THE STUDY OF CELL SURFACE PHENOTYPES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

by

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ABSTRACT

Size-exclusion high performance liquid chromatography was developed to study surface membrane proteins of malignant lymphocytes. The membrane proteins were separated in accordance to their molecular sizes or complexed sizes and displayed, in conjunction with SDS-PAGE, as two-dimensional matrices on which the location of proteins is determined by both retardation in the HPLC column and the mobility on the SDS-PAGE gels. Any association or induced-interaction between proteins are reflected by predictable changes of the retention times, thus by the location on the two-dimensional matrix.

Individual membrane proteins in the mixture were identified or separated by the changes of their retardation in the column when mAb-Ag complexes formed by applying specific monoclonal antibodies to the extracts of vectorially labelled cells. A number of surface markers have been identified and several new components are characterized. This study has demonstrated that (1) SE-HPLC is a potent method for analysis of complicated mixtures, particularly when a complete separation is not necessary (2) the technique has advantages for the study of non-covalent interactions between proteins, particularly valuable for antibodies of low affinity (3) the method is powerful for compositional study and initiative investigation of the complexity of mixtures.

By using this technique in conjunction with SDS-PAGE, B-CLLs were found to have heterogeneous surface phenotypic presentations with respect to both quantity and quality. The HPLC-SDS PAGE study revealed that the expression levels of individual surface components in B-CLL patients were at a correlated fashion and, across the patient panel, present a continuous spectrum of such relationships, confirmed the earlier reports of immunological studies by Maddy et.al. that individual B-CLL patients can be ranked in a sequence according to their expression level of CD45 isoforms and that this sequential variation of CD45 is in correlation with levels of sIg and CD21. In addition, this study discovered a group of uncharacterized proteins, band 4.1 (160KD), band 4.3 (135KD) and band 2 (300KD) which are also correlated with CD45RA, CD21 and sIg. A comprehensive reportiore of B-CLL phenotypes has been established with respect to the membrane protein expression.

Cell activation study was conducted to investigate the relationship between surface glycoproteins and cell differentiation. TPA-induced B-CLL cells were shifting their phenotypes from the 230KD-(of CD45)dominated form (1A type) to 200KD- and 180KD-dominated form (1B type), with correlated reduction of CD21, band 4.1, 4.3 and band 7.1 and induction of band 2, further substantiated the conclusions drawn from the *in vitro* unactivated B-CLLs, PLL and T-CLL. Parallel study was also carried out in normal tonsilar B, B+T cells. Fluorescent activated cell sorter (FACS IV) was employed as a complementary means in this study. The study confirmed the previous observation and defined three new surface components closely associated with cell differentiation and strongly supports the idea that heterogeneity of phenotypes reflects the *in vivo* differences of cell maturity, and proposes that these new components are associated with certain stages of malignant cell maturation. The significance of such profiles and their relationships were discussed with respect to the cell differentiation, the relationship to PLL, HCL and normal B lymphocyte.

As preliminary studies, non-ionic detergent Triton X-100 was examined for solubilization of cell membrane proteins. Antibody-induced capping of B-CLL lymphocytes was also conducted to investigate the interactions and relationships of surface proteins and cytoskeleton, preliminary results were presented and discussed.

To my parents and grandmother

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ABBREVIATION

ALL -- Acute lymphocytic leukaemia

Ag -- Antigen

APS -- Ammonium persulphate

B-CLL -- B cell chronic lymphocytic leukaemia

BM -- Bone marrow

cALLA -- Common acute lymphoblastic leukaemia antigen

CD -- Antibody cluster of differentiation

cDNA -- Complementary deoxyribonucleic acid

cIg -- Cytoplasmic immunoglobulin

CLL -- Chronic lymphocytic leukaemia

Con A -- Concanavalin A

DTT -- Dithiothreitol

EBV -- Epstein-Barr virus

EDTA -- Ethylenediaminetetraacetic acid

$F(ab')_2$ --Antigen-binding fragment of immunoglobulin

FACS -- Fluorescence activated cell sorter

Fc -- Crystallizable fragment of immunoglobulin

FCS -- Foetal calf serum

FITC -- Fluorescein isothiocyanate

HCL -- Hairy cell leukaemia

HEPES -- N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid

HPLC -- High performance liquid chromatography

IEF -- Iso-electrical focusing

Ig -- Immunoglobulin

KD -- Kilodalton

LCA -- Leucocyte common antigen

LSG -- Leucocyte sialoglycoprotein

mAb -- Monoclonal antibody

MHC -- Major histocompatibility complex

Min -- Minute

MRBC -- Mouse red blood cell

mRNA -- Messenger ribonucleic acid

MW -- Molecular weight

NS-1 -- P3-NS1-1, non-secretor mouse myeloma cell line

PAGE -- Polyacrylamide gel electrophoresis

PLL -- Prolymphocytic leukaemia

PMSF -- Phenylmethyl sulphonyl fluoride

PWM --Pokeweed mitogen

RBC -- Red blood cell

rpm -- rotation per minute

SDS -- Sodium dodecyl sulphate

SE -- Size exclusion

sIg -- Surface immunoglobulin

SRBC --Sheep red blood cell

T-CLL -- T cell lymphocytic leukaemia

Tdt -- Terminal deoxynucleotidyl transferase

TEMED -- N,N,N',N'-tetramethylethylene diamine

TPA -- 12-O-tetradecanoyl-phorbol-13-acetate

TRAP -- Tartrate-resistant acid phosphatase

Tris -- Tris (hydroxymethyl) aminomethane

TX-100 -- Triton X-100

WBC -- White blood cell

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CHAPTER 1 INTRODUCTION

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CHAPTER 1 INTRODUCTION

1. Structure and Constituents of Plasma Membranes

Eukaryotic cells are organised with sophisticated membrane systems. The plasma membrane envelops the cell and separates it from its surroundings. The internal membrane systems divide the cellular organelles into different functional compartments with distinct biochemical enviroments. The biochemical importance of the membrane was gradually realized in the 1940's and 50's when biochemical studies revealed that some enzymatic reaction systems in mitochondria and chloroplasts could proceed only if the organells or membraneous envelops of the organelle membrane are maintained intact. In some cases, disruption of the membrane integrity could completely abolish the enzymatic properties.

Two major classes of molecules consisting of membranes are lipids and proteins. The proportion of lipids and proteins in eukaryotic membranes (Singer and Nicolson, 1972) varies among different species and different organelles. Carbohydrates are also found in great abundance in eukaryotic plasma membranes, linked to membrane proteins and lipids where they face the exterior of the cell.

The contemporary model for membrane structure, the Fluid Mosaic Model, was proposed by Singer and Nicolson in 1972 (Singer and Nicolson, 1972). The model emerged from several earlier proposals including those of E. Overton at the turn of the century, of Danielli and Davson (1935) and J.D. Robertson (1959). The Singer-Nicolson model proposes that the biological membrane is constructed of two layers of phospholipid molecules (bilayer) which are oriented with their polar ends facing outwards to aqueous surroundings while the non-polar hydrocarbon tails pointing to the interior of the bilayer, interacting with each other by hydrophobic forces which stabilize the phospholipids matrix. The fluid mosaic model suggests that the membrane lipids are in a mobile, fluid state and free to diffuse laterally and that proteins are suspended individually or in aggregates in globular units either penetrating the bilayer (integral proteins) or attached to the surface of the lipids (peripheral proteins). Recent studies show that lipids in the immediate vincinity to the proteins are immobilized to certain extent and 'trapped' in a fixed shell

surrounding the membrane proteins, imposing restrictions on the lipid fluidity. Integral proteins comprise about 70-80% of the total membrane proteins.

The carbohydrate groups attached to membrane proteins and lipids form two groups of complexed molecules, glycoproteins and glycolipids, respectively. The carbohydrate residues of glycoproteins and glycolipids are found to be almost exclusively on the outer surface of the plasma membrane. According to the Singer-Nicolson model, interactions between the carbohydrate groups can form a network that anchors the membrane proteins and restricts their movement. The glycoproteins are demonstrated to play an important role in the function of receptor and immune recognition.

In the cytoplasmic side of cells, there is a group of protein polymers comprising distinct arrays of complexes, the cytoskeleton. Microfilaments, microtubules and intermediate filaments are among the major elements of this complicated network. The first indication that cytoskeleton may be associated with some membrane proteins came from observations on the capping phenomenon in lymphocytes (Tilney and Detmers, 1975). In the plasma membrane of a cell capable of cap formation, actin is found to be directly associated with the cap structure. Nicolson and Painter (1973) showed that the spectrin molecule may be directly connected to the bases of the red blood cell glycoproteins on the cytoplasmic face of the plasma membrane.

2. Haematopoiesis, Neoplasia and Surface Markers of B Lymphocytes

2.1. Haematopoiesis

Lymphocytes develop from pluripotent haemopoietic stem cells, which give rise to all of the blood cells, including erythrocytes (red cells), leucocytes (white cells) and platelets. The leucocytes are engaged in cell recognition and immune response to provide defensive functions. On the basis of morphology and function, the leucocytes can be divided into four subgroups: granulocytes, monocytes, T and B lymphocytes. The first two provide non-specific defence while the T and B lymphocytes are involved in cell recognition and specific immunity.

In mammals, the haematopoietic stem cells are primarily detected in the embryonic yolk sac before being located in the liver (in foetuses) and bone marrow (in adults) which are refered to as primary lymphoid tissues. Although most of the lymphocytes will not survive after they develop in a primary lymphoid organs, some of their progeny migrate from these haematopoietic tissues to, or via thymus (T cell), secondary lymphoid organs--mainly the lymph nodes, spleen and gut-associated lymphod tissues where the cells proliferate and differentiate into lymphocytes and from where the lymphocytes travel via blood stream to the peripheral lymphoid organs where T and B lymphocytes are specified and react with foreign antigens.

Lymphocytes of B lineage are believed to be committed to the production and processing of immunoglobulin molecules. Under normal circumstances, the B cells are maintained to a steady number of cells with a high daily turnover rate (10^8 in the mouse (Ling, 1983)). The B lymphocytes, according to the expression of their surface phenotypic markers, can be subdivided into many different subsets, representing different stages of maturity/differentiation or functional capacity in the immunity. This study concerns two major surface proteins which are widely regarded to be associated with such staging--immunoglobulins and CD45 antigens. Immunoglobulins, which distinguish B lymphocytes from T lymphocytes, are demonstrated to be closely associated with certain functional stages of B cell development. CD45 (used to be called as the Leukocyte-common Antigen, L-CA) is another major cell surface molecule of all lymphoid and myeloid cells (Scheid and Triglia, 1979; Sarmiento et.al., 1980). The antigen comprises a family of glycoproteins with broad heterogeneity in apparent molecular weight dependent upon cell types (Omary, et.al., 1980; Woollett, et.al., 1985). Combining with other surface markers (or the clusters of differentiation, CD), it becomes possible to elaborate the diversity of B lymphocytes and their development.

2.2. Neoplasia

Almost all human cancers are derived from a single cell, i.e. monoclonal origin as suggested by the expression of a single Ig light chain (Aisenberg and Block, 1976) and unique Ig-idiotype specificities (Hamblin et.al., 1980). It is conceivable that they are, at least initially, relatively homogeneous in cell type characteristics which

are determined by the cell lineage and developmental states of the involved cells. However, distinct phenotypic features are often observed in malignant cells as the cells transformed from individual precursor cells may continue some limited differentiation. Malignancies in haematopoietic system share these features and have a broad range of potential 'target' cells as the system contains several diverse cell types.

Neoplasias of the haematopoietic system are believed to be a result of defective differentiation as well as an uncontrolled proliferation (Caligaris-Cappio et.al., 1984; Anderson et.al., 1984; Dighiero and Binet, 1987) when homeostatic control is disrupted by a malignant transformation. The outcome of such a disruption is the imbalanced accumulation of malignant cells of clonal origin and these cells are unable to differentiate past a certain stage of maturity, referred to as 'maturation arrest'.

Chronic lymphocytic leukaemia (CLL) was first described by Turk (1903). The CLL lymphocytes are characterized by their morphological features as small, resting cells together with surface phenotypic features defined by monoclonal antibody, lectins and erythrocyte rosetting techniques. These characterizations also give rise to the classification of other leukaemias, namely, 'prolymphocytic leukaemia' (PLL), 'hairy cell leukaemia' (HCL) and some types of lymphomas. The differences of these leukaemias will be described later.

The mechanisms of the malignancy are still obscure and often contradictory evidence is presented. Generally, however, it is believed that maturation arrest and uncontrolled proliferation are responsible for the neoplasia, either independently or together. It suggests that the leukaemia has a counterpart in the normal lymphocytes, i.e. arising from a normal population of haematopoietic cells, whose development is deterred by some external factors, such as the presence or absence of certain growth factors which impair the normal haematopoiesis, or internal factors such as genetic defects. Evidence is accumulating in supporting the 'normal counterpart' theory. CD5 cluster (T1, Leu1, T101) is a pan-T monoclonal antibody and is consistently found in most B-CLL cells (Boumsell et.al., 1978; Wang et.al., 1980), therefore, confirmed the presence of dual B- and T-cell marker in CLL with heterologous anti-T sera. A small subpopulation, corresponding to about 2-5% of B cells in

lymph nodes, was found CD5 positive, together with characteristics of low sIg and formation of rosettes with mouse erythrocytes (Bofill et.al., 1985; Antin et.al., 1986), corresponding to the features of B-CLLs. Therefore, it was suggested that this subpopulation was the normal counterpart of the B-CLL lymphocyte. Brooks et.al. (1981) described a monoclonal antibody, FMC7, and showed that most of the B-CLL cells were negative whereas most B prolymphocytic leukaemic cell and hairy cell leukaemic cells, which are largely $CD5^-$, were positive. This finding leads to a hypothesis that the $FMC7^+$ subpopulation could be the normal counterpart of both prolymphocytic and hairy cell leukaemias. However, the surface phenotype of the leukaemic cell is not necessarily matched to that of the target cell because the transformed individual precursor cells may still undergo some differentiation, developing distinct phenotypic features, i.e. there may be a partial maturational arrest. The phenotype of leukaemic cells reflects the lineage relationships of the target cell together with the developmental level at which the dominant subclone has been detained after its limited further development.

