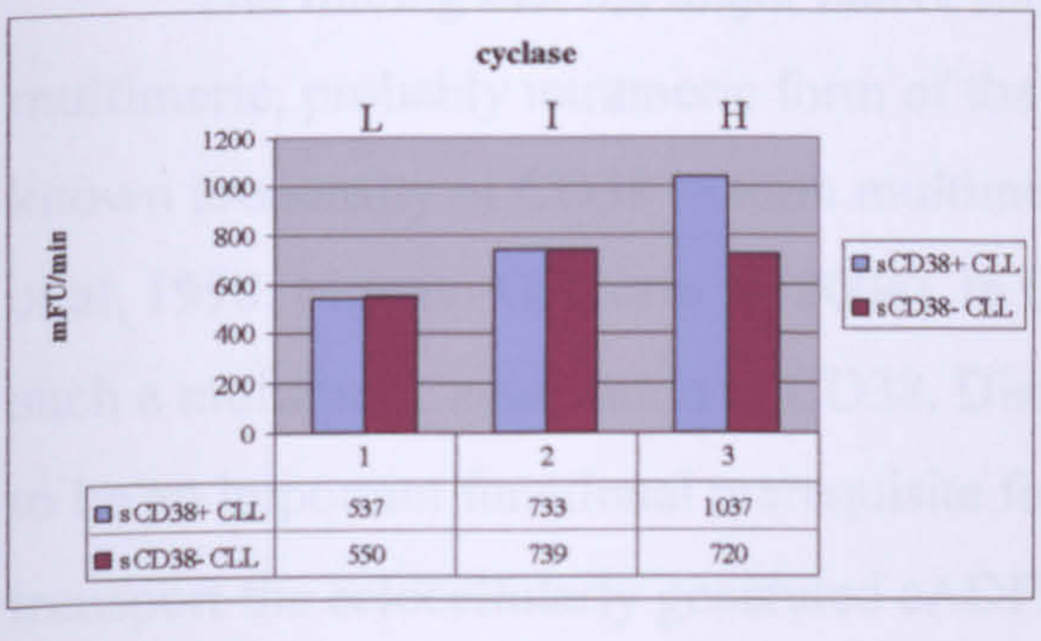


Fig.3.6. Intragel assay for measuring cyclase/hydrolase activities of CD38 in whole-cell lysates and immunoprecipitates of PCL cells. 10^8 PCL cells were lysed in 3% CHAPS and CD38 was immunoprecipitated with Ab-4 (2 μg/sample). Whole cell lysates (WCL) and immunoprecipitates (IP) were run on a 10% SDS-PAGE (1.5% SDS, no β-mercaptoethanol) at 4C. Pre-stained molecular weight markers (MW) were also applied; ∅ represents empty lane. After electrophoresis, the gel was cut into two and the two pieces incubated with 3 mM NHD+ (cyclase) and 0.2 mM εNAD+ (hydrolase) substrates for 20 minutes at 37C. Fluorescent bands were visualised on a UV transilluminator. Only hydrolase reaction with the CD38 monomer (37 kD) of the IP from PCL.cells produced a fluorescent band (circle); cyclase reactions (NHD+) were negative. Some of the MW markers were also fluorescent (64 kD). In order to localise the fluorescent CD38 band better, MW markers were subsequently stained with Coomassie-blue (CB).

3.3.6. The enzyme activities of different MW forms of CD38

To assess the enzymatic activity of the different molecular forms of CD38, immunoprecipitates were separated by SDS-PAGE under non-reducing conditions and the enzyme activity from different parts of the gel analysed. The gels were sliced into 3 sections (<64kD, 64-116 kD, >116 kD) and proteins recovered according to the syringe maceration extraction procedure (Scheer et al, 2001) (see Methods). Cyclase and hydrolase activities in the three fractions were measured by the fluorimetric assay.



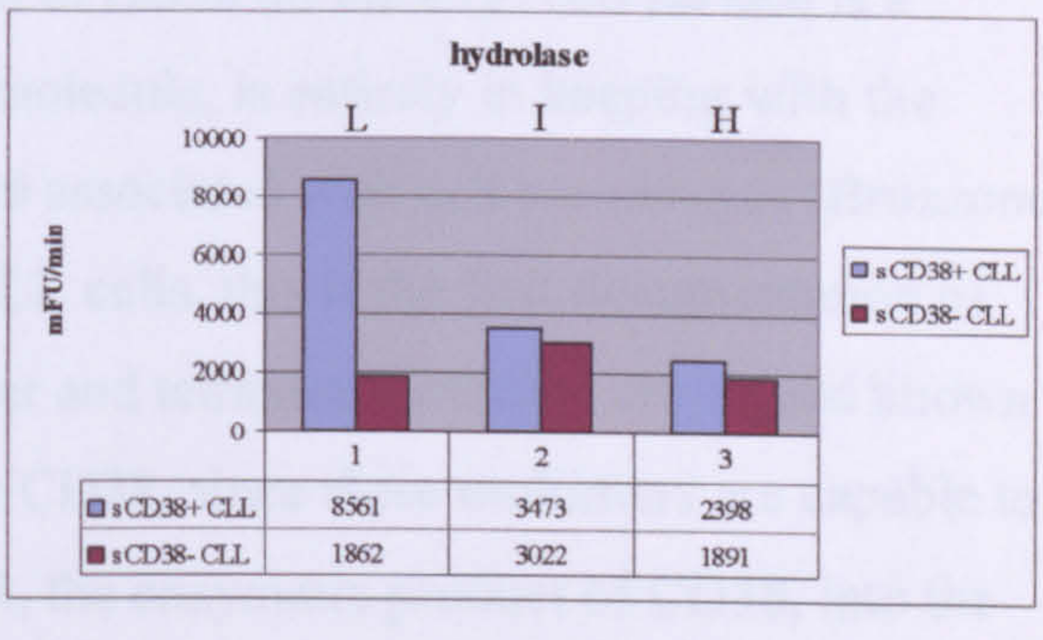


Fig.3.7. CD38 enzyme activities in eluates from SDS-gels. CD38 immunoprecipitates from sCD38+ and sCD38- CLL were run on SDS-PAGE and proteins recovered from the gels according to the SME procedure. Cyclase and hydrolase activities were determined in the low (L) (<64 kD);intermediate (I) (64-116 kD); and high (H) (>116 kD) MW fractions. All 3 fractions showed both cyclase and hydrolase activities. Activity is expressed as mFU/min. The picture represents one of three consecutive experiments.

For technical reasons, (different buffer volumes needed to solubilize the minced gel slices of different MWs; material loss during mincing; proteins are differentially elutable from the gel) the results did not allow quantitative comparisons. What is clear, however, that all 3 fractions showed both cyclase and hydrolase activities. It is tempting to speculate that the activity in the HMW fraction is attributable to the HMW forms discussed earlier. The activity in the LMW fraction could be derived from either the 45 kD monomer or the ~60 kD form described in Chapter 2, and the activity in the intermediate fraction from

CD38 dimers. These latter forms were rarely detected in Western blots probably because the non-covalent, ionic and hydrophobic bonds of the dimers are disrupted during SDS-PAGE, producing monomers.

3.4. DISCUSSION

The aim of this Chapter was to extend the findings of Chapter 2 by examining the location and enzymatic functions of different MW forms of CD38. Several approaches were used to achieve this goal.

The finding that the major native form of CD38 on the CLL cell surface is a multimeric, probably tetrameric form of the molecule, is entirely in keeping with the known propensity of CD38 to form multimers associated with cell membranes (Bruzzone et al, 1998; Moreno-Garcia et al, 2004). In CLL cells, this is the first documentation of such a multimeric association of CD38. Dimer and tetramer formation are indeed known to be an important functional prerequisite for CD38, since these multimers are capable to transport the ectocellularly generated cADPR, the enzymatic product of CD38, into the cell interior (see General Introduction). Since the tetrameric forms found in CLL cell lysates largely resist reduction, it is probable that these are the product – at least to a certain extent – of an intracellular crosslinking reaction by transglutaminase (Umar et al, 1996).

A novel, interesting and unexpected finding was the occurrence of the tetramers on the surface of CLL cells classified as "CD38-negative" by FACS with the HB-7 antibody. It seems likely that this ~205 kD tetramer was the molecular form detected by the Ab-4 antibody in the FACS experiments (Chapter 2) as a positivity on the surface of sCD38- CLL cells.

These data clearly show that the present classification of CLL on the basis of cell surface expression of CD38 has its limitations and has to be viewed in context of the antibody and methodology used. It has been already indicated in Chapter 1 that the choice of the antibody has an impact on the results and inferences drawn in the clinical setting. The data in Chapter 2 and Chapter 3 made also clear that the same caution has to be exercised in the experimental setting, studying CD38 under different conditions and by

various methodologies. As shown, CD38 has a number of different molecular forms and is present at different cellular locations, thus these different reactivities are to a certain extent understandable.