However, sometimes more is implied than just a block to normal differentiation or normal sequential gene expression. In both animals and man, there are two outstanding features in cancer cells that oncofoetal or foetal phase specific genes are expressed (Alexander, 1972; Sheridan et.al., 1976) with the presence of many other parameters of cell structure and function, which is considered as a consequence of 'gene derepression' (Odell, 1977). As a result, the dominant subclones in the malignant cells may lack the sequential expression of membrane properties which would normally be anticipated in an activated proliferating progenitor cell with progressive maturation or, in the main, the neoplastic cells may present surface phenotypes with some distinct features associated with defective maturation or genetic bases.

The aim of this study is to exploit such distinct membrane properties by analysing the cell surface polypeptides and phenotypic heterogeneity, as a whole, of leukaemic lymphocytes. The correlations between some membrane proteins and possible implication of such correlations have been examined and discussed. By discovering the progressive expression of these surface receptors, it is attempted to understand certain relationships with respect to differentiation or maturation of the cells.

Biochemical methodology has been employed as a major approach to the project, in conjunction with immunological techniques, particularly the fluorescence activated cell sorter (FACS). Size exclusion high performance liquid chromatography (HPLC) has been developed to fulfill the project. Monoclonal antibody was used, in combination with HPLC, to identify specific surface receptors and to examine their interactions with other membrane components as well as possible cytoplasmic components.

2.3. Surface Markers of B Lymphocyte

2.3.1. Immunoglobulins

Immunoglobulin molecules consist of two identical heavy chains (H) and two identical light chains (L). In higher vertebrates, there are five classes of H chains- α, δ, ϵ, γ and μ, comprising five different classes of antibodies, IgA, IgD, IgE, IgG and IgM, respectively. There are two types of light chains, κ and λ. Combinations of different heavy and light chains not only generate five classes of antibodies but also a number of subclasses of IgG and IgA immunoglobulins.

Both heavy and light chains contain two segments of distinct compositions, a variable region at their amino-terminal ends and a constant region at the carboxyl-terminal ends. It is the variable regions of H and L chains that act as the antigen-binding site. The variability of the amino-acid sequence of the variable regions gives rise to the diversity of the antibody. These features have been demonstrated to have important genetic implications.

The study on genomic DNA shows that a single C gene segment encodes the constant (C) region of an Ig chain but each heavy chain V region is coded for by three gene segments, V, J and D while light chain V region by V and J. The random combination of these variable region genes makes a substantial contribution to the antibody diversity.

The choice of the particular gene segments that encode the class of heavy chain produced is a commitment of the B cell differentiation, function and the order of the CH genes in the germ line. During B cell development or antigen challenge, there is a switch in the constant region of the genes from making one class of antibody to another. Synthesis of Ig u heavy chains is first detected in large, cycling cells known as 'pre-B' cells. These proliferating cells lack Ig light chain and the synthesised u heavy chains remain in the cytoplasm. As the cells divide into small, non-proliferating or 'resting' pre-B cells, the gene rearrangement of the Ig light chain loci initiates synthesis of complete IgM molecules, giving rise to small 'immature' or 'primary' B cells featured a low level surface IgM expression. These 'immature' B cells, then, depart from the

primary lymphoid organs-the bone marrow and circulate to the primary follicles of the peripheral or secondary lymphoid organs during which the heavy chain switching occures and results in the transcription and translation of other heavy chain isotypes. It has been found that, at this stage, the majority of the circulating B cells possess both surface IgM and IgD although surface IgG and IgA might be expressed along with sIgM (Abney et.al., 1978). These cells, capable of producing surface immunoglobulin, are regarded as 'mature' or 'immunocompetent' cells but remain in a resting and quiescent state undergoing constant re-circulation between the secondary lymphoid tissues unless these circulating cells are challenged by antigens.

Once the resting B cells are stimulated with antigen, they are triggered to undergo the antigen-dependent development and differentiate to either antibody-secreting plasma cells (terminally differentiated B cells) or to small 'memory' cells. During the B cell differentiation, surface Ig expression is changed to the production of secretory immunoglobulin and continuously switching of Ig isotype is simultaneous with the process, which is associated with the maturation of immunocompetent B cells expressing sIgM and sIgD via an immunoblast stage to plasma cells secreting low affinity IgM with the loss of sIgD.

Neoplasia is generally monoclonal origin, i.e. developed from a single cell at any one of the preceding stages of differentiation of cells of the series. The typical sIg profile found in CLL is $sIgM^{+}$ $sIgD^{+}$, with occasional other isotypes. The expression of sIgG together with sIgM and/or sIgD was observed but its incidence and significance are controversial (Stevenson, et.al., 1981; Kumararatne and Ling, 1983). The expression of sIg on CLL cells is markedly reduced (Digliero, G., 1980). CLL cells are found to contain some amounts of cytoplasmic Ig (Han et.al., 1982). The cytoplasmic Ig may be IgM or IgM + IgD and may be associated with a more heterogeneous sIg phenotype (Han et.al., 1982). Interestingly, CLL lymphocytes which express sIg weakly usually secrete free light chain either exclusively or in great excess over Ig in culture (Hannam-Harris et.al., 1982). However, there are no conclusive associations between Ig phenotypes and particular lymphomas although excess free light chain secretion of CLL may indicate immaturity but the indication is not compelling because the phenomenon could be resulted from a sluggish response of H-chain synthesis in neoplastic state.

2.3.2. CD45 Antigen

CD45 antigen is a group of high molecular weight glycoproteins, exclusively confined to cells of haematopoietic origin (Scheid and Triglia, 1979). Various names are applied to this family, including B-200, T-200, Leukocyte Common Antigen and the mouse allotypic marker Ly-5. Recently, the antigen has been assigned with the cluster of differentiation number CD45 in the Third International Leucocyte Typing Conference (McMichael et.al., 1987).

CD45 is one of the most abundant cell surface proteins in lymphocytes, comprising approximately 10% (Williams and Barclay, 1985) of total cellular proteins. Several members of this family have been described and there are at least four forms detected by SDS-PAGE with apparent molecular weight ranging from 170KD to 240KD. The selective expression of individual members of the antigen is controlled in a cell-type-specific fashion that is conserved throughout the mammalian evolution (Trowbridge, I.S., 1978; Newman et.al., 1984; Woollett et.al., 1985; Maddox et.al., 1985). B lymphocytes only express the highest molecular weight form of the antigen while thymocytes express the lowest (see references above). T lymphocytes exhibit a more complicated pattern of expression and, usually, a combination of several members of the family with the predominance of lower molecular weight forms depending upon cell subset (Lefrancois and Bevan, 1984; Woollett et.al., 1985; Lefrancois and Goodman, 1987). Moreover, there are evidence showing that the different patterns of the CD45 expression can change upon cell activation. Recently, the gradual understanding of the molecular basis of the CD45 family has greatly facilitated the elucidation of the molecular diversity of the antigen and its heterogeneous expression.

Based on the nucleotide sequences of cDNA clones, the primary structures of rat and mouse CD45 molecules have been predicted. It has been found, by Northern blot analysis and S1 mapping assay, that there are three different size classes of mouse CD45 mRNAs although the precise molecular basis for the heterogeneity of the CD45 family is not yet fully understood. A complete amino acid sequence of a member of the human CD45 antigen deduced from cDNA sequences has recently been established (Streuli et.al., 1987).

Streuli et.al. (1987) deduced the complete amino acid sequence of a member of the human CD45 antigen from cDNA sequences and have shown that there are at least five different forms of CD45 mRNAs generated by a single human CD45 gene. Three cDNA clones, LCA.260, LCA.1 and LCA.6/2, are isolated from human tonsil and SB cell line cDNA library and the LCA.260 and LCA.1 sequences have extensive deletions relative to the LCA.6 sequence. Another human LCA cDNA clone, LCA.111, is the same as LCA.260 except for a few differences ascribable to polymorphism. The three distinct structures defined by the clones LCA.6, LCA.260/LCA.111, and LCA.1 suggest that there are at least three different classes of human CD45 mRNAs.

By analysing a genomic DNA clone isolated from a human placental DNA library (Lawn et.al., 1978), seven exons, tentatively called exons A, B, C, D, E, F and G, were located in this cloned genomic DNA. The comparison of the CD45 cDNAs and gene sequences has proven that the variable CD45 mRNA structures are generated by differential splicing of three exons, A, B and C. Therefore, a single human CD45 gene is responsible for multiple forms of mRNAs that encode proteins of different structures. These different mRNAs are generated by differential usage of three exons by alternative splicing. Each exon can be either included in or excluded from a CD45 mRNA independently of the other two exons. In principle, the number of different forms of the CD45 mRNA and proteins can be up to eight. So far, there is no any convincing evidence suggesting differential usage of any other exons. Although the estimated number of the CD45 mRNA is eight, some of the different forms should have similar molecular weights because of the similar sizes of the three variable exons, together with the enormous sizes of the CD45 mRNAs and proteins. SDS-PAGE might not be able to distinguish some mRNAs and their products, which may account for the fact that only four different forms are seen by SDS-PAGE.

Intriguingly, exons A, B and C are expressed in a cell-type-specific fashion, B lymphocyte uses all three variable exons and expresses a member of CD45 antigen with the largest molecular weight; thymocyte splices between exons D and the exon for the leader sequence, deleting exons A, B and C and expresses the lowest molecular weight form of the CD45 while T lymphocyte appears to adopt a number of combinations and expresses several chains of CD45 with various molecular weights.

The structure of the CD45 glycoproteins has been infered from the study of cDNA clones for human, mice and rats. All three species possess very similar structures. The mature protein is 1118-1304 amino acids long depending upon species and individual chains of CD45, the human CD45 is a protein of 1304 amino acids. The whole molecule is oriented in the membrane bilayer with the amino terminus pointing outside of the cell. Based on hydrophobicity analysis, the potential transmembrane region of the molecule is 22 amino acids long, located in amino acid positions 553-574 from the amino terminus. The extracellular domain has 552 residues and the large cytoplasmic domain has 707 residues. The extracellular domain possesses a signal sequence of 23 hydrophobic amino acids at the beginning of the protein sequence, which is very likely the signal peptide for the transfer of the protein across the cellular membrane. Comparison between human, mice and rats CD45 sequences shows that the cytoplasmic domains are highly conserved with approximately 85% homology but the extracellular portion is less conserved with only about 33% homology except the signal peptide which is very well conserved (about 90% homology), as is the membrane-spanning peptide. In human, the extracellular domain of CD45 has an insertion of 161 amino acids near the NH2-terminus. The cysteine-rich sequences in the extracellular domain are highly conserved, which may suggest that higher-order structure, rather than the primary structure, is important for functions of the extracellular portion of the CD45 molecules.

Both potential N- and O-linking sites are notably abundant in CD45 peptides, most of which are segregated within the extracellular domain. The O-linked glycosylation sites are probably encoded within the exon for the leader sequence and exons A-E found at the amino-terminal end of the molecule. In human CD45, the potential N-linked glycosylation sites on the extracellular domain are variable (Streuli et.al., 1987), which is consistent with the observed molecular weight range of the antigen. It appears that most of the O-linked carbohydrates is located at the end of the amino terminus (Barclay et.al., 1987) while the N-linked is distributed along the whole extracellular domain. The extensive glycosylation could play significant roles in the function of the antigen as the extracellular segment of the molecule lacks sequence conservation.

There are two types of antibodies to CD45 antigen with respect to their specificities: antibodies that are reactive with common epitopes for all members of the CD45 family for a given species, and antibodies that recognize restricted epitopes which are designated as CD45R (Cobbold et.al., 1987). In the light of the genetic basis of the CD45 family, the specificity diversity is likely to be generated through variable exon splicing. It is most likely that the common epitopes are coded for by exons common to all members while the restricted epitopes can be generated either by sequences encoded by the variable exons or by cell-type-specific modification, such as glycosylation, of the regions encoded by either variable or common exons.

The cell-type-specific expression of CD45 members suggests that the use of the variable exons must change in a programmed way during cell differentiation. In murine haematopoietic cell developmemt, CD45 (Ly-5) is detected on the first recognizable committed B-cell precursor by monoclonal antibodies 14.8 (Kincade et.al., 1981), RA3-6B2 (Coffman, 1982) and 100C5 (Dumont et.al., 1983). 14.8 recognizes restricted CD45 determinants, precipitating the higher molecular weight forms of the antigen. RA3-6B2 is one of the few anti-Ly-5 (CD45) antibodies that identify only the B-cell-restricted determinants. However, events before the acquisition of Ly-5 (CD45) are less certain. In B lineage lymphocytes, cells preceding the large pre-B cells are found to exhibit a phenotype of $Lyb2^{+}$ and $CD32^{+}$ but $CD45^{-}$ (Holmes et.al., 1986).

Interestingly, the expression of the CD45 family members appeares to change upon cell activation. In T cell, changes of CD45 expression have been demonstrated when cells are stimulated with either concanavalin A (Con A) or in a mixed lymphocyte reaction (Lefrancois and Bevan, 1984). Evidence also shows that CD45 carbohydrate structures differ amongst leukocyte populations and both T and B cell CD45 carbohydrate structures change upon activation (Brown and Williams, 1982; Childs et.al., 1983; De Petris and Takacs, 1983; Lefrancois and Bevan, 1984; Lefrancois et.al., 1985). T-cell clones have been found usage of variable exons dissimilar to those by peripheral T cells (Lefrancois and Bevan, 1984; Lefrancois et.al., 1986; Lefrancois and Goodman, 1987).

The tumour cells differ from normal B-cells in frequently expressing the T-cell forms of CD45 molecules, i.e. the lower chains of the antigen. It has been shown that chronic lymphocytic leukaemia (CLL) exhibits a broad spectrum of the expression combinations of CD45 members, ranging from CD45RA-predominant to CD45RO-predominant--typical of T-cell features (Smith et.al., 1985; Roxburgh and Cooper, 1987). Changes of CD45 expression in neoplastic cells (Maddy et.al., 1989) have been documented when TPA is used to activate the cells. TPA is found capable of converging the CD45 expresson of B-CLL cells towards a CD45RO-predominant (180KD) state, i.e. acquisition of CD45 features similar to T lymphocytes.

2.3.3. Other Surface Markers

(1) CD5

Only a minor population of adult B cells express CD5 antigen and this 67KD molecule was originally regarded as T cell marker (OKT1, Leu1). However, most of the B-lineage CLL lymphocytes are CD5 positive (Royston et.al., 1980). $CD5^+$ B-cells were first detected in the lymph nodes around the seventeenth week of gestation (Bofill et.al., 1985) and then become evident in the primary follicles of the spleen, but are only occasionally observed in BM (Bofill et.al., 1985; Caligaris-Cappio and Janossy, 1985). $CD5^+$ B-cells are largely confined to peripheral lymphoid organs. The murine equivalent of this antigen is Ly-1 which has been established as a marker defining a distinct B cell lineage (Hayakawa et.al., 1983). The fact that normal human B cells express mouse erythrocyte receptors (MRBC, see below) together with CD5 suggests that they are probably identical populations or at least overlapping in the expression of two markers. Both $MRBC^+/CD5^+$ B cells and their murine counterparts share the features of sIgM and sIgD positive and of either or light chain. It has been proposed that $MRBC^+/CD5^+$ B cell population may represent a separate lineage observed at different stages of differentiation and may be the normal counterpart of B-CLL (Dighiero and Binet, 1987). CD5-positive B cells appear to be associated with autoimmune disease (Plater-Zyberk et.al., 1985).

(2) Common Acute Lymphoblast Leukaemia Antigen (CALLA) (CD10)

This antigen was first defined by the monoclonal antibody J5 (Ritz et.al., 1980) and is detected on 70% cells of acute lymphocytic leukaemia and on normal bone marrow cells which express Tdt and HLA-DR. The antigen is not present on normal peripheral blood lymphocytes and is considered as a marker of the B-committed progenitor and pre-B stages of development. The molecular weight of this antigen is 98KD.

(3) CD19

The cluster of CD19 antibody contains B4, HD37, 4G7 and SJ25-C1, all of which recognize a B-lineage-restricted protein of apparent molecular weight of 95KD. The antigen appears at about the same time of immunoglobulin gene rearrangement or earlier and is expressed by majority of blood B cells, B cells within germinal centres. The antigen is retained throughout differentiation in the primary tissue until the plasma cell stage. CD19 is the only specific antigen which is expressed on B-progenitors and present until the terminal plasma cell stage.

Most B-cell malignancies are CD19 positive, including B-cell precursor, CLL and most lymphomas. Evidence from phenotypes of cells of acute lymphoblastic leukaemias of non-T type suggests its expression preceeds cALL antigen (CD10).

(4) CD20

The antigen defined by the CD20 cluster of antibodies is a phosphorylated protein of 35KD. The prototype antibody in this cluster is B1 (Stashenko et.al., 1980). The antigen is demonstrated to be essentially restricted to the B-lineage cells, although there is disagreement about whether it is found on dendritic cells. CD20 antigen is strongly expressed in B cells as detected by immunoflurescent assays and its expression may quantitatively vary with cell maturation (Gordon et.al., 1984) and tends to be lost upon cell activation.

(5) CD21

CD21 is a single chain protein of apparent molecular weight 140KD when reduced (Lida et.al., 1983) and 120KD when not reduced. The prototype antibody is B2 (Nadler et.al., 1981). The antibodies of this cluster (B2, BL-13 and HB5) stain some T-ALL cells and follicular dendritic cells strongly, but are otherwise considered as restricted to B cells. B cells in the mantle zone stain strongly and B cells in lymphoid tissue are found to express significantly higher CD21 than B cells in blood. CLL B cells are positive although never strong with respect to immunoflurescence. The expression of CD21 appears to be later in B cell differentiation than that of CD20 antigen and is present throughout the B lineage from the pre-B stage. The antigen disappears when B cells are activated (Boyd et.al., 1985).

CD21 has been found to serve as receptors both for Epstein-Barr virus and for the complement component C3d (Lida et.al., 1983; Shaw et.al., 1986). Some anti-CD21 antibodies are capable of inducing a strong proliferative response in the presence of T cells (Nemerow et.al., 1985).

(6) CD22

This antigen is defined by antibodies HD6, HD39, 29.110 and SHCL-1 etc. (Nadler, L.M., 1986). The immunoprecipitation shows two diffuse bands of apparent molecular weights of 130KD and 140KD, both highly glycosylated. The molecular weights of both bands on SDS-PAGE are dependent on reduction and non-reduction of the

samples. Majority of blood B cells (75%) bear the antigen but tissue B cells and malignant cells show complicated patterns. Some pre-B lines are positive as are a proportion of ALL cells and the antigen is present in the cytoplasm in some ALLs and early B-cell precursors (Dorken et.al., 1987) although the later are at a more advanced differentiational level. CD22 is expressed in about 25% CLL lymphocytes while most lymphomas and hairy cell leukaemias react with anti-CD22 antibody. The same as CD21, this antigen is lost when cells are activated. The expression and distribution of this antigen present a complicated pattern, therefore, a direct correlation between its expression and cell differentiation is less compelling.

(7) MHC Antigens

MHC antigens are expressed on the cells of all higher vertebrates. The two principal classes of the MHC antigens are Class I and Class II, comprising a set of cell-surface glycoproteins. MHC Class I consists of a 45KD glycoprotein transversing the plasma membrane. The antigen is ubiquitously expressed on the surface of almost all nucleated somatic cells and of highly polymorphic characteristics. A small protein of 11.5KD, $ß_2$-microglobulin, is noncovalently associated with the extracellular domain of the MHC Class I molecule. MHC Class II antigen is another highly polymorphic cell-surface glycoproteins but has a much narrower tissue distribution, being expressed by most B cells, some T cells, some macrophages and macrophagelike cells. The MHC Class II glycoproteins are composed of two non-covalently bonded polypeptides--an α chain of 35KD and a ß chain of 28KD. Both chains are oriented transmembrane bilayer.

(8) Mouse Erythrocyte Receptors

Receptors for mouse red cells (MRBC) are detected on small proportion of B cells in the bone marrow, blood and peripheral lymphoid organs (Stathopoulos and Elliott, 1974; Gupta et.al., 1976) but not on monocytes, granulocytes or T cells. In the blood and tonsils, $MRBC^+$ cells usually also express both sIgM and sIgD, but in bone marrow and foetal liver, cells which are MRBC positive do not exhibit any surface Ig (Kagan et.al., 1979). The MRBC receptor, therefore, is regarded as a marker of an early stage of B cell development, expressing with or before the appearence of cytoplasmic u chain

and conserving up to the immuno-competent stage. Most B-CLL is MRBC positive but PLL and HCL are found unreactive with MRBC.

3. Size Exclusion High Performance Liquid Chromatography

3.1. Principle

Size exclusion liquid chromatography (SEC) (Porath and Flodin, 1959) is also often referred to as gel chromatography, gel filtration and gel permeation chromatography. The technique, described in detail by Yau et.al. (1979), is a potent method for the separation and characterisation of proteins, enzymers and other macromolecular substances. Recently, as the improvement of packings and the packing techniques, the size exclusion LC has been widely applied to a broad variety of sample types for different separation problems.

Size exclusion chromatography (SEC) separates molecules according to their effective size in solution. Solute size in SEC is determined by molecular hydrodynamic radius which is the radius of gyration of the solute in the mobile phase. Sample molecules of differnt hydrodynamic radii are retarded by a porous packing particle to different extents. A mixture of sample molecules passes through the packing particles; if some of the molecules are too big to enter the pores, they are totally excluded from the porous passages and eluted directly through the column along with the mobile phase, appearing first in the chromatogram. Those small molecules which are able to permeate almost the entire particle and likely to go through the whole porous channel will be retained the most and eluted most slowly. Solutes of intermediate sizes are less able to access to the walls of the pores because of steric hindrance and retained less by the pores. The extent they are retarded by the packing is dependent on their relative sizes. As a result, in SEC separation big molecules have shorter retention and come out earlier than small ones. Conceivably, most of the sampling molecules will be eluted before solvent molecules which are usually very small and entirely permeate the pores. Therefore, the retention of solvent molecules on the column, t0, is the time for mobile phase molecules to move from one end of the column to the other.

Theoretically, separation in SEC is strictly based on the molecular size. Effects from other mechanisms, such as adsorption, are usually undesirable and minimum and can be avoided through proper combination of substrate and mobile phase.

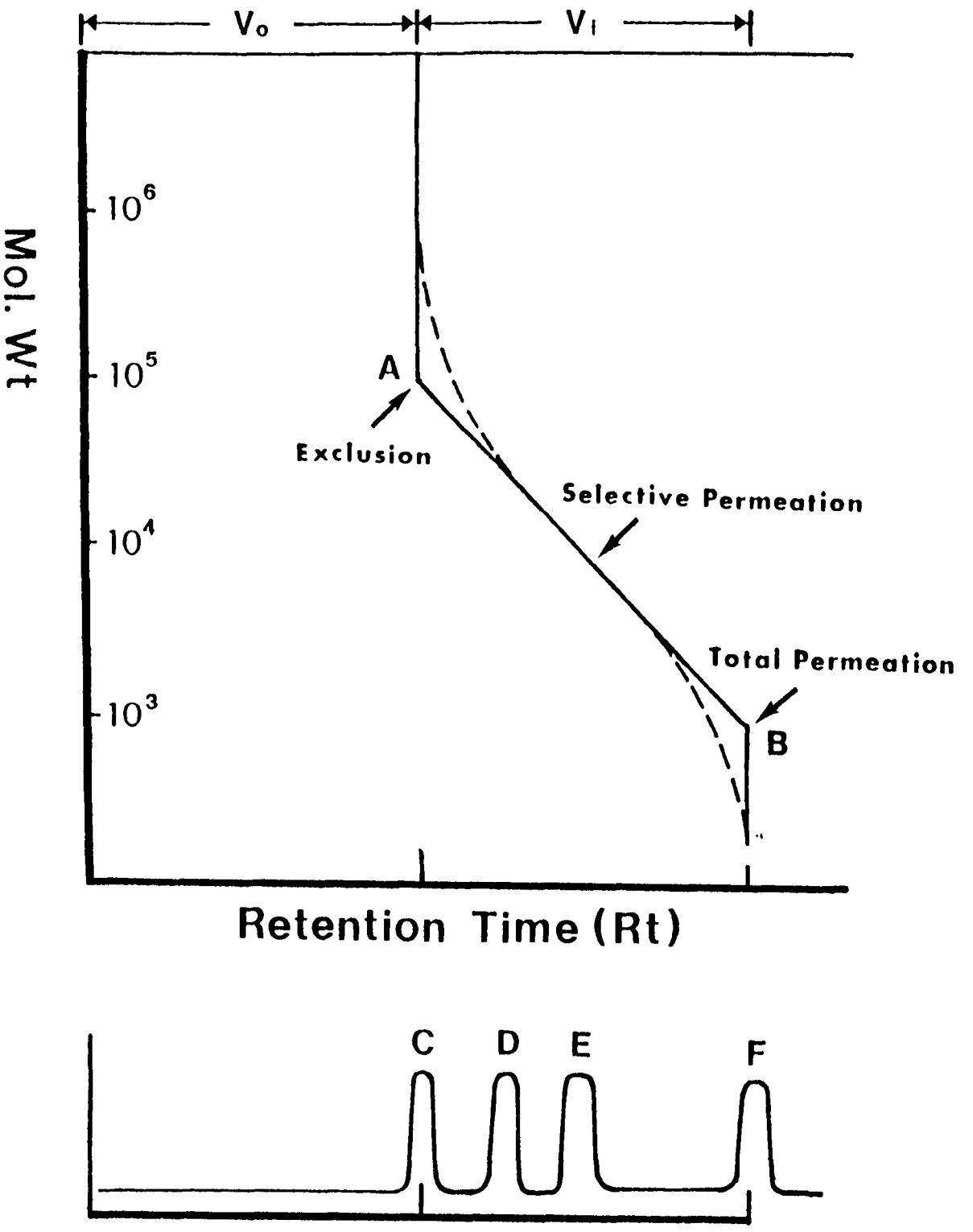

Fig 1

Fig 1. Hypothetical calibration plot of a size exclusion column to show the fractionation characteristics. Molecules bigger than 10^5 or smaller than 10^3 will be eluted as single peaks C or F, respectively. Only molecules of proper sizes (within the range of selective permeation) are fractionated and separated. The figure is adopted from Snyder and Kirkland (1979) with modifications.