Although a large body of data have become available on the function of CD38 in different non-human and human tissues and cell lines, the function of CD38 in CLL remains to be clarified. In other cell types, however, its major functions are directly linked to its enzymatic product, cADPR which acts as a second messenger mobilizing Ca++, and thereby bringing about most of the physiological effects of CD38 – cell proliferation, gene transcription, motility, signalling (Funaro et al, 2000). This was the reason it seemed important to examine the enzymatic function of CD38 in CLL cells and to compare CD38 subgroups in this respect. Our finding that cells from both subgroups have distinct cyclase and hydrolase activities is in line with the data in Chapter 2, that CLL cells previously classified as "CD38-negative" do express CD38. The observation that the bulk of the whole-cell enzymatic activity is confined to the cell membrane is in keeping with the propensity of CD38 to be associated with cell membranes (Moreno-Garcia et al, 2004) and gives rise to the idea that the major enzymatically active forms of CD38 in CLL cells are the membrane-bound multimers detected on the surface by the radiolabelling experiments.

The protein elution studies presented in this Chapter – although not allowing quantitative conclusions – indicate that the HMW fractions of CD38 have both cyclase and hydrolase activities. In other cell types, these tetramers were shown to have higher cyclase/hydrolase ratios than the 45 kD monomeric form (Umar et al, 1996). Detailed further studies allowing quantitative comparisons and enzyme kinetic analyses were beyond the scope of this work and will be the subject of future studies.

To study the intracellular topology of CD38, the subcellular fractions of CLL cells were studied. One of the major findings in these experiments were that a HMW form of CD38 was found predominantly in the membrane fraction. Since the radiolabelling experiments demonstrated the presence of a ~205 kD, but not the 172 kD form on the cell surface, it seems likely – as already suggested in Chapter 2 – that the 172 kD molecule is a membrane-associated proteolytic fragment of the full CD38 tetramer. As it has already been pointed out, multimer formation occurs predominantly in the cell

membrane-associated forms of CD38 because of the instrumental role of its transmembrane portion in the mutual adherence and association of monomers (De Flora et al, 1997).

The concomittant presence of the 172 kD band in the cytosolic fraction might be due to contamination of this fraction with intracellular microvesicles and the ~60 kD form can correspond to a proteolytic product of CD38 tetramers released into the cytosol from sites of intracellular proteolysis. The presence of the 45 kD form in the mitochondrial fraction of sCD38+ CLL cells is in line with the known occurrence of CD38 in mitochondria (Yamada et al, 1997).

CHAPTER 4

CD38 AS A MARKER OF CELL PROLIFERATION IN CLL

4.1. INTRODUCTION

Following the clinical, biochemical and structural studies of Chapters 1-3, it seemed important next to consider the function of CD38 in CLL. In particular, it was necessary to consider why expression of surface CD38 (as detected with the HB-7 antibody) is correlated with a bad prognosis. In the light of the extensive literature demonstrating a role for CD38 in cell proliferation (see below), it appeared reasonable to hypothesize that sCD38 expression might in some way be related to active cell cycling, which in turn might contribute to "bad" disease.

Although CLL is often regarded as an accumulative rather than proliferative disorder, in about 25% of all CLL cases there are clearly features of a progressive, proliferative condition with B-symptoms, short survival and therapy resistance. The small but significant proliferating pool maintaining and feeding the rest of the clone is believed to be located in so-called proliferation centres in lymphoid tissues and bone marrow (Dormer et al, 1983). In these areas, T-cells, cytokines and extracellular matrix probably all contribute to the proliferative process.

The proliferative capacity of the clone has been shown to have prognostic significance in different types of non-Hodgkin's lymphomas (Hall et al, 1998). In CLL, a higher percentage of S-phase cells were shown to be correlated with tumour bulk and poor outcome (Orfao A et al, 1992). Also, markers of increased cell proliferation such as thymidine kinase activity and sCD23 were also documented to be correlated with adverse prognosis (Hallek et al, 1999; Lampert et al, 1999). Other CLL subsets known to have a worse outcome such as cases with trisomy 12 have been shown to be associated with increased cell proliferation as measured by the Ki-67 antigen (Garcia-Marco, 1996). Recently, in studies based on telomere length and telomerase activity, Damle et al (2004) and Caligaris-Cappio (2004) et al reported that unmutated CLL cases have undergone a much higher number of cell divisions than have mutated ones. Other proliferative features of unmutated CLL were documented

by showing higher thymidine kinase values in this subset (Magnac et al, 2003). Since unmutated CLL is known to have a bad prognosis and also to be closely associated with CD38 positivity (see Chapter 1), these data also point indirectly to a possible relationship between CD38 expression and cell proliferation in this disease.

There is a large body of data on the relationship between CD38 expression, cell proliferation and cell-cycle regulation in human and non-human cells. Masuda et al recorded oscillations of ADP-ribosyl cyclase activity in the unicellular organism, Euglena gracilis during cell cycle, with the highest values at the time of cell division (Masuda et al, 1997). Franco et al (2001) found that in human fibroblasts, CD38 enzyme activity is associated with elevated Ca++ levels and increased proliferation. In another series of experiments, Zocchi et al (1998) showed that transfection of CD38 into HeLa and 3T3 cells increases intracellular Ca++, cell proliferation and decreases doubling time.

In studies of normal human lymphoid tissues, Golay et al (1994) reported that in tonsillar B cells, the actively proliferating subset is highly CD38-positive. It has also been documented that cADPR, the enzymatic product of CD38 cyclase activity, augments the colony-forming capacity of human haematopoietic precursors (Zocchi et al, 2001). In CD34+ human haematopoietic stem cells, Holm et al (1999) found that cytokine-induced proliferation is confined preferentially to the CD38+ subset.

In CLL cells, CD38 and cell proliferation have been studied by a number of different groups. Deaglio et al found that ligation of IL-2-upregulated CD38 on CLL cells results in increased cell proliferation and plasmablastic morphology (Deaglio et al, 2003). They speculated that, in CLL lymphoid tissues, CD38 might function as a signalling molecule capable of bringing about proliferative processes upon appropriate extracellular stimuli. Others documented that stimulation of cell proliferation in CLL cells by loxoribine (an immunostimulant acting on TLR7) is accompanied by an upregulation of a number of different markers including CD38 (Goodman et al, 1994). Granziero et al showed that survivin – a factor enhancing proliferation and having also an anti-apoptotic effect – is expressed mainly in CD38-positive cases (Granziero et al, 2001). Boechtler et al reported on the higher proliferative activity of CD38+/CD19+ CLL cells in bone marrow (Boechtler et al, 2001). There are also a number of reports that CD38 expression can increase in CLL, a phenomenon

paralleled by a switch to a more progressive course (Chevallier et al, 2002; Hamblin et al, 2002; Kroeber et al, 2002). All these data therefore support the hypothesis that surface CD38 expression in CLL may be associated with enhanced cell cycle activity.

In this work, Ki-67 immunoreactivity is used to estimate the number of CLL cells in cell cycle. Ki-67 is the name of a clone of mouse antibodies raised against the human L428 Hodgkin's disease cell line, reacting with a nuclear antigen present in cycling cells in all phases of the cell cycle other than G0 (Gerdes et al, 1984). This antibody has been widely used to assess the size of the proliferative fraction in a range of normal and haemic and non-haemic tumours (Franklin et al, 1985; Burger et al, 1986; Gatter et al, 1986; Gerdes et al, 1986). In non-Hodgkin's lymphomas, the percentage of Ki-67 positivity has been shown to correlate with histological grade and prognosis (Gerdes et al, 1997; Grogan et al, 1988; Hall et al, 1988). In low-grade non-Hodgkin's lymphomas, Ki-67-positivity has been shown to be associated with worse prognosis (Hall et al, 1988). In CLL, Cordone et al (1992b) showed that Ki-67 activity is correlated with prolymphocyte count, stage and aggressiveness of the disease. Other groups also reported the association of Ki-67 positivity and adverse clinical features in CLL (Orchard et al, 1996, Astsaturov et al, 1997).

Two approaches were used in this Chapter to examine the relationship between sCD38 and cell cycling. In the first, CLL cells co-stained for surface CD38 and nuclear Ki-67 were examined by flow cytometry and the relationship between CD38 expression and cycling activity was analysed by a number of approaches (see below). In the second, CLL lymph nodes were stained for CD38 and the reactivity of proliferation centres assessed by APAAP immunocytochemistry.