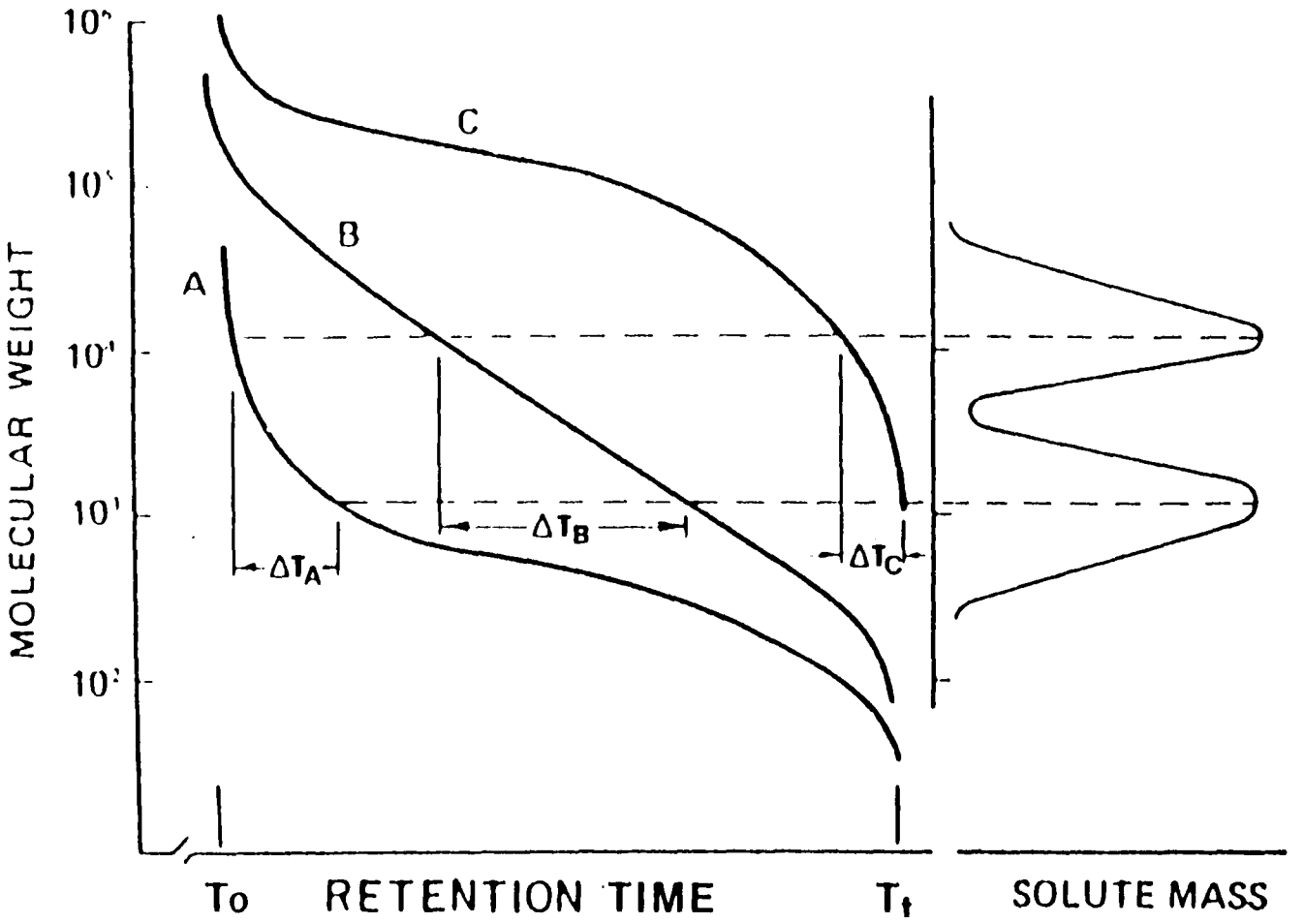

Fig 2

Fig 2. Selection of the proper size exclusion packing for the optimum separation of two adjacent bands. T_0 is the total exclusion time and T_t is the total permeation. Columns A, B and C have different characteristics of selective permeations. The retention distances (ΔT) of two molecules, 10^3 and 10^4, respectively, by three columns are $\Delta T_B > \Delta T_A > \Delta T_C$. See the text for details. The figure is adopted from Snyder and Kirkland (1979) with modifications abcde

3.2. General Consideration of the Separation

3.2.1. Calibration Plots

A calibration plot is a graph of sample molecular size versus relative retention for a particular packing, which reflects the ability of the packing or the column to separate different molecules on the basis of their molecular weights. Fig 1 shows a hypothetical plot of sample molecular weight versus retention time Tr. Point A is a total exclusion point where molecules too large to enter the pores of the packings are entirely excluded, eluted as a single band at the front of the chromatogram with a retention time of T_0. Molecules with smaller molecular weight than B, the point for total permeation, would permeate the packing particles totally and will be retained for a time corresponding to retention time Tt. Molecules with molecular weight between A and B will gradually spend longer time in the column as their size decreasing and the retention time (volume) points construct a plot, the calibration plot. The molecular weight range between the total exclusion and the total permeation is called the fractionation range of a column where a column performs best.

It is conceivable that in a certain molecular weight range, a wider fractionation range would yield better separation. Although there are several theoretical parameters for the efficency of separation, the measuring of band center spacing is probably the most straightforward; the further apart the two bands, the better their separation is. Compounds are separated best when their molecular weights are within the column fractionation range. Fig 2 shows calibration plots of three columns with different packings, compounds a and b, with molecular weights of 10^3 and 10^4, respectively, are separated on these three columns, A, B and C. For packings A and C, both compounds are near either exclusion limits or permeation limits of both columns, respectively, so that the spacing between the bands is small, i.e. the differences of the retention times, TA and TB, are small and the separation is poor. With column B, the size of the interested compounds falls within the fractionation range and the resultant band center spacing, TC, is large. Therefore, the separation is improved.

Choice of an appropriate column, or pore size, is the key issue when embarking an analysis of proteins. Once the optimum pore size is determined, satisfactory

resolutioncan be achieved by combining two or more columns with the same pore size. Although an appropriate calibration plot for a given packing is usually provided by the manufacturer with the column, it is desirable for the individual columns to be tested and calibrated under experimental conditions.

3.2.2. Mobile Phases

The major consideration of the mobile phase choice is its ability to dissolve the sample molecules and low viscosity at the operation temperature. In SEC, the resolution is usually not affected by the mobile phase.

Compatibility with the column packing is another consideration for the mobile phase. With the silica-based packings, a wide selection of solvents can be used but pH must be maintained within the range of 2-8.5 for aqueous solvents because dissolution of siliceous particles will occure beyond this pH range.

Sometimes, interactions (i.e. adsorption) between the sample molecules and the packing surface can be a problem and it is the case particularly with silica-based packings where retention of solute molecules, in addition to the desired size exclusion, is often observed. Elimination of such unwanted retention can be achieved by using a mobile phase that is much more strongly competitive for the active sites of adsorption than the solutes. Adsorption can be judged by the elution of sample molecules after the total permeation volume (Vo). Band tailing may sometimes be an indication but is not reliable. Appropriate ionic strength is effective in eliminating adsorption on silica substrates in aqueous medium and normally 0.1-0.5 M is recommended. However, caution must be excercised in selecting the mobile phase ionic strength as the effect on retention is complicated and often short of prediction. Careful examination is needed for individual systems and samples.

In the aqueous mobile phase, the type of anion used has a significant influence on undesired adsorption of solute molecules to siliceous surface during chromatography, by probably the effect of counter-ions. Phosphate and sulfate seem to be more effective than monovalent anions, such as chloride. With an unmodified silica packing, a weakly acidic ion exchange phenomenon may occure at pH > 4-5 because the acidic

SiOH residues of the silica can interact with charged groups of the solutes. Therefore, a surface-modified silica packing is much preferred and, even so, the mobile phase should still be adjusted to obtain the optimum separation.

3.2.3. Temperature

For most of the biological analysis of SEC, column operation at ambient temperature should be satifactory. However, for samples of low solubility or mobile phase with high viscosity, temperature may need to be increased. Generally speaking, column efficiency and resolution can be improved at above-ambient temperature by the lowering of the viscosity of the aqueous mobile phase, but the sample molecules must be sufficiently stable under such a temperature.

With most of low-concentration detergent extracts of membrane proteins, HPLC fractionation under room temperature is able to generate satisfactory resoluiton with convenience.

3.2.4. Sample Size

Both the sample volume loaded onto a column and the sample weight in the volume can affect the final resolution which can be theoretically predicted. Too big a volume of sample results in band broadening, while overloaded sample weight causes deteriorating of column efficiency, resulting in asymetrical bands. Typically, sample volume in the 25-250 ul range is recommended for high efficiency columns. As a general rule, larger volumes of more dilute sample solution rather than smaller volumes of more concentrated samples are preferred for chromatographic analysis.

In the separation of membrane protein preparations, sample size should be well controlled to avoid poor resolution and low efficiency, because of the use of detergent and of macromolecules involved, which may present higher viscosity. A special caution must be taken that viscosity decreases sharply upon drop of temperature. Empirically, the sample solution charged should not have a viscosity greater than twice that of the mobile phase.

Size exclusion chromatography is capable of delivering powerful initial separation and an overall picture of sample composition when a proper combination of the operation parameters is achieved. Among many advantages, those distinct ones are listed below:

(1). SEC is one of the gentlest method in protein separation, without involvement of any high-energy forces or chemical interactions during the process. This is essential particularly when higher structures of proteins are of importance, such as in the separation of membrane surface receptors. This is also true when non-covalent interactions of proteins are concerned. As SEC is a gentle process, samples do not suffer from loss or reaction, therefore, recovery is usually high.

(2). With the help of calibration plots, molecular weights of unknown components can be easily worked out. Therefore, SEC is particularly useful for unknown samples that a single run is normally able to provide sufficient information on the molecular weight distribution and the composition. This technique is potent for an exploitory investigation.

(3). As the separation usually falls within the fractionation range, the retention of any known molecules is predictable, which is characteristic of SEC, and saves time when collection of only interested fractions is sufficient. And also, it is possible that a series of injections of different samples can be carried out with a proper time interval whereas without risking to overlap one chromatogram with another. Under the same principle, repeated loading of the same sample at predetermined times can make collection of big amount of a particular fraction possible.

(4). The study of protein interactions can be facilitated by the SEC, especially the in vitro induction of such complexes by using specific agents, i.e. antibody. Because of its gentle nature of the technique, such complexes should stay complexed during separation and their retentions are predictable under the basis of calibration plots.

(5). With the modern HPLC, the column packing does not tend to accumulate strongly retained compounds and the columns usually do not degrade and they perform reproducibly over a long period.

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CHAPTER 2. GENERAL ASPECTS OF METHODS AND MATERIALS

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All chemicals and reagents used in this study were obtained from Sigma, BDH, Pharmacia and Fisons unless otherwise stated. 'Analar' grade was selected for most of the purpose. % composition refers to volume/volume.

1. Preparation of the Cells

1.1. Leukaemic Lymphocytes

Freshly collected blood from leukaemic patients was loaded on pre-warmed (20^{o} C) Ficoll-Hypaque (Pharmacia) and centrifuged at 800 g for 20 min. The lymphocytes were partitioned and harvested from the interface with a thin pipette. The cells, then, were washed free of plasma and platelets in an isotonic HEPES buffer consisting of 133 mM NaCl, 1.3 mM KCl buffered to pH 7.4 with 10 mM HEPES (RBC) and the cells were transfered to the similar buffer but with 5 mM $MgCl_2$ and 2 mM $CaCl_2$ (WBC) pH 7.4, prior to lactoperoxidase-catalysed surface protein iodination. For the antibody treatment of intact cells or capping assay, the cells were washed with RPMI1640 medium supplemented with 2% Foetal calf serum (FCS). The viability was routinely > 95% by acridine orange/ethidium bromide staining (Parks et.al., 1979)

1.2. Normal Lymphocytes

Normal lymphocytes were prepared from tonsils freshly obtained after tonsilectomy:

A. The tonsil lymphatic tissue was scraped out with a scalpal blade and suspended in RBC pH 7.4 buffer containing 50 U/ml pencillin and 50 ug/ml streptomycin (Flow Lab.). Cell clumps were dispersed by repeated passage through a syringe without a needle and the suspension left in room temperature for 20 min to allow the settlement of large particles. This procedure can be repeated until a clump-free supernatant was produced. The cells were washed three times in the same buffer until the supernatant became clear.

B. Dead cells were removed on prewarmed Ficoll-Hypaque (20^{o} C) for 20 min at 800 g (Davidson and Parish, 1975). Viable mononuclear cells were collected from the interface and given two washes at 20^{o} C to get rid of residual Ficoll contamination.

C. Monocytes were depleted by incubating the cells on glass Petri dishes at 37^{o} C for 1 hour in RPMI1640 supplemented with 10% human serum, 50 U/ml penicillin and 50 ug/ml streptomycin at the concentration of $1x10^{7}$ cells/ml. Non-adherent cells were collected , washed with WBC buffer pH 7.4 and resuspended in RPMI1640-10% FCS medium containing 0.1% NaN3 and 50 U/ml penicillin + 50 ug/ml streptomycin at approximately $2x10^{7}$/ml.

D. T lymphocytes were removed by rosetting with neuraminidase-treated sheep erythrocytes (SRBC). A 10% haematocrit of SRBC (about $3x10^{9}$/ml) was washed twice with the RBC pH 7.4 buffer and pelleted by centrifugation at 1000 g and resuspended in 3 ml WBC buffer pH 7.4. 100 ul neuraminidase (*Vibrio Comma*, Behringwerke AG, Marburg, 1iu/ml) was added, and the suspension incubated at 37^{o} C for 20 min. After being washed twice in WBC buffer pH 7.4, the cells were resuspended in 15 ml RPMI1640 containing 10% FCS, 0.1% NaN3 and 50 U/ml pennicillin and 50 ug/ml streptomycin at about $1x10^{9}$/ml. 5 ml lymphocyte suspension ($2x10^{7}$) was mixed with 5 ml SRBC suspension ($1x10^{9}$), gassed with 10% CO_2-in-air and centrifuged at 400 g for 5 min to allow the cells to settle down. The mixture was left on ice overnight. The pellet was then gently dispersed and resuspended and loaded over Ficoll-Hypaque, centrifuged at 400 g for 30 min to remove the rosetted T cells.

E. The cells in the interface were collected and washed in the WBC buffer pH 7.4 twice. The purified B lymphocytes were checked for T cell contamination and viability by using monoclonal antibody to T-cell markers and acridine orange/ethidiium bromide staining, respectively. The T cell contamination was routinely less than 5%. The viability was > 95%.

2. Surface Labelling of Lymphocytes

2.1. Iodination by Lactoperoxidase Catalysis

Cells were suspended in 0.9 ml ice-cold WBC buffer pH 7.4 with cell numbers up to $1.5x10^8$. 20 ul 0.5 M glucose, 5 ul 0.1 mM $K^{127}I$ and 0.06 unit glucose oxidase (*Aspergillus niger*--Sigma) were added into the cell suspension at 4^o C. 1.5 units lactoperoxidase (*Bovine milk*--Sigma) was added followed immediately by the addition of 100 u*Ci* $Na^{125}I$ (Amersham). The cell suspension was immediately incubated in 37^o C water bath for 20 min. The cells were then washed twice with ice-cold WBC buffer pH 7.4 containing 5 mM $K^{127}I$, to remove unbound iodine, once with RBC buffer pH 7.4 and then resuspended in 200 ul RBC buffer pH 7.4.

2.2. Tritiation of Surface Glycoproteins

The tritiation method employed in this study was of the neuraminidase/galactose oxidase treatment based on Gahmberg and Hakomori (1973). To $5-8x10^7$ cells in 1 ml WBC buffer pH 7.4 were added 25 ul neuraminidase and 50 ul galactose oxidase and the cell suspension was incubated at 37^o C for 20 min. The cells were pelleted and washed twice in WBC buffer pH 7.4, once in WBC buffer pH 8.0 and resuspended in 1 ml WBC buffer pH 8.0. The cells were then incubated at 20^o C for 10 min with 2 m*Ci* tritiated sodium borohydride. Unbound tritium was washed away with WBC buffer pH 7.4, then RBC buffer pH 7.4. The tritiated cells were suspended in 200 ul RBC buffer pH 7.4.

3. Triton X-100 Extraction of Cells

200 ul cell suspension was added with an equal volume of Triton X-100 extraction solution consisting of 1% Triton X-100 (Sigma), 1 mM PMSF, 0.5 TIU aprotinine, 1 mM EDTA and 5 mM iodocetomide in RBC buffer pH 7.4. Iodocetamide was present in the Triton X-100 extraction medium to prevent formation of artifactual disulfide bonds. After being on ice for 10 min, insoluble material was spun off at 2000 rpm (900 g) for 10 min.

4. Size Exclusion Chromatography

The description of the SE-HPLC technique will be detailed in **Chapter 4**.

5. Gel Electrophoresis and Protein Staining

5.1. SDS-PAGE

The HPLC fractions were prepared for SDS-PAGE by adding an equal volume of SDS sampling buffer and dithiothrietol (DTT) to 100-175 ul fraction at a final concentration of 50 mM and the mixture was heated to boiling for 4 min.

One dimensional SDS-PAGE in the discontinuous buffer system of Laemmli (1970) was used for the separation of the proteins. The slab gel apparatus was constructed as described by Studier (1973). The polyacrylamide gels (150 mm X 140 mm) were cast between two glass plates (180 mm X 180 mm), separated by 1.5 mm thick perspex spacers. The plates and spacers were clamped together by metal spring clips and sealed on three sides with 1% agrose. A perspex sample comb was used to produce 16 sample wells, each 6 mm wide, 15 mm deep and 3 mm apart in a 10 mm length stacking gel.

5.1.1. The Fomular of the SDS-PAGE solutions

Acrylamide Stock Solution (23%)

30 g Acrylamide (BDH)
0.8 g N,N'-Methylene Bisacrylamide (BDH)
Add 100 ml H_2O

If the solution is MADE UP to 100 ml, according to the following recipe, the concentration of the gradient running gel will be 8-15%.

Running Gel Buffer

19.2 g Trizma Crystals, pH 8.7 (Sigma)---- 1.5 M Tris

0.4 g SDS (BDH)---------- 0.8% (W/V)

Adjust pH to 8.8 with HCI , make up to 100 ml

Stacking Gel Buffer Stock Solution

6.06 g Tris

0.4 g SDS

Adjust pH to 6.8 with HCI , make up to 100 ml

Electrode Buffer (pH 8.3)

3.03 g Tris

14.41 g Glycine

1 g SDS

Make up to 1000 ml

10% Ammonium Persulphate (APS)

SDS Sampling Buffer

0.125 M Tris-HCL (pH 6.8)

20% (W/V) SDS

30% Glycerol

0.002% (W/V) Bromophenol Blue

1mM EDTA

6-11.5% Gradient Running Gel Solution

	6% (ml)	11.5%
Acrylamide Stock Sol.	4.0	7.5
Running Gel Buffer	3.75	3.75
H_2O	7.15	3.65
APS	0.1	0.1

TEMED	5 ul	5 ul

Stacking Gel Solution

Acrylamide stock solution	3.3 ml
Stacking gel buffer	5.0 ml
H_2O	11.5 ml
APS	0.2 ml
TEMED	14 ul

Overlaying Buffer

Running Gel Buffer	2 ml
H_2O	6 ml

5.1.2. The polyacrylamide gel solution and the buffer are described as follows:

Three different concentrations of the running gels were used in this study depending on requirements. Running gels of 15% and 8-15% or 6-11.5% linear gradients of acrylamide were polymerized by the addition of ammonium persulfate and N,N,N',N'-tetramethylethylene diamine (TEMED). The running gel solutions with each concentration were prepared separately and placed in gradient-forming chambers. Using a peristatic pumping system, the gradient gel solutions were mixed and directed between the glass plates. The gel top should be adjusted to horizontal, and a layer of 10 X diluted running gel buffer was layered carefully on the gel surface and, usually, the polymerized gels were stored away in a cold room overnight for a complete polymerization.

Before use, the overlaying solution was removed with a syringe and the gel surface was washed once with stacking gel solution lacking TEMED. The stacking gel (4% acrylamide) was poured onto the running gel and the comb inserted. Within about 20 min, the stacking gel polymerized and the comb and the lower spacer were removed. The gels then were affixed to a standard electrophoresis tank filled with electrode buffers. Samples were loaded on with a 100 ul Hamilton syringe. For big samples, they could be introduced in two loadings. The first loading was usually 110 ul and was electrophoresed for a while at a high current of 60 mA to allow the sample to go into the stacking gel and then the rest of the sample was introduced. It is preferable to run the samples at a high current in the stacking gel to avoid diffusing until the bromophenol blue dye front reached the running gel when the current was lowered down to 45 mA. Running time was approximately 4 hours.

5.2. Fixation and Staining of Proteins

After electrophoresis, proteins were visualised either by Coomassie Blue or Silver staining.

A. Coomassie Blue Staining: proteins on the gels were fixed and stained with 0.025% (W/V) Coomassie Brilliant Blue R250 (Sigma) in acetic acid : methanol : water (10:45:45) solution overnight at room temperature and destained with acetic acid : methanol : water (10:20:70) for approximately four hours at 50° C.

B. Silver Staining: gels were fixed for 8 hours in acetic acid: methanol: water (2:5:13) solution and rinsed in distilled water for 1 hour with at least one change of water. The gels were then soaked in 7 ug/ml dithiothreitol (DTT) solution in water for 2 hours with continuous agitation, transferred into 0.15% silver nitrate without rinse, incubated for two hours. After being rinsed rapidly twice with water, the silver stained gels were developed with 100 ul of 40% formaldehyde in 200 ml of 3% potassium carbonate under continuous agitation. As the image was appearing and at an appropriate stage of the development, half of the developing solution was poured off and 10 ml of 2.3 M citric acid put in to stop the reaction. The stopping procedure should be carried on for at least 30 min. The gels could be either stored in distilled water for a short period or vacuum-dried between two sheets of cellophane (Bio-Rad Ltd). The dried gels were presented as a transparent matrix.

6. Fluorography

Radioactive-labelled molecules on the gels were detected by autoradiography or fluorography. Gels of iodinated proteins were exposed to pre-flashed X-ray film (Kodak X-Omat H Film) or higher sensitivity Hyper film-MP (Amersham), both backed with an intensifying screen (Ilford Fast Tungstate) in a metal cassette at -70^{0} C for periods of up to five weeks. Gels containing tritiated materials, after being fixed and stained , were rinsed for 15 min in distilled water and incubated in Autofluor (National Diagnostics, US) for 1 hour. Gels were vacuum-dried onto Whatman No.17 filter paper at 60^{0} C. Autofluor-processed gels can not be dried on cellophane sheets. Dry gels were exposed to pre-flashed X-ray films backed with an intensifying screen, at -70^{0} C.

Exposed films were developed in Kodak LX 24 developer at 5.6 X dilution for 4 min at room temperature.

7. Antibody-Induced Capping of CLL Cells

The lateral movements of cell surface antigens of B-CLL were investigated. $1x10^8$ cells suspended in 1 ml 10% FCS-RPMI1640 medium containing 50 U/ml penicillin and 50 ug/ml streptomycin (Flow Lab) were reacted with monoclonal antibody, incubated on ice for 50 min or at room temperature for 15 min. The cells were washed twice with ice-cold phosphate buffer saline (PBS) pH 7.4 and were further incubated with goat anti-mouse Ig-FITC ($F(ab)_2$ fragments) in 37^o C for various periods of up to 24 hours with continuous rotation. Cells without treatment of the first antibody were cultured with goat anti-mouse Ig $F(ab)_2$-FITC as a control. Cells were washed and resuspended in WBC buffer pH 7.4.

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CHAPTER 3. STUDY OF TRITON X-100 EXTRACTION

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CHAPTER 3. STUDY OF TRITON X-100 EXTRACTION

1. Introduction

Before embarking upon a solubilization procedure, two questions must be considered: the first is how effective is the solubilization and the second is how the activity and function of the solubilized proteins are affected by the detergent employed during the procedure. The criterion of solubility is, by its nature, operational and really depends upon the requirement of the subsequent analysis. The most commonly quoted criterion is the retention of a protein or activity of a protein in the supernatant after sedimentation at 1.05×10^5 g for 1 hour with reference to the density of the medium. In this study, the solubility of proteins and protein complexes is defined at the operational way of performing the HPLC size exclusion column, i.e. supernatant of centrifugation under minimum gravity which sediments materials unapplicable to a HPLC column.

The choice of an appropriate detergent is probably the most complex issue as no single detergent has ever emerged to be ideal for all purposes. Triton has been found to solubilize many ligand-binding proteins with the retention of functions (Strauss et.al., 1979). Triton X-100 usually does not appear to induce conformational changes in proteins leading to loss of their biological properties(Dorken and Koehler, 1961; Burkhard and Stolzenberg, 1972; Cuatrecasas, P., 1972; Meunier et.al., 1972; Umbreit and Strominger, 1973; Simons et.al., 1973; Rubin and Tzagoloff, 1973). As Triton X-100 appears to be very inefficient in breaking protein-protein interactions, most proteins preserve their quaternary structures in the presence of high concentrations of Triton X-100 (Helenius and Simons, 1972; Snary et.al., 1974; Crumpton and Hayman, 1974). From these points, Triton X-100 is suitable for this study.

This chapter explores the characteristics of Triton X-100 solubilization of lymphocyte membrane proteins. The effectiveness of the detergent and its concentration are carefully examined. In comparison, some other non-ionic detergents, including Brij96 and Brij99, are also under investigation.

2. Materials and Methods

Cell surface proteins were either radioiodinated or tritiated as stated in **Chapter 2**. Contaminating erythrocytes were removed as follows: cells were pelleted in WBC buffer pH 7.4, 1 ml 10x diluted WBC buffer pH 7.4 was added to the pellet and the cells were immediately dispersed and left on ice for 20 seconds. The cell suspension was quickly transferred to 10 ml normal concentration WBC pH 7.4 buffer. The cells were then pelleted and resuspended in the same buffer. This procedure can be repeated as necessary. Normally 80-90% of the white cells were viable by acridine orange/ethidium bromide staining after this treatment.