4.2. METHODS

4.2.1. CLL cells and patient characteristics

Cryopreserved peripheral blood samples from 29 patients with well-characterized CLL were used. The male/female ratio was 2.3 and the mean age 64 years; 15 cases were CD38-positive (>20% CD38+/CD19+ cells) and 14 CD38-negative. To minimize the relative numbers of contaminating cells, cases with higher lymphocyte counts were preferentially used (WBC >40 x 10^9/L in 23/29 of cases).

4.2.2. Antibodies

The following antibodies were used: anti-CD38-PE (HB-7, Becton Dickinson), anti-Ki-67-FITC (DAKO, F0788), anti-CD19-PE-Cy5 (Santa Cruz, sc-18896) and class-specific control antibodies: IgG1-PE (BD), IgG1-FITC (DAKO) and IgG1-PE-Cy5 (Santa Cruz).

4.2.3. Permeabilization and FACS staining of CLL cells

These were performed according to the method of Jacob et al (1991). Briefly, 2 x 10^6 CLL cells were first incubated for surface CD19 and CD38 with the anti-CD38-PE and anti-CD19-PE-Cy5 antibodies and the isotype controls (all in 1:10 dilution) in RPMI/BSA for 15 minutes at room temperature in the dark. After two washes in PBS containing 1% BSA, cells were permeabilized and stained for intranuclear Ki-67 by incubation in 50 µl Hanks solution with 0.3% saponin and 2% FCS containing the Ki67-FITC antibody or the isotypic control (1:10) for 30 minutes at 4C. After two washes in PBS with 0.1% saponin and 2% FCS, cells were resuspended in PBS and analysed on a Becton Dickinson FACSscan flow cytometer. In each case, 10 000 events were acquired; analysis was performed by the CellQuest software.

4.2.4. Analysis of FACS data

In order to exclude cellular debris, apoptotic cells and monocytes, the lymphocytic gate was set up manually. In order to exclude T-cells, known to have distinct cycling activity (Cordone et al, 1992a), the lymphocytic gate was applied to a forward scatter/CD19-PE-Cy5 dot plot and the CD19+ population gated on. This gate was then applied to a CD38-PE/Ki-67-FITC dot plot, in which the actual percentages of positive cells were determined. Small versus large lymphocytes were gated on the forward scatter/side scatter plot after applying the CD19+ gate; Ki-67 values within the two subpopulations were obtained from CD38-PE/Ki-67-FITC dot plots. Data were analysed by the CellQuest software.

4.2.5. Immunohistology

Formalin-fixed, paraffin-embedded CLL lymph node tissues (n=3) were stained for CD38 by the following procedure. After clearing and rehydration, slides were boiled

in 10 mM sodium citrate buffer (pH 6) for 10 minutes and blocked with 10 mg/ml BSA before overnight incubation with AT-1, HB-7 and Ab-4 antibodies. These amounts of antibodies were chosen after titration over a range of concentrations. Sections were then incubated with biotinylated goat anti-mouse antibody followed by ExtraVidin alkaline phosphate and exposure to substrate (Fast Red/Naphthol AS MX phosphate/levamisol). Slides were counterstained by haematoxylin.

In addition, fixed frozen sections (n=3) were stained in an identical way. These tissues were obtained from the Candis tissue bank with the approval of the Liverpool Research Ethics Committee.

The peripheral blood CD38 reactivity of CLL cells was known in one of these cases (sCD38+), which is shown on Fig.4.8.

4.2.6. Statistical analysis

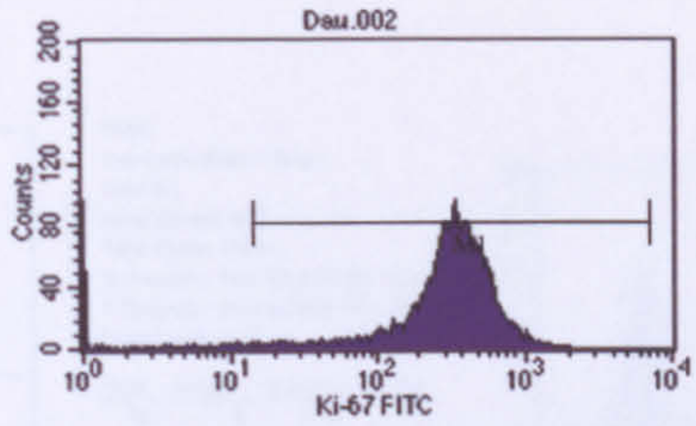
Comparing means of Ki-67 percentages in the CD38+ vs CD38- subgroups and means of CD38 percentages in the Ki-67+ vs Ki-67- subgroups were calculated by the Mann-Whitney test. To correlate surface CD38 and nuclear Ki-67 expression, the Pearson correlation was applied. Comparison of CD38 expression in the Ki-67+ vs Ki-67- subsets was performed by the paired-samples T-test. For comparison of Ki-67 expression in small vs large CLL lymphocytes, the independent-samples T-test was used. All analyses were performed by the SPSS software package.

4.3. RESULTS

4.3.1. Positive controls

Before testing CLL cells, in order to establish a positive control, the above methodology was applied to cell types known to have a high proliferation rate. Daudi cells and blasts from AML and ALL cases were used for this purpose. As shown on Fig.4.1, all 3 cell types show bright positive staining with the anti-Ki-67 antibody, strongest in the Daudi cells (>90%), while the AML and ALL blasts expressed 69% and 29% respectively. These results are in accord with previous literature (Hall et al, 1988).

Daudi cells

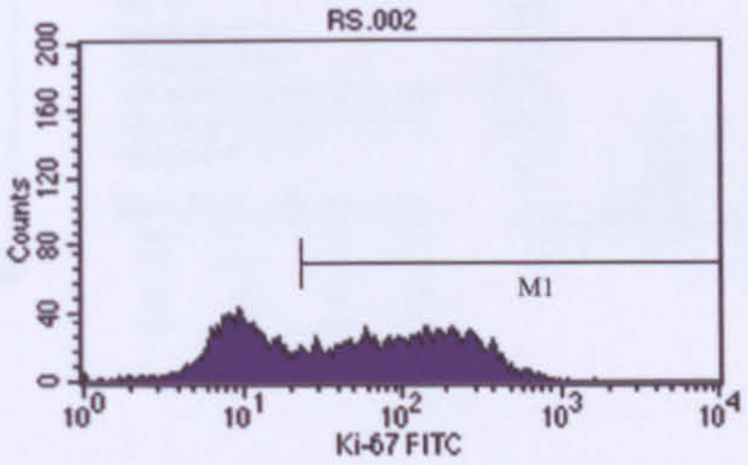


Histogram Statistics

File: Dau.002
Acquisition Date: 10-Mar-3
Gate: G4
Gated Events: 9589
Total Events: 10000
X Parameter: FL1-H Ki-67 FITC (Log)

Marker	Left, Right	Events	% Gated	% Total	Mean
All	1, 9910	9589	100.00	95.89	342.88
M1	15, 7365	9316	97.15	93.16	352.77

AML

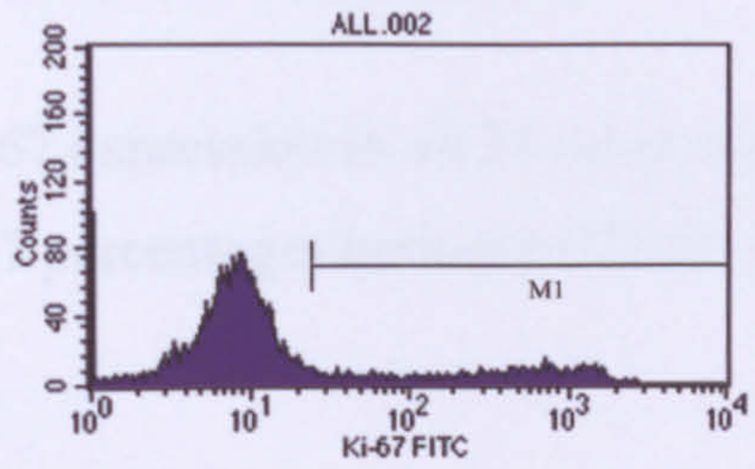


Histogram Statistics

File: RS.002
Acquisition Date: 10-Mar-3
Gate: G4
Gated Events: 9612
Total Events: 10000
X Parameter: FL1-H Ki-67 FITC (Log)

Marker	Left, Right	Events	% Gated	% Total	Mean
All	1, 9910	9612	100.00	96.12	97.28
M1	25, 9910	5840	60.76	58.40	153.11

ALL



Histogram Statistics

File: ALL.002
Acquisition Date: 10-Mar-3
Gate: G4
Gated Events: 9889
Total Events: 10000
X Parameter: FL1-H Ki-67 FITC (Log)

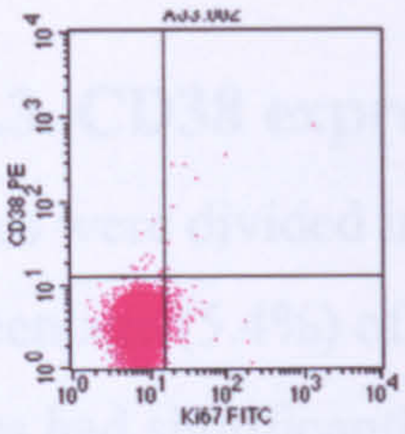
Marker	Left, Right	Events	% Gated	% Total	Mean
All	1, 9910	9889	100.00	98.89	110.76
M1	26, 9910	1973	19.95	19.73	521.59

Fig. 4.1. Flow cytometric detection of Ki-67 in saponin-permeabilized leukocytes: positive controls. Cell types known to be highly proliferative (Daudi cells, AML and ALL blasts) were chosen for this purpose. All three conditions show marked Ki-67 positivity, strongest in the Daudi cells with more than 90% of the cells being positive.