The effectiveness of Triton X-100 extraction was investigated as illustrated in Table 1. The iodinated cells were resuspended in RBC buffer pH 7.4 at $2x10^8$ per ml. A volume of the suspension was mixed with an equal volume of Triton X-100 detergent solution with a concentration of twice the final one, and incubated on ice for 10 min. The cells were then centrifuged at 1000 g for 10 min. The supernatants were applied for analysis on 15% polyacrylamide SDS gels after reduced with 50 mM DTT.

The Triton X-100 insoluble pellets were subjected to further extractions. They were dispersed and washed twice in Triton X-100 extraction medium of the appropriate concentration to minimize protein contamination from the supernatants. The pellets were then suspended in 100 ul SDS extraction medium with an addition of 10 ul DNAase I to digest the DNA released from the nuclei. The mixture was incubated at room temperature for 60 min. EDTA, PMSF and iodocetamide were present to prohibit proteolysis. The insoluble matter was spun out and the supernatant was subjected to SDS-PAGE analysis on 15% polyacrylamide gels.

Here the presence of DNAase I also serves to destabilize F-actin filaments, thereby loosening the actin filaments, and to release any trapped materials (Hitchlock et.al., 1976; Blikstad et.al., 1978).

Table 1. The solubilisation of membrane proteins by 0.025% Triton X-100

	Final Triton X-100 Concentration				
			0.025%		
	0.5%	0.05%	①	②	③
Cell Susp	1 vol	1 vol	1 vol	1 vol	1 vol
RBC buffer				1 vol	
1% TX-100	1 vol				
0.1% TX-100		1 vol			
0.05% TX-100			1 vol	2 vol	1 vol

(1) Incubation time: 10 min
(2) TX-100(V) / cell(V) = 2 / 1, 10 min incubation
(3) Incubation time: 30 min

3. Results

0.01% Triton X-100 extracted virtually nothing as shown by the autoradiograph (Fig 1) and the Coomassie Blue stained gel (Fig 5). The cells after the extraction were still intact as seen by optical microscopy although more fragile when washed. A further solubilization of the cell pellet with 0.5% Triton X-100 resulted in the identical bands as those cells extracted directly by 0.5% Triton X-100.

0.05% was a turning point for the solubilization (Fig 1 and Fig 5). It was able to solubilize all of the components which 0.5% does (Fig 1). But the extraction was incomplete and the re-extraction of the pellets yielded more of the same components. Above 0.1% Triton X-100, no significant increase of the solubilization was observed. A 43KD iodinated component, which was one of the predominant bands in the extract, was effectively extracted by 0.5% Triton X-100 but a significant amount was still left in the insoluble fractions (Fig 2).

As 0.05% was found to be the turning point, 0.025% Triton was tested under different conditions as illustrated in Table I to examine the solubilization in detail. Fig 3 shows that 0.025% Triton X-100 failed to release the membrane proteins as nearly few proteins were detected by the Coomassie Blue staining and the autoradiographs. The gels only revealed a minor amount of surface membrane proteins in the supernatant, implying that the cell membrane was not broken up by the Triton which was confirmed by microscopic examination. It was conceivable that the inability of 0.025% Triton X-100 to break down the membrane bilayer may be due to insufficient free Triton X-100 molecules to disrupt membrane lipids. Therefore, an experiment was conducted to examine the effect of the ratio of Triton to cell numbers at 0.025% concentration. Radiolabelled-cell suspension, split into two equal parts, was treated with Triton X-100 at a final concentration of 0.025% but one part was at cell suspension : Triton=1:1 (v/v) while another one at cell suspension : Triton=1:2 (v/v) (Table 1). The mixtures were incubated on ice for 10 min under identical conditions. The autoradiographs showed no significant differences between the two treatments. However, if the 0.025% Triton X-100 cell suspension (cell suspension : TX-100=1:1) was incubated for a period of 30 min, three times of the normal incubation, it was found that protein solubilization was markedly increased and higher molecular weight components were released as shown by the autoradiographs (Fig 3).

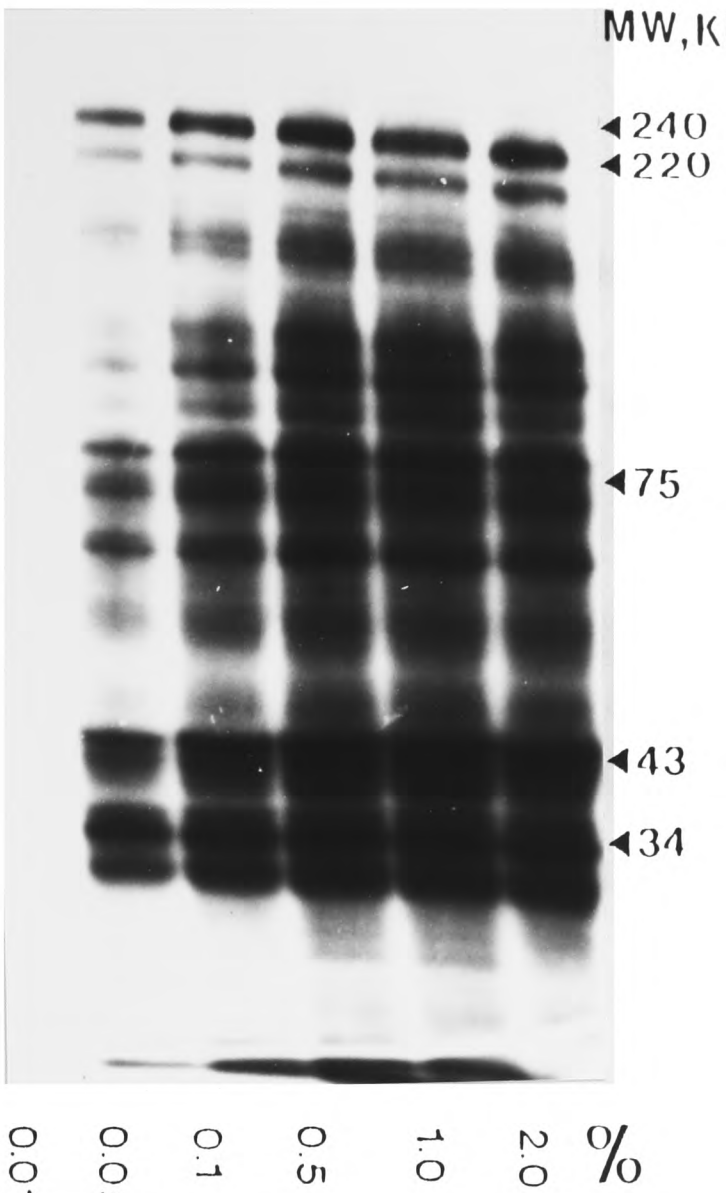

Fig 1

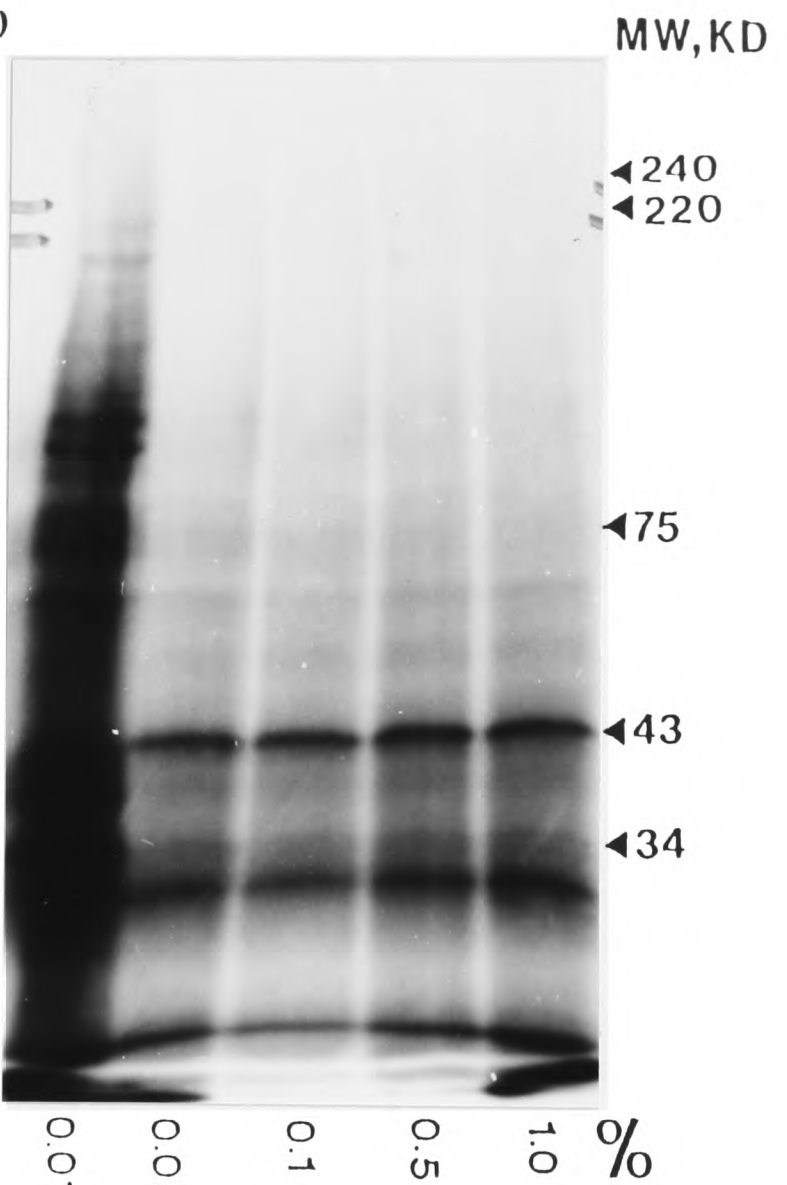

Fig 2

Fig 1. Solubilization of ^{125}I radiolabelled B-CLL cell membrane proteins by a series of Triton X-100 concentrations. The extracts were analysed by SDS-PAGE on a 15% polyacrylamide gel. The fluorography was recorded on Kodak X-ray films. Human erythrocyte membrane proteins were used as molecular weight markers.

Fig 2. ^{125}I radiolabelled materials of Triton X-100 insoluble pellets. Insoluble pellets after solubilization with different Triton X-100 concentrations (the bottom figures) were extracted with SDS extraction medium.

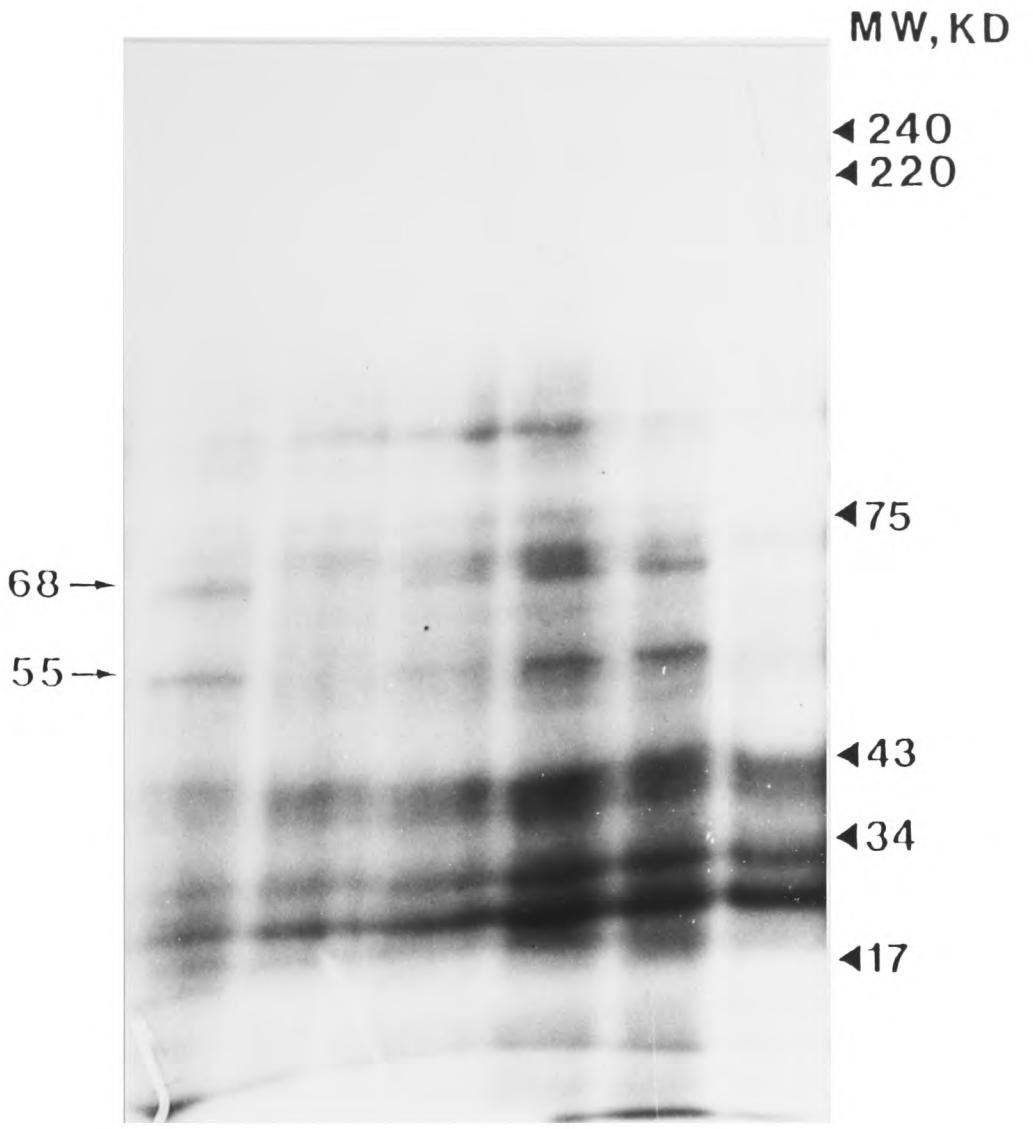

Fig 3

Fig 3. Solubilization of ^{125}I radiolabelled membrane proteins by 0.025% Triton X-100 under various conditions. (1) 10 min incubation (2) 0.025% TX-100, 10 min re-extraction of (1) (3) TX-100(V) : cell(V) = 2 : 1, 10 min incubation (4) 30 min incubation (5) 0.05% TX-100 (6) 0.05% TX-100 re-extraction of (5). The samples were reduced and separated by SDS-PAGE on a 15% polyacrylamide fix gel.