4.3.2. Detection of Ki-67 expression in sCD38+ and sCD38- CLL cells

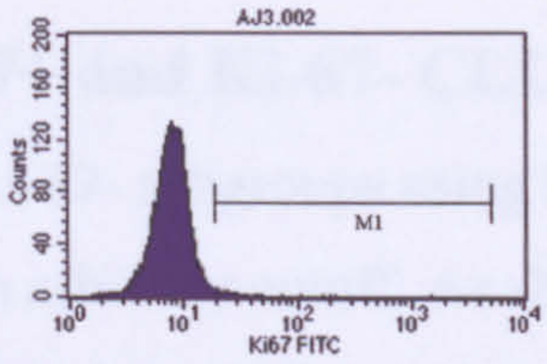
After establishing a positive control, the same procedure was applied to previously cryopreserved sCD38+ and sCD38- CLL cells from peripheral blood. 29 cases were studied, 15 being sCD38+ and 14 sCD38-. Fig.4.2 shows characteristic staining patterns of sCD38+ and sCD38- CLL clones. It is noteworthy, that in the sCD38+ clones, the majority of Ki-67+ cells coexpress CD38.

CD38-



Panel:
Acquisition Date: 6-Aug-3
Gate: G3
Gated Events: 9835
Total Events: 10000
X Parameter: FL1-H Ki67 FITC (Log)
Y Parameter: FL2-H CD38 PE (Log)
Quad Location: 17, 12

Quad	Events	% Gated	% Total
UL	18	0.18	0.18
UR	9	0.09	0.09
LL	9683	98.45	96.83
LR	125	1.27	1.25

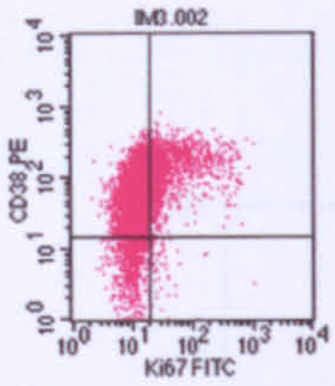


Histogram Statistics

File: AJ3.002
Sample ID: AJ3
Patient ID: AJ3
Panel:
Acquisition Date: 6-Aug-3
Gate: G3
Gated Events: 9835
Total Events: 10000
X Parameter: FL1-H Ki67 FITC (Log)

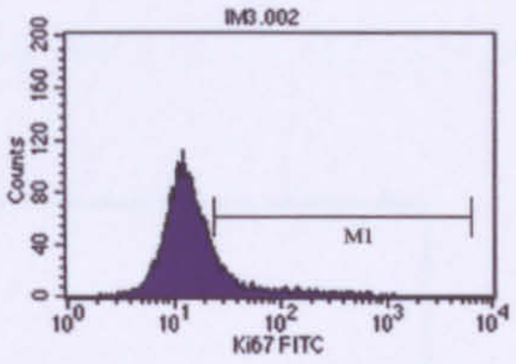
Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean
All	1, 9910	9835	100.00	98.35	8.43	7.79
M1	19, 5280	65	0.66	0.65	56.71	30.97

CD38+



Panel:
Acquisition Date: 3-Aug-3
Gate: G3
Gated Events: 9729
Total Events: 10000
X Parameter: FL1-H Ki67 FITC (Log)
Y Parameter: FL2-H CD38 PE (Log)
Quad Location: 22, 14

Quad	Events	% Gated	% Total
UL	7311	75.15	73.11
UR	1570	16.14	15.70
LL	805	8.27	8.05
LR	43	0.44	0.43



Histogram Statistics

File: IM3.002
Sample ID: IM3
Patient ID: IM3
Panel:
Acquisition Date: 3-Aug-3
Gate: G3
Gated Events: 9729
Total Events: 10000
X Parameter: FL1-H Ki67 FITC (Log)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean
All	1, 9910	9729	100.00	97.29	21.59	14.54
M1	25, 6671	1226	12.60	12.26	81.75	51.62

Fig.4.2. Ki-67 staining patterns of CD38- vs CD38+ CLL cells. The picture shows characteristic staining of sCD38+ and sCD38- CLL cells on CD38PE(Y axis)/Ki-67-FITC (X axis) dot plots and Ki-67-FITC histograms. Two typical cases from the 15 sCD38+ and 14 sCD38- clones are shown.

Fig.4.3 shows Ki-67 expression in all 29 cases and in sCD38+ vs sCD38- cases. The difference of Ki-67 percentages between CD38+ and CD38- subgroups was significant ($p<0.01$).

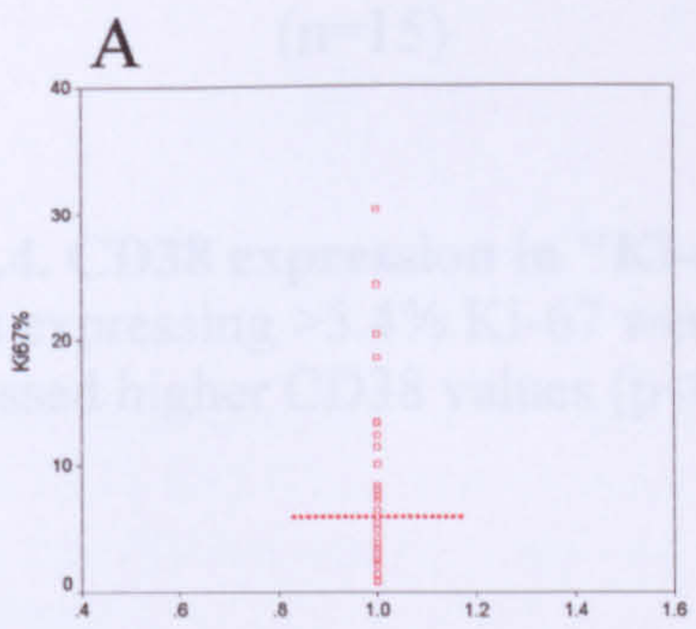


Fig.4.3. Detection of Ki-67 expression in CLL cells. (A) Distribution of Ki-67 percentages in all 29 clones studied; **(B)** Comparison of Ki-67% values in sCD38+ vs sCD38- cases. CD38+ cases express higher Ki-67 values ($p<0.01$).

4.3.3. CD38 expression in Ki-67+ and Ki-67- CLL clones

Cases were divided into Ki-67+ and Ki-67- subgroups using the median Ki-67 percentage (5.4%) of all 29 cases as an arbitrary cutoff. As shown in Fig.4.4, Ki-67+ cases had significantly higher CD38 values ($p<0.02$).

Fig.4.4. CD38 expression in "Ki-67-positive" and "Ki-67-negative" CLL clones. Cases expressing >5.4% Ki-67 were considered as "Ki-67-positive". Ki-67+ cases expressed higher CD38 values ($p<0.02$).

4.3.4. Relationship between surface CD38 (HB-7) and Ki-67 expression

Next, the correlation between expression of sCD38% and Ki-67% values in all 29 cases was examined. As shown in Fig.4.5, there was a linear correlation between surface CD38% and Ki-67%. Although the relationship was not very close, it reached statistical significance ($p<0.04$).