The analysis of the insoluble materials demonstrated that two radioactive surface components were tightly associated with the pellet cytoskeletal mesh at a significant quantity (Fig 2). One has an apparent molecular weight of 43KD and another of about 28KD. Components of similar molecular masses have also been found in the supernatants, but whether they are identical to those of the pellets are still an open question. The 43KD component co-migrated with the red cell actin. Two weaker radioactive components were also detected in the pellet, their molecular weights were 68KD and 56KD, respectively. On the Coomassie Blue stained gels, more bands were detected (Fig 4), including a 70KD, a 45KD, a 33KD, a 28KD and a 17KD, respectively. By overlaying the autoradiographs with the related Coomassie Blue-stained gels, it was found that the 43KD radiolabelled band and the 45KD Coomassie Blue-stained band were distinct molecules. The radioactive band co-migrated with the red cell actin on SDS-PAGE but not the Coomassie Blue stained one. The 43KD iodinated component was one of the major bands in both Coomassie Blue stained (Fig 5) and autoradiographs while the 45KD band was, if any, weakly iodinated and its total quantity is at a much lower level than 43KD. The 43KD could be G-actin. The 28KD band was the only band overlaid with each other on autoradiographs and Coomassie Blue-stained gels. 17KD and 70KD components were not surface-iodinated (Fig 2).

It was of interest that several radioactively labelled components tended to be easily released by low concentrations of Triton X-100. These components consisted of a 68KD, a 55KD, a 43KD and a 28KD (Fig 3). Radiolabelled cell pellets, which had been extracted with 0.025% Triton X-100, were subjected to a second extraction with the same Triton X-100 medium, or solubilized by 0.5% Triton X-100. The SDS-PAGE analysis found that both 68KD and 55KD bands were absent from both the second 0.025% Triton X-100 and the 0.5% Triton X-100 extractions (Fig 3). The bands were entirely released by the first 0.025% Triton X-100 treatment. In a separate study, it was confirmed that both components were not associated with the Triton X-100 insoluable pellets.

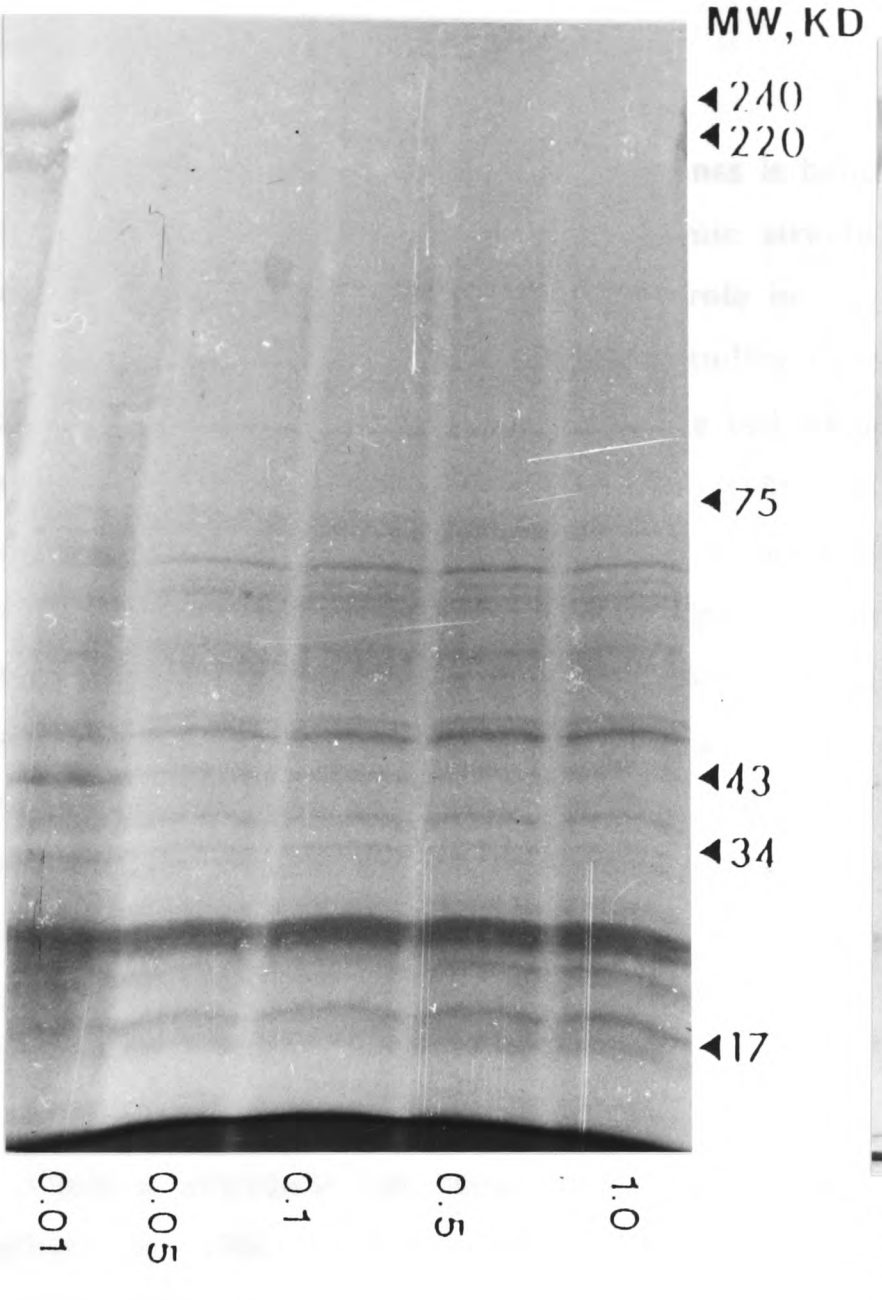

Fig 4

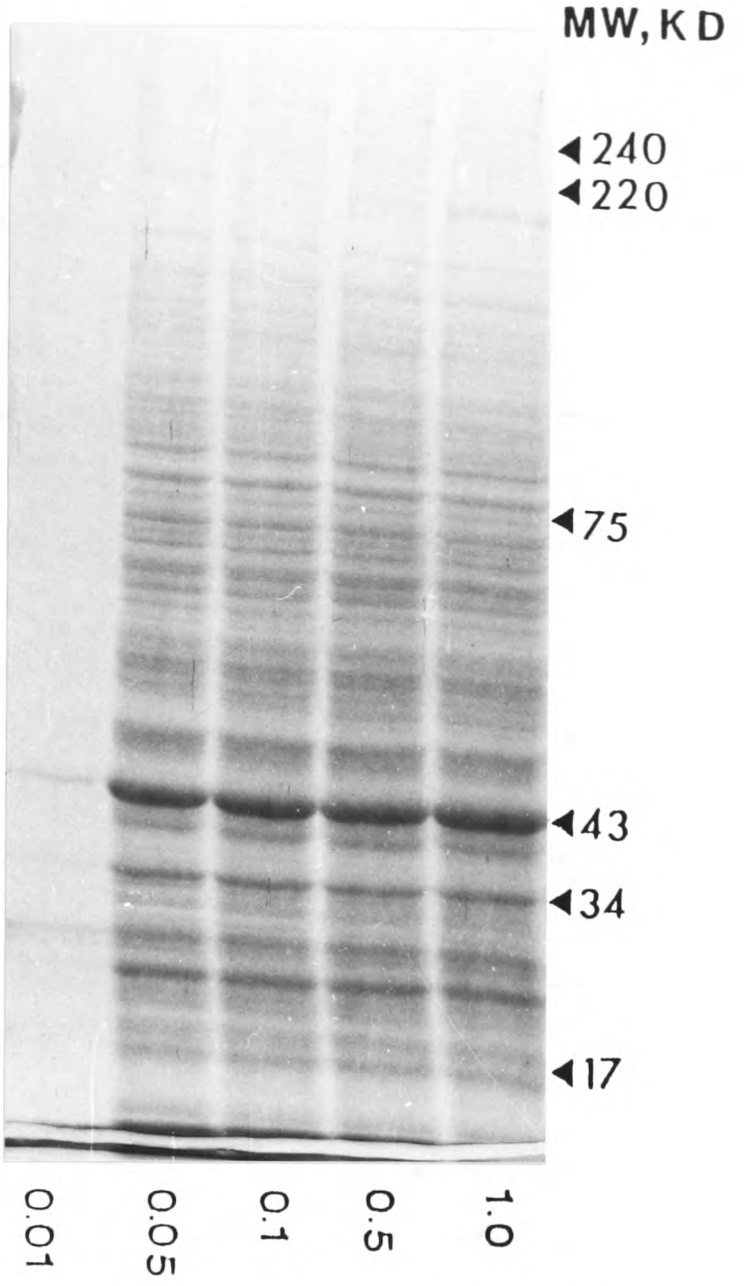

Fig 5

Fig 4. Coomassie Blue stained Triton X-100 insoluble proteins extracted by SDS, corresponding to Fig 2, note the difference of the radiolabelled 43KD band and the Coomassie Blue stained band.

Fig 5. Coomassie Blue stained Triton X-100 soluble proteins extracted by different TX-100 concentrations. Its corresponding fluorograph is Fig 1. It is noted that 0.01% failed to solubilize any proteins.

4. Discussion

The cytoplasmic face of plasma membranes is believed to be the meeting place for the transmembrane proteins and the cytoplasmic structures. The interaction between these two cellular structures plays an important role in conducting signal transduction and cell responses to its surroundings. Extensive studies have been conducted on the structures and constituents of the cytoskeleton in the red blood cell which serves as an excellent model for such purposes (Branton et.al., 1981). Whether the knowledge of the red blood cell cytoskeleton can be extended to nucleated cells has remained a question. However, numerous studies have demonstrated that these cells possess abundance of filamentous cytoskeletal networks within the cytoplasm and these networks are associated with the transmembrane surface proteins at the inner face of the bilayer (Bourguignon and Singer, 1977; Hirokawa and Tilney, 1982). The understanding of solubilization characteristics of membrane proteins might be capable of depicting such relationships and interactions. This study has examined the concentration effect and perturbation of non-ionic detergent on the lymphocytic plasma membrane and the cytoskeleton-associated fraction.

Once a detergent has been selected, an empirical rather than a logical process (Racker, E., 1983), two groups of factors need to be elaborated to ensure the reproducibility and the consistency of the solubilization. One is the ionic strength and the pH of the medium employed in the experiment. Precipitates could occure when anionic detergents are used at an acidic pH, which, though, is not common for non-ionic detergents, like Triton X-100, unless buffers with a very high ionic strength are present. The second factor is the detergent-protein ratio. We found that up to $1.5X10^8$ cells can be efficiently solubilized by 200 ul 0.5% Triton X-100 and further increase of either detergent concentration or the detergent-protein ratio does not result in any increased extraction. It should be born in mind that if the concentration of proteins to be solubilized is close to that of the detergent, the free detergent concentration after solubilization is highly dependent on the initial detergent-protein ratio.

In exercise of solubilization, caution must be taken as contaminants can be found in non-ionic detergents such as Triton X-100 and Brij35 (Ashani and Catravas, 1980; Chang and Bock, 1980), and they vary between different batches of the detergents and after storage in aqueous solution. One of the most significant contamination for this study is the oxidising compounds which react with sulphydryl groups of proteins and

also with carbonyl compounds. Inclusion of iodocetamide in the extraction medium can partly prevent the formation of such artefactual disulfide bonds.

The Coomassie Blue stained proteins on SDS-PAGE demonstrates that the concentrations of Triton X-100 below 0.05% are unable to break the membrane bilayer and release proteins from the cells except a few surface proteins solubilized at a very small quantity, which will be discussed below. The gels exhibited few surface and cytoplasmic components in the Triton extracts of below 0.05%. At 0.05%, the membrane bilayer is apparently broken down, abruptly releasing of cytoplasmic proteins and membrane surface proteins. In our system, 0.05% is found to be the minimum concentration capable of effectively dislodging the membrane proteins from the lipid matrix.

Although 0.025% failed to disrupt the membrane bilayer it perturbates and destabilizes the lipid bilayer and dissociates some external membrane proteins. The solubilization of 68KD and 55KD components indicates such an effect and, hence, shows that both molecules are originated from the external peripheral face of the membrane. It is rational that the membrane lipid bilayer gradually sequesters the detergent molecules and the monomeric form of the detergent binds to hydrophobic segments of the membrane proteins, which probably accounts for the fact that some detergent can cause the expansion of membrane area (Seeman, P., 1966; Kwant and Van Steveninck, 1968).