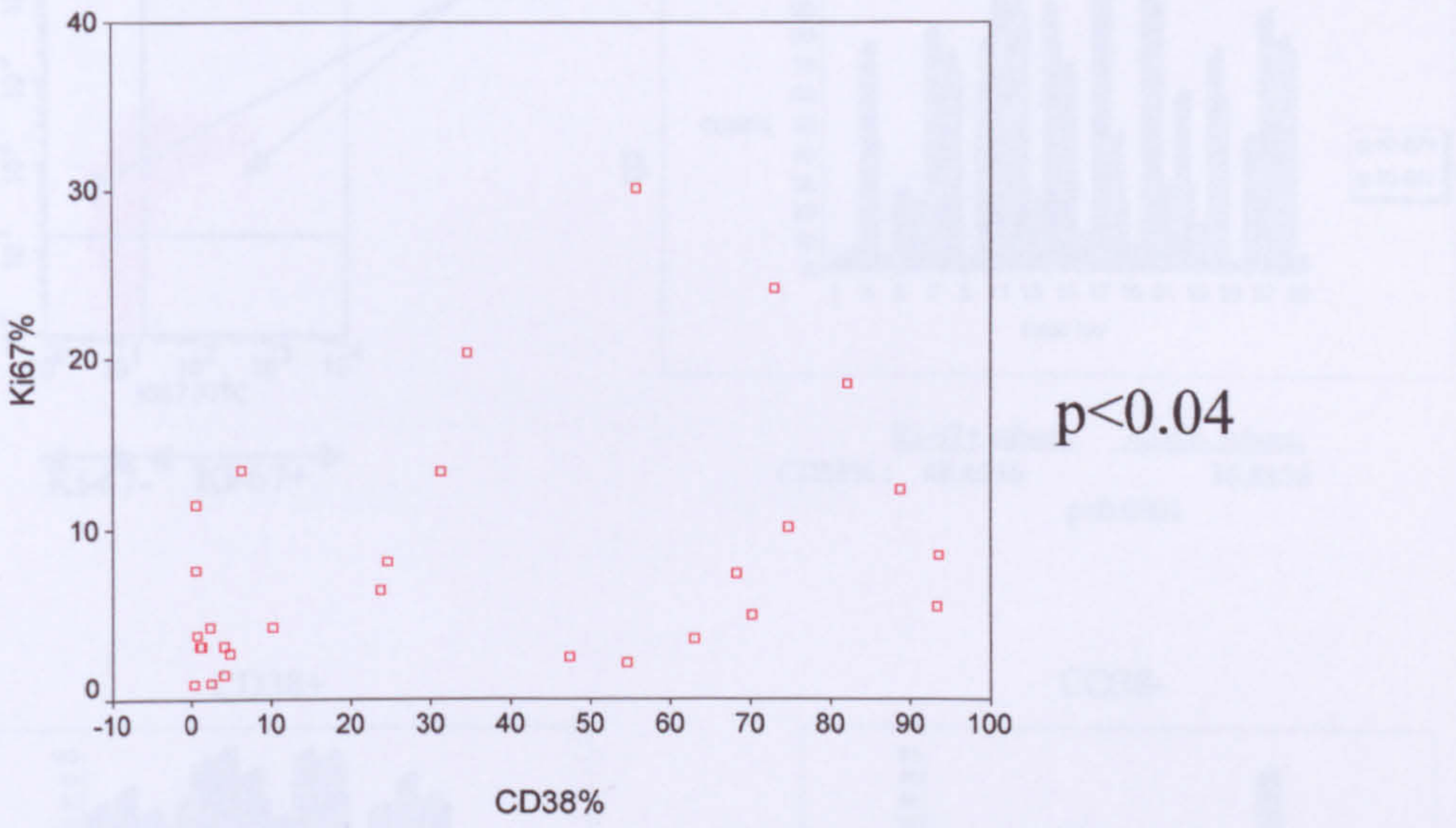


Fig.4.5. Relationship of surface CD38 expression and Ki-67 expression in CLL. Both CD38 and Ki-67 are expressed as percentages of CD19+ cells. A tendency for linear correlation can be observed reaching statistical significance ($p<0.04$; Pearson correlation).

4.3.5. CD38 expression of the Ki-67+ vs Ki-67- subpopulations in individual CLL clones

In this analysis, I addressed if there is any difference in CD38 expression in the Ki-67+ (cycling) vs Ki-67- (non-cycling) subpopulations in a given clone. As shown in Fig.4.6, cells in the cycling compartment had greater CD38 values; the difference was highly significant ($p<0.0001$).

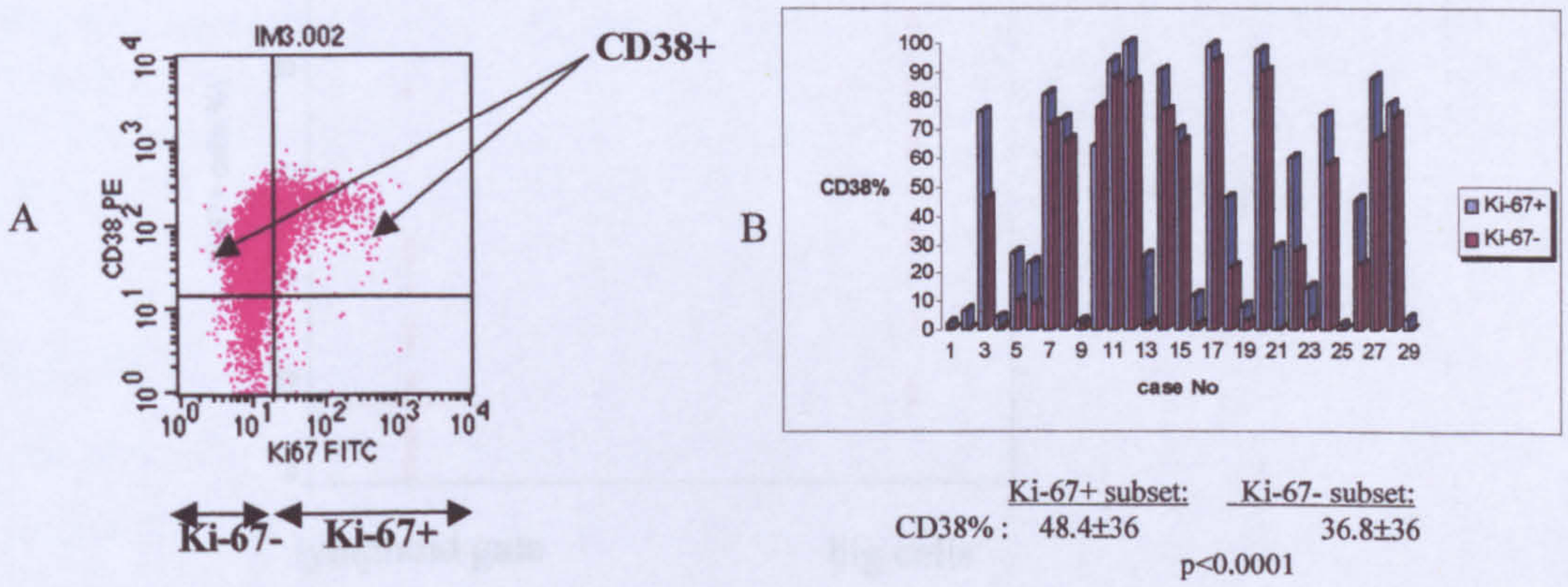


Fig.4.6. CD38 expression in the cycling vs non-cycling subpopulations of CLL clones. (A) The numbers of CD38+ cells within the Ki-67+ and Ki-67- cell populations were determined on Ki-67-FITC/CD38-PE dot plots after applying the CD19+ gate. The percentage of CD38+ cells within these two subsets were then compared: **(B)** in all cases studied; **(C)** in sCD38+ cases; and **(D)** in sCD38- cases. In all comparisons, CD38 percentages were significantly higher in the Ki-67+ subsets. Blue columns represent Ki-67+, brown columns Ki-67- subpopulations.

4.3.6. Ki-67 expression in small vs large CLL cells

Ki-67 expression was compared between small and big CLL lymphocytes using the mean forward-angle light scatter as a measure of cell size (see Methods). As shown on Fig.4.7., big cells express significantly higher levels of Ki-67 ($p<0.0001$).

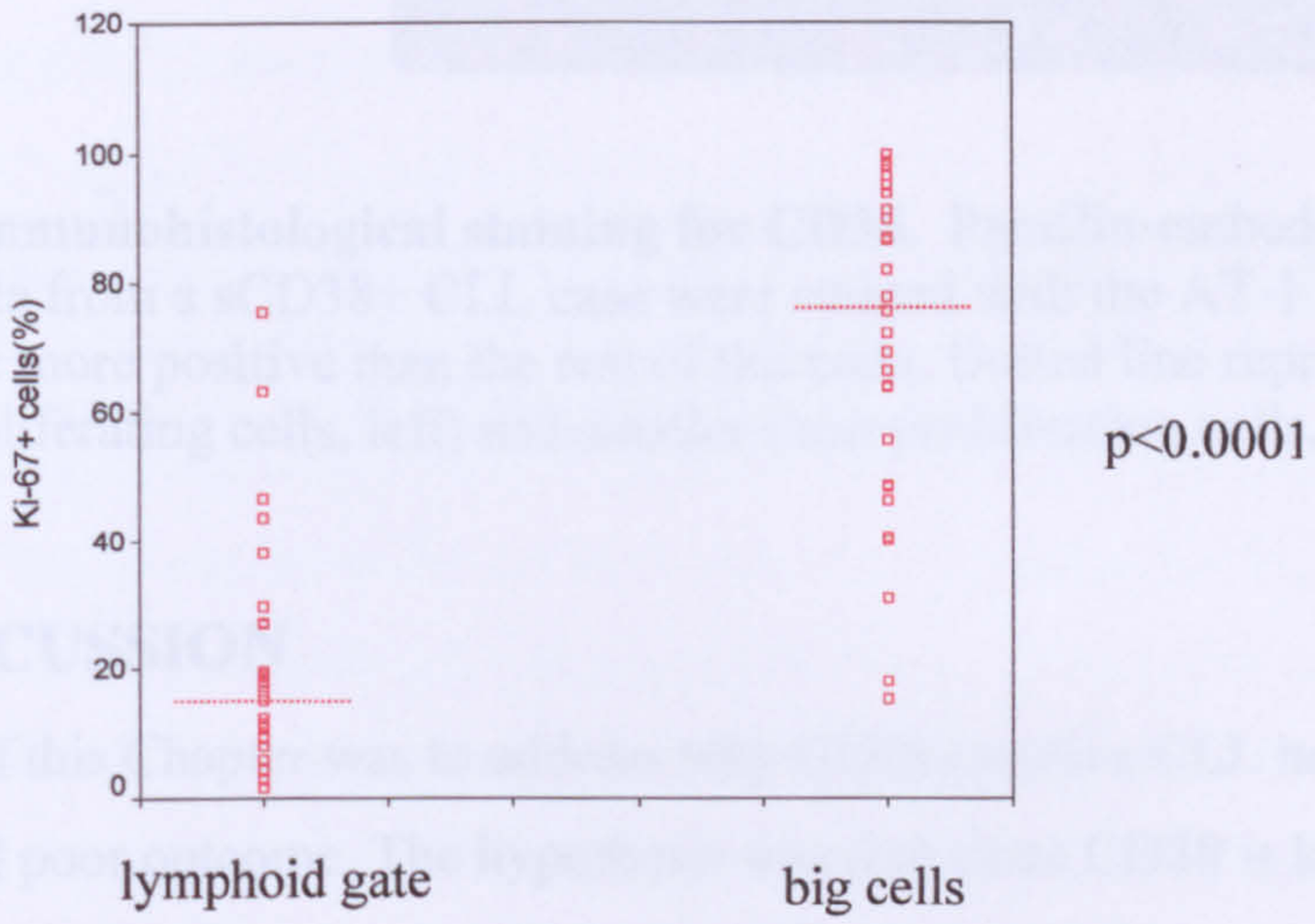


Fig.4.7. Comparison of Ki-67 expression of small vs large CLL cells. Small and large CLL lymphocytes were gated on as described in the Methods section. Ki-67 percentage was significantly higher in the large-cell population ($p<0.0001$).

4.3.7. Immunohistological staining for CD38

Of the 3 antibodies tested (HB-7, AT-1, Ab-4), only AT-1 produced clear staining of paraffin-embedded material. With this antibody, cells in the proliferation centres were markedly more positive than those in other parts of the infiltrated CLL nodes. (Fig.4.8)

In an attempt to obtain staining with all 3 antibodies, frozen sections were also examined. No clear staining of CLL cells was detected with any of these antibodies.

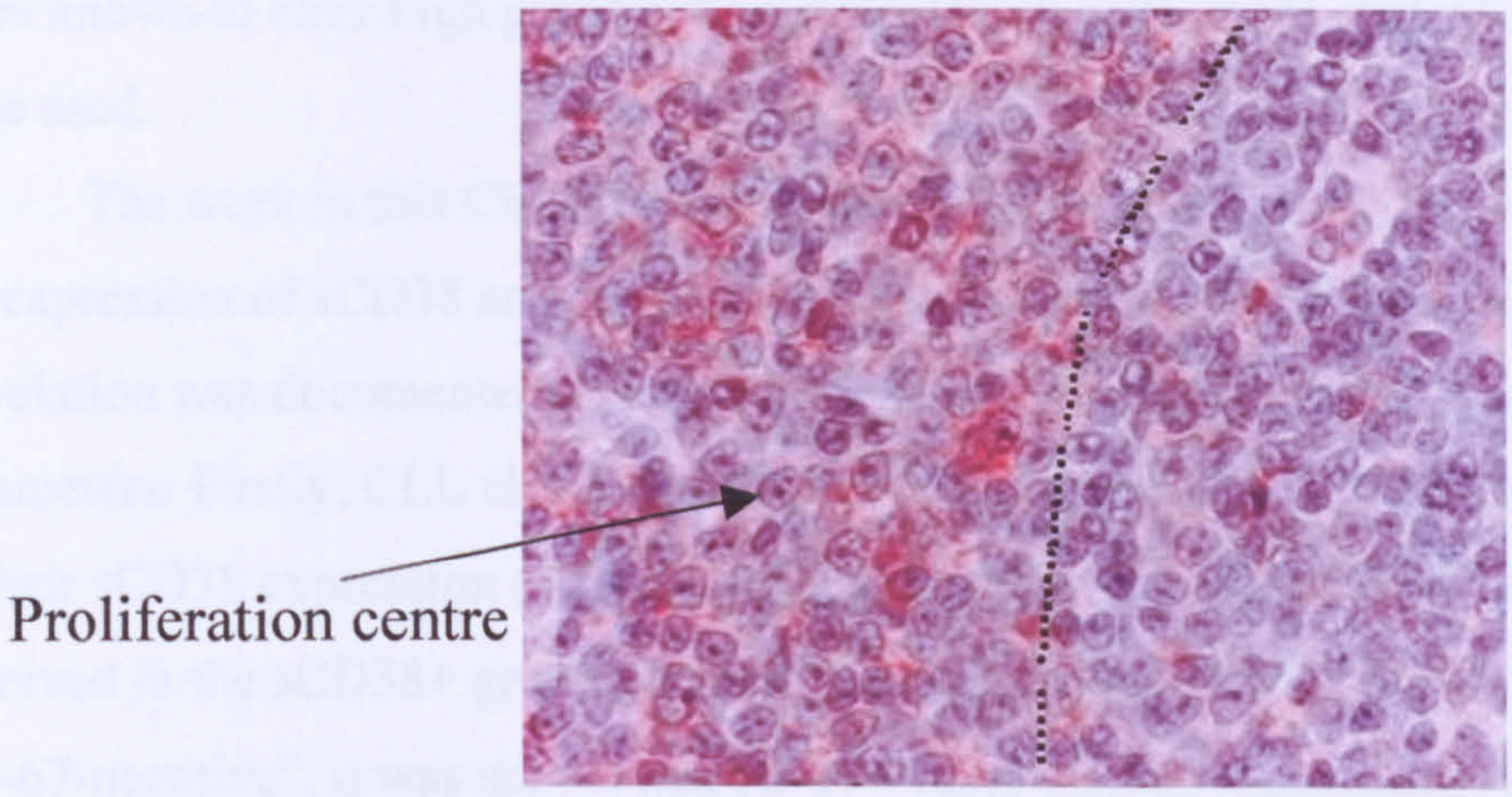


Fig.4.8. Immunohistological staining for CD38. Paraffin-embedded sections of a lymph node from a sCD38+ CLL case were stained with the AT-1 mAb. Proliferation centres are more positive than the rest of the node. Dotted line represents the border of larger (proliferating cells, left) and smaller (non-proliferating cells, right).

4.4. DISCUSSION

The aim of this Chapter was to address why CD38-positive CLL has a progressive course and poor outcome. The hypothesis was that since CD38 is known to have an impact on cell proliferation in other cell types, the expression of this molecule on the surface of CLL cells might also be indicatative of proliferative activity.

To answer this question, CLL cells were stained for the proliferation marker Ki-67, simultaneously with surface CD38 and CD19. The permeabilization technique used is known to preserve well cell morphology and surface antibody staining, thereby enabling selection of different cell populations for analysis (Jacob et al, 1991). Since it has been documented that there are significant numbers of cycling T-cells in the peripheral blood of CLL patients (Cordone et al, 1992a), cells were also stained for surface CD19 in a triple-colour immunofluorescent method, making it possible to exclude non-B cells in the course of the analysis. To avoid contamination with normal B-cells, another possible source of proliferative activity (Cordone 1992a), cases with high lymphocyte counts (usually >40 x 10^9/L) were employed. For the detection of surface CD38, the antibody HB-7 was used, the reactivity of which is known to be correlated with outcome (see Chapter 1). Ki-67 has been reported to be upregulated in the course of apoptosis (Baisch, 2002); indeed, we found that excessive amounts of apoptotic cells or cellular debris can produce false positivity. Therefore, in all experiments, these were gated out before further analysis. As positive controls, cell

types known to have high proliferation rates, Daudi cells, AML and ALL blast cells were used.

The work in this Chapter demonstrated for the first time a correlation between the expression of sCD38 and that of Ki-67 in CLL cells (Matrai et al, 2003). This correlation was documented by a number of different approaches to compare these two parameters. Firstly, CLL clones were classified as "positive" or "negative" according to their sCD38 expression (20% cutoff) and significantly higher Ki-67 values were observed in the sCD38+ group. Secondly, by grouping cases as "Ki-67-positive" or "Ki-67-negative", it was shown that Ki-67+ cases display significantly higher CD38 expression than do Ki-67- ones. Thirdly, after directly correlating sCD38 and Ki-67 expression, a linear relationship between these two variables could be documented.

Next, taken into account that every CLL clone is heterogeneous with regard to Ki-67 and CD38 expression, we tested whether the Ki-67+ and Ki-67- subpopulations within a given clone show any difference in CD38 expression. The results showed that cells in the Ki-67+ ("cycling") compartment of the clone expressed significantly higher levels of CD38 than those in the Ki-67- ("non-cycling") subset.

Finally, since bigger cell size can be a sign of activation and proliferation, and because it has been previously shown in this Department that larger CLL cells tend to be CD38-positive (Manocha et al, 2003), we compared Ki-67 expression in large versus small CLL lymphocytes. We found that large CLL cells express significantly higher levels of Ki-67 than small ones, indicating a greater proliferative activity in those cells. As it has already been referred to, this is in keeping with the finding of Cordone et al, who found that there are significantly higher numbers of Ki-67+ cells in CLL/PL (1992b).

Since the main proliferative compartment in CLL is probably not the peripheral blood but the lymphoid tissues, sections of lymph nodes from CLL patients were also stained for CD38 to assess the relationship of cell proliferation and CD38. Preferential expression of CD38 was observed in the proliferation centres, consisting of large cells, probably prolymphocytes and blasts.

Regarding Ki-67 reactivity observed in the present study as compared with previous investigations, the majority (>85%) of the CLL clones were found to express Ki-67 in 0-15% of the cells; this range of positivity is in keeping with previous studies (Cordone et al, 1992b; Hall et al, 1988, Drach et al, 1989). Interestingly, the higher Ki-67 positivity observed in CD38+ vs CD38- clones (9.2% vs 3.5%, resp.) shows a good

correspondence with the Ki-67 values reported by Cordone et al in CLL/PL vs "small cell" CLL (11.7% vs 2.3/2.9%)(Cordone et al, 1992b). Although direct comparison of the present work with that of Cordone et al is not possible because of differences in methodology and since direct morphological assessment was not a part of the present work, it nevertheless is tempting to speculate that the reason for this similarity is that most CLL/PL cases are also CD38+, considering also the known correlation between large cell size and CD38-positivity in CLL (Manocha et al, 2003).

Approximately 10% of the cases studied here showed unexpectedly high (20-30%) Ki-67 values which, at first sight, seems difficult to reconcile with the well-established low proportion of proliferating cells in the blood in CLL. With this regard, it should be kept in mind that this antibody recognises also cells in the G1 phase of the cell cycle, whereas DNA measurements detect only cells in S-phase. Other investigators have also reported high Ki-67 values in a subset of CLL patients, like values between 10-20% by Drach et al (1989) and Hall et al (1998) and Cordone et al described percentages as high as 50% in CLL/PL (1992b). The present results therefore emphasize that high levels of Ki-67 positivity (up to 30%) can be observed in CLL and indicate that, in contrast to the wide perception of CLL as an indolent, slowly progressive and non-proliferative disorder, a not insignificant subset of cases possess substantial numbers of cells in active cell cycle in the peripheral blood. This is also in keeping with the recent recognition reached by a non-radioactive, stable isotopic labeling technique that CLL clones can have a cell turnover rate up to 1-10% per week (Messmer et al, 2002).

How CD38 up-and downregulation relates to the re-circulation of cycling CLL lymphocytes between lymph nodes and peripheral blood, needs to be clarified. There are data that CD38 expression in the bone marrow is higher than in the peripheral blood of the same clone (Ghia et al, 2003). There are also reports on the selective elimination of CD38- (or CD38+) subsets after chemotherapy (Hamblin et al, 2002).

In summary, the present data show correlation between CD38 expression, cell cycle activity and cell size in CLL and therefore provide evidence for the initial hypothesis that high sCD38 expression indicates greater cell cycling activity and that this is at least partly why CD38 is an adverse prognostic marker in CLL and CLL/PL. Since the completion of this work, other groups have reported similar findings (Hambley et al, 2004). Further studies are needed to elucidate the exact relationship between CD38 expression and cell cycle regulation in CLL.

CONCLUSIONS AND FUTURE WORK

The Thesis makes the following novel points. –

- Nearly all sCD38+ CLL clones (as detected by HB-7) have unmutated IgVH genes, whereas CD38- CLL clones can be either mutated or unmutated
- Both sCD38+ and sCD38- CLL cells contain CD38 protein and message
- sCD38- CLL cells possess little or no 45 kD monomer, but various other molecular forms (~27 kD, ~60 kD, ~205 kD) of CD38 are found in both CD38 subsets
- Among these forms, the 205 kD and 45 kD molecules are present at the cell surface, and most of the CD38 enzymatic activity of CLL cells is attributable to these surface-bound forms
- sCD38- CLL cells (by HB-7) possess immunoreactive and enzymatically active CD38 on their cell surface (by Ab-4)
- Detection of CD38 is influenced by the method of cell preparation and the monoclonal antibodies used
- The cell cycling subpopulation in the CLL clone preferentially expresses CD38

Since CD38 is firmly established as an adverse prognostic factor in CLL, these results indicate that it is the possession of the 45 kD monomer that endows the malignant cells with the biological function – most likely cell proliferation – that results in the poor prognosis of CD38+ disease.

The present findings suggest a number of questions for future investigation. These include:-

- What is the cell biological role of the 205 kD form present on the surface of CLL cells?
- What is the mechanism of the involvement of CD38 in CLL cell proliferation?
- How specific are the above findings to CLL cells?

Possible approaches to these questions will be briefly outlined below.

The biological role of the 205 kD CD38 present in sCD38- (and sCD38+) CLL cells.

Because it has recently become clear that CD38 is required for chemokine induced motility (Partida-Sanchez, 2001), and because work in this Laboratory has shown that CLL-cell motility on, and transmigration through, HUVEC are not related to conventional CD38 positivity, it is tempting to speculate that the 205 kD form described above might contribute to CLL cell motility in response to chemokines. Since chemokines induce a CD38-dependent Ca^{2+} rise, it would be interesting to examine Ca++ fluxes in sCD38+ and sCD38- CLL cells exposed to chemokines. Also, it would be interesting to examine the effect of pharmacological inhibitors of cADPR on the chemokine-dependent motility of both CLL subtypes on and through HUVEC.

Involvement of CD38 in CLL cell proliferation

Possible approaches here would be to use known stimulators of CLL cell proliferation to see whether surface sCD38- CLL cells can proliferate in response to these stimuli. It would also be of interest to determine whether induction of proliferation is associated with upregulation of CD38. In addition, the effect on proliferation of pharmacological inhibition of CD38 should be assessed.

Study of molecular forms of CD38 in normal B cells

Although CLL cells were compared with plasma cell leukaemia cells in the present study, it would be interesting to know, whether the different molecular forms of CD38 identified in CLL cells are also present in normal circulating B cells. The absence of alternative forms in THP monocytic cell lysates (Fig.2.2.) raises the possibility that normal B cells might also lack some of these forms. If this is so, it would be of interest to stimulate normal B cells in ways mimicking exposure to autoantigen and follow the change in the patterns of CD38 expression. It would also be possible to stimulate CD38+ CLL cells in this way and examine consequent changes in the molecular forms.

Also, of course, the story of CD38 in CLL could evolve in other and unexpected ways. Whatever these ways turn out to be, the present studies indicate that the molecule is more than just a surface marker of poor prognosis disease.

APPENDIX

1. Extraction and reverse transcription of total RNA from CLL cells

Following thawing of cryopreserved cells or purification by FACS sorting or with immunomagnetic beads, CLL cells were washed and resuspended in a small volume of PBS. The cells were lysed in TRIzol (Invitrogen, Paisley, UK) reagent (1ml/10^7 cells) at room temperature for 10-15 minutes and RNA extracted by the addition and mixing with chloroform (200µl/ml of TRIzol). Following centrifugation (13,000g, 15 minutes, 4^0C) the aqueous phase was removed and RNA precipitated by the addition of isopropanol (500µl/ml of TRIzol) and incubation at room temperature for 10 minutes. The RNA was pelleted by centrifugation (13,000g, 15 minutes, 4^0C) and after removal of the supernatant the pellet was washed by resuspension in 70% ethanol (1ml/ml of TRIzol). The RNA was collected by centrifugation as before, air dried and dissolved in DEPC-treated H_2O (typically 40µl/10^7 CLL cells).

Aliquots of total RNA (between 1-5µg) were incubated at 65^0C for 5 minutes then placed on ice. These were then reverse transcribed with ImProm-II Reverse Transcriptase (0.5µl; Promega, Southampton, UK) and the supplied buffer, using an oligo$(dT)_{15}$ primer (20pmol/reaction) in the presence of 10mM dNTPs and RNAsin (~30units; Promega) in a volume of 20µl. Following incubation at room temperature for 5 minutes, 37^0C for 60 minutes and 70^0C for 10 minutes, the resulting first-strand 'cDNAs' were diluted to 50µl in H_2O and stored at –20^0C.

2. Standard RT-PCR

PCR primers flanking exon 3 (absent in the known splice variant) were designed from the published sequence (accession number D84276) to amplify CD38 products from 'cDNA'. Thus, CD38FOR1 (5'- GAA CTC AGA CCG TAC CTT GCA AC) and CD38BACK1 (5'- ATG GTG GGA TCC TGG CAT AAG TC), were expected to amplify products of 438bp from the full-length transcript, or 303bp from the alternatively spliced transcript.

Neither the sense (from exon 2), nor the antisense primers (from exon 7) were intron spanning. The integrity of RNA/'cDNA' preparations was confirmed by additionally amplifying an actin fragment using the primers ACTINFOR (5'- CTG GAC TTC GAG CAA GAG AT) and ACTINBAC (5'- TCG TCA TAC TCC TGC TTG CT) designed from sequence accession BC014861.

The Actin PCR primers had been validated prior to the start of this project and were in regular use in the Department. The efficacy and fidelity of the CD38 primer pair was confirmed, after optimising the PCR conditions, by cloning (into pGEM-T-easy; Promega) and sequencing (Lark Technologies, Saffron Walden, UK) the single product visible on an ethidium bromide stained agarose gel.

Separate aliquots of 'cDNA' were used to amplify CD38 and actin fragments with 2.5-5 units of Taq polymerase (Promega) in the supplied buffer plus 0.2mM of each dNTP, 2.5mM Mg^{2+}, and 20pmol of each primer in a final volume of 50µl. The reactions were carried out in an 'Omnigene' thermal cycler (Hybaid, Ashford, UK) using a 'Touchdown' protocol (Don et al, 1991) in which the annealing temperature was reduced from 60^0C to 55^0C over the first 12 of a total of 30 cycles. The denaturation (94^0C), annealing and extension (72^0C) steps each lasted 1 minute and there was a final incubation at 72^0C for 10 minutes. Each set of reactions also included a no-template negative control for each set of primers. Results were only accepted if these reactions failed to amplify detectable products.

Aliquots (typically 10µl) of the PCR products were assessed by electrophretic separation on 1% agarose gels, visualised by ethidium bromide staining, and hybridisation to radio-labelled (NEBlot kit; New England Biolabs, Hitchin, UK and α^{32}P-dCTP; Amersham Biosciences, Amersham, UK) gene-specific probes after Southern transfer to nylon membranes using a routine protocol (Sherrington et al, 1994). The probes were prepared from fractions of the plasmids that had been sequenced to verify that they contained the relevant gene fragments. The sizes of the products were estimated by co-electrophoresis with a mixture of HindIII-restricted λ DNA and HaeIII-restricted ϕX174 DNA (New England Biolabs) and the fidelity of their origins was confirmed by the positive hybridisation signals obtained after autoradiography.

3. Real-Time PCR

Real-time/quantitative RT-PCR reactions were carried out using an 'Opticon 2 DNA engine' (MJ Research/GRI, Braintree, UK) thermal cycler. The accumulation of the PCR products was followed by measuring the fluorescence generated by the incorporation of SYBR Green I. Data were accrued and analysed by the 'Opticon Monitor 2' software supplied with the thermal cycler. All reactions within an experiment were carried out in duplicate and included no-template negative controls for each set of primers used. The fidelity of the amplified products was monitored by measuring their melting temperatures and, in the initial experiments - designed to optimise the conditions, confirmed by electrophoresis, as described above for standard RT-PCR. The presence of well defined single peaks at the established melting temperatures of the respective PCR products at the end of a run, was accepted as evidence that accumulation of a single PCR product was being measured during the reaction. The machine was programmed to measure the fluorescence of the target gene fragments at a temperature greater than the melting temperature of primer-dimers. The primer-dimer melting temperatures were established for each set of primers in a series of control experiments.

The primer pairs used for amplification of CD38 and actin gene fragments were identical to those used for standard RT-PCR. In some experiments, an additional 'housekeeping' gene was included to exclude the possibility that differential actin expression levels were clouding the interpretation of the results. Thus, a fragment of the ribosomal RNA L27 was amplified using a set of primers previously validated in the Department. These were L27FOR (5'-GAC GCA AAG CTG TCA TCG TG) and L27REV (5'- GCA GTT TCT GGA AGA ACC AC) designed from sequence accession BC007273.

The following protocol was established from a series of preliminary developmental experiments. The reactions were carried out in a volume of 25µl using equal aliquots of 'cDNA', 1pmol of each relevant primer and a proprietary 2x master-mix (DyNAmo^{TM}; Finnzymes, Keilaranta, Finland) containing buffer, dNTPs, SYBR Green I, $MgCl_2$ (giving 2.5mM final concentration), and modified ('hot-start') Tbr DNA polymerase. The reactions, in 96-well plates, were cycled as follows; 95^{0}C for 10 minutes x1, 94^{0}C for 30s, 64^{0}C for 30s, 72^{0}C for 40s and 81^{0}C for 1s followed by fluorescence measurement x45 then 72^{0}C for 10 minutes x1, the temperature was then ramped from 65-98^{0}C in

0.3^{0}C steps and held for 1s after each step while the fluorescence was measured to establish melting temperatures. Finally the reactions were incubated at 72^{0}C for 10 minutes.

Prior to analysis, the melting curves were assessed to ensure that single products were being measured at 81^{0}C (fluorescence measurement temperature), and the negative control wells were checked to ensure that nothing other than primer-dimers were present.

For each experiment a 'cycle threshold' (Ct) was established, on software-generated graphs of baseline-subtracted fluorescence vs cycle number data, at a point where the signal surpassed the background noise and at which all products were accumulating in the exponential phase of amplification. The Ct values were exported to Excel™ (Microsoft) spreadsheets for further analysis. The Ct values for CD38 transcripts were normalised to the actin, and in some case also to L27, values and plotted for evaluation.

4. IgVH gene sequence analysis

Total RNA was extracted from CLL cells using Trizol reagent (Life Technologies, Paisley, Scotland), and 1-μg aliquots were reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Promega, Southampton, United Kingdom) and an oligo(dT)15 primer. The resulting cDNAs were used to isolate clonally expressed VHDHJH sequences (covering the entire length of the gene to CDR3) in 2 sets of VH gene family-specific polymerase chain reactions (PCRs). In the first set of reactions, the sense primers were consensus sequences derived from framework region 1 (FWR1)3 of each of the 7 families. In the second set of 6 reactions, the sense primers were from the leader regions (families 1 and 7 having a common primer).. In all reactions, the same combination of 3 constant region-specific (α, ϒ, μ) antisense primers was employed.. The 50-μL reactions (20 pmol of each primer, 1.5 mM MgCl2, 100 μM of each dNTP, and 2.5 U Taq polymerase in supplied buffer from Promega) were cycled 30 times using a touch-down protocol, with the annealing temperature reduced from 63°C to 57°C over the first 12 cycles, and then analyzed by electrophoresis. PCR products for a given family, which were duplicated in both sets of reactions, were cloned. Between 2 and 4 clones derived from each PCR were sequenced commercially with a vector-specific primer. The sequences were accepted as representing the CLL cell clone if there was

identity between and within the FWR1 and leader sequence-derived PCR clones. The VH gene alleles were identified and the degree of sequence divergence from the nearest germline counterpart was determined using V-BASE.

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