The 43KD band is associated with the cytoskeletal matrix although it can be extracted into the supernatant at a significant quantity. Both the soluble and insoluble 43KD components comigrating with the erythrocyte actin on SDS-PAGE but they showed slightly differing patterns on gel. With regard of its apparent molecular weight, the band, or part of the band, could be G-actin. The two 43KD components from soluble and insoluble fractions might be two different forms of actin. However, this 43KD component is heavily iodinated and comprises one of the major bands in the lymphocytic membrane. Actin has been found to express on the surface of B-CLL lymphocyte (Bachvaroff et.al., 1980). It is not yet known whether this band is entirely attributed to actin or some other bands as well. In a membrane preparation study with mild non-ionic detergent Tween-40 in this investigation, a 43KD component was observed as a predominant component associated with Triton X-100 insoluble material from the prepared membrane. This band also is surface-iodinated and a 34KD and a 28KD radioactive bands were found to co-pellet with it as well.

Moore and Dedman (1982) identified a number of polypeptides in extracts of various tissues. These polypeptides had molecular weights of 67KD, 34KD, 30KD and 11KD on SDS-PAGE. Recently a group of polypeptides with similar molecular weights have been identified in Ca^{+}-chelator extracts of lymphocytic plasma membrane and intestinal epithelial brush border membranes (Owens and Crumpton, 1984; Gerke and Weber, 1984). One common feature of these polypeptides is that they are all preferentially associated with the non-ionic detergent insoluble complex of the membrane (Mescher et.al., 1981; Davies et.al., 1984). The complex comprised predominantly actin and proteins of 120KD, 68KD (termed p68), 33KD and 28KD. The 68KD component identified in this study suggests its exterior origin or even transmembrane because of its association with the insoluble fraction and capable of being iodinated. In term of the molecular mass, this component is identical with p68 described by Owens and Crumpton (1984) but is contrast to their observation that p68 is allocated in the cytoplasmic side of the plasma membrane.

Knowledge of the plasma membrane protein-cytoskeleton matrix function is very limited. Structurally, the matrix may serve to stabilize the membrane bilayer and by such stabilization to maintain high surface tension. The matrix may also, functionally, mediate the interactions between membrane proteins and cytoplasmic organelles by providing anchorage points for transmembrane proteins and cytoskeletal filaments. Abnormalities discovered in neoplastic cells may support such assumptions and suggest possible correlations between structural defects of the cytoskeleton and functional anomalies of the membrane receptors.

In conclusion, Triton X-100 concentrations not below 0.1% are able to efficiently solubilize most of the membrane proteins of lymphocytes. Further increase of TX-100 concentration above 0.1% does not significantly increase extraction. 0.5% is chosen as the routine concentration as (1) it is the mostly quoted concentration (2) it has wide tolerance to variation of cell number.

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CHAPTER 4. SELECTION AND CALIBRATION OF COLUMNS

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CHAPTER 4. SELECTION AND CALIBRATION OF COLUMNS

1. Introduction

The central element of the size exclusion (SE) chromatography is the column as the separation is largely controlled by the packing materials used. Traditionally, SE-chromatographic analysis of synthetic organic polymers has been made with cross-linked, semirigid polystyrene gel packings. The disadvantage of these semirigid polystyrene particles is that column input pressure must be limited to a relatively low level which restricts their application in modern SE-HPLC. Recently, small, rigid inorganic silica-based packings have been of increasing interest and utility. Better performance has been possible after chemical bonding organo-silanes to derivatize the silica surface is used (Phannkoch et.al., 1980; Schmit et.al., 1980; Roumeliotis and Unger, 1981). This modification deactivates the silica surface and provides the chemical inertness for protein separation. In order to overcome dissolution problems which reduce the useful operating lifetime of the column and generate voids in the packed bed, Stout and DeStefano (1985) found that porous silica microspheres treated with zirconium salt and then a hydrophilic organo-silane can sustain wider pH range and give longer lifetime of column performance.

With certain types of samples, different packing materials of similar pore size could generate different separation while for a particular column, different combinations of analytical paremeters will result in different resolutions. Of the most important paremeters, the ionic strength of mobile phase and flow rate are usually examined and adjusted before embarking on a routine investigation and will have distinctive effects on the behaviour of the column.

2. Materials and Methods

Following columns from different manufacturers were tested during the course of the initial work:

(1) Du Pont Zorbax bioseries GF-250 and GF-450. Both columns are surface-stabilized, hydrophilic gel filtration columns. The columns are expected to produce optimum results when typical aqueous buffer solution under pH range of 3.0-8.5 is employed as a mobile phase. The column characteristics are:

GF-250: Particle size: nominal 4-5 um
Pore size: 150 A
Optimum MW: 10-250KD (for spherical molecules)
Column configuration: 250 mm x 9.4 mm ID

GF-450: Particle size: nominal 6.0 um
Pore size: 300 A
Optimum MW: 25-900KD (for spherical molecules)
Column configuration: 250 mm x 9.4 mm ID

(2) E. Merck's (F.R.Germany) Lichrospher 100 Diol and 500 Diol columns. These two columns are surface modified amorphous silica which is a reaction product of silica with (3-(2,3-epoxypropoxy) propyl) trimethoxysilane. The column characteristics are as follow:

100 Diol: Particle size: 5 um
Column configuration: 250 mm x 4.0 mm ID

500 Diol: Particle size: 10 um
Column configuration: 250 mm x 4.0 mm ID

The columns were carefully connected, using the shortest tubing to optimize the performance as discussed in **Chapter 1**. At least 200 ml mobile phase was run through the column whenever a new buffer was employed.

Gilson model 302-303 piston pumps, fitted with a 10SC pump head, were used for this study. Recommended flow rates for GF-250 and GF-450 columns are from 0.5 to 2.0 ml/min. Although resolution is flow rate dependent (Yau et.al., 1979) a typical flow rate of 1 ml/min was chosen for the daily chromatography although other settings were examined. ARheodyne Model 7125 syringe loading sample injector, fitted with a 100 ul sample loop, was used for loading samples.

The refill speed is a parameter which determines the time required for the piston return stroke. The refill speed is normally set at its highest value which on Gilson model 302-303 is 9. However, the mobile phase must be carefully degassed when a high refill speed is applied.

Precompression setting is essential to minimize pumping pulsations. The duration required for the precompression is a function of (a) the operating pressure (b) the compressibility of the mobile phase (c) the pump head model and (d) the flow rate as well. The setting (0-9) is in linear relation to the working pressure for Model 10SC pumping head if the delivered liquid fixed. The setting effect is evaluated on the detector baseline. For the subsequent work, setting 5 is found adequate for most of the situations.

0.2 M phosphate buffer saline (pH 7.4) containing 0.5% Triton X-100 (or other non-ionic detergent relavant to the detergent in the cell extraction medium) was used as the mobile phase (eluent). The buffer was freshly made, warmed up to about 60^{0} C with Triton X-100 added dropwise. The buffer was then cooled down to 20^{0} C. 0.005% sodium azide was used as an anti-microbial agent. The buffer was filtered through Nylon 66 membrane filters with a 0.045 um pore size (Anachem, UK) and then degassed in an ultrasonic bath for 20 min. The column was equilibrated with the newly prepared buffer.

Table 1 and 2 in the **Appendix III** list the standard proteins used for the calibration and their retention times in the abovementioned buffer with or without detergents. All proteins were dissolved in the phosphate buffer saline pH 7.4. The protein concentration was monitored by spectrometery and adjusted to similar levels for easy comparison of the out-put signals. In order to avoid column blockage all samples were subjected to centrifugation at 1000 g for 4 min.

100 ul sample was loaded to the column. The elution of the proteins were monitored by a Gilson HM holochrome UV/VIS detector at 243 nm which was found to be a window for the protein detection with the presence of Triton X-100 and the eluting profiles and the retention times were recorded.

Various working conditions and columns were tested as described in the result. The operating temperature of the columns was ambient, usually between 20-25^{0} C.

3. Results

100 Diol and 500 Diol columns were examined under the recommended conditions using 0.05 M phosphate buffer pH 7.4 running at the flow rate of 0.2 ml/min and 0.5 ml/min, respectively. 100 ul standard protein sample, dissolved in the same medium as the mobile phase, was injected and the eluted proteins were detected by the UV monitor at 243 nm. The elution profiles were recorded for calculating the retention times which were measured as the distance between a peak and the injection of a sample. Table 1 shows the retention times of the standard proteins. Log of molecular weight was plotted against the retention time in minute as in Fig 1c (100 Diol not shown) with respect to the use of ' Time ' fractionation mode. The data presented here were the means of three independent runs, the maximum deviation of the retention time did not exceed 0.1 min. For different runs in the same batch, the operation pressure varied within 1 bar.

Two Zorbax columns were tested under the recommended conditions by using the same standard proteins as for the Diol columns. The resultant calibration plots of GF-250 and GF-450 are shown in Fig 1 (a and b). Comparing the Log MWs *versus* retention time (min) plots of two sets of columns, two Du Pont columns apparently give better separation and slightly improved linearity (Fig 1d). Between GF-250 and GF-450, the latter one has better performance at fractionating high molecular weight components as shown by Fig 1d. Fig 2 shows the resolution of the standard protein mixture by a GF-450 column eluted with 0.2 M phosphate buffer pH 7.4 under the flow rate of 1 ml/min. Therefore, the GF-450 was shosen for further examination under different flow rates and ionic strengths. Fig 1f shows the effects of the mobile phase ionic strength on the protein retardation on the column. The changes of protein retardation demonstrated the marked differences of separation when buffers with different ionic strengths were used. In 0.1 M phosphate buffer pH 7.4, proteins were eluted in about 6 min and proteins were greatly compressed within 4 min, resulting in poor resolution, particularly for big molecules.

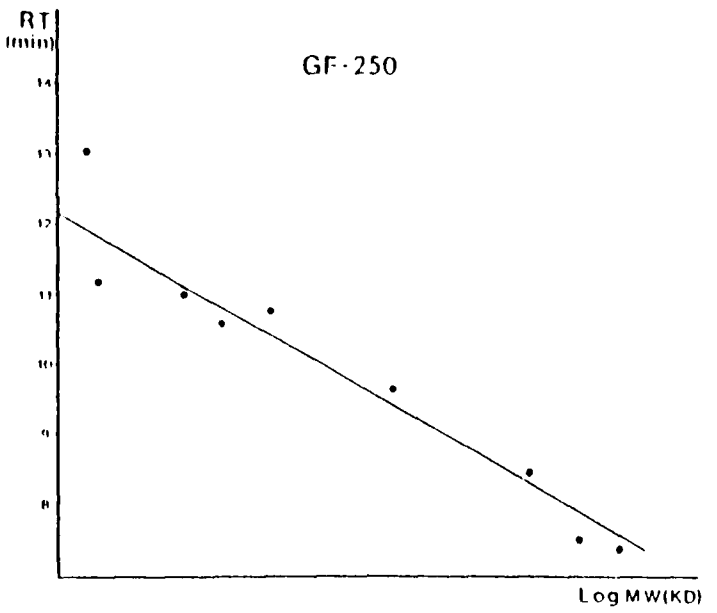
The retention of proteins was also affected by the flow rates used. It was found that the efficacy of separation was in the order of 0.5 ml/min, 1 ml/min and 1.5 ml/min, but dropped down at 2 ml/min as judged by the plot slop. For further analysis, mixtures of standard proteins were applied to the column under different conditions as examined above, the UV absorbance profiles suggested that 0.2 M phosphate buffer of pH 7.4 with

1 ml/min flow rate is an appropriatecombination. Fractionation of actual cell membrane extracts confirmed this setting.

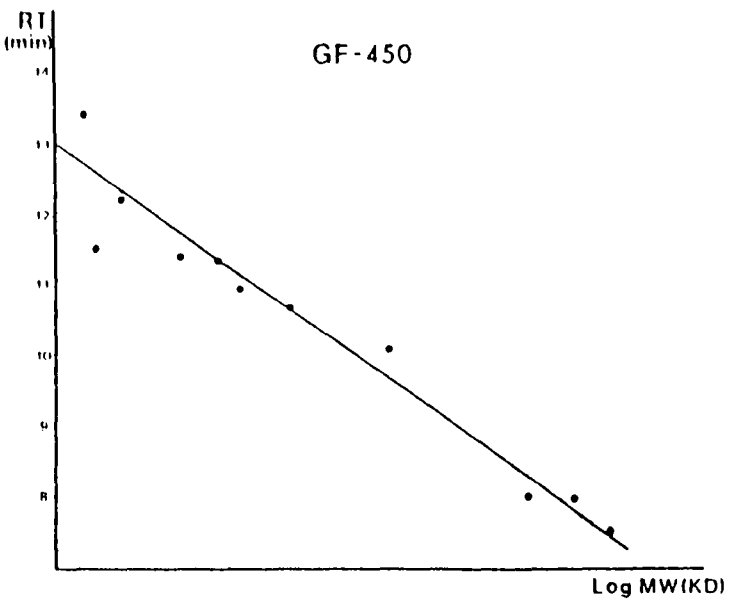
The presence of non-ionic detergents yielded no significant effect on the retention of the standard proteins tested. Fig 1e shows that the retention times of proteins with or without 0.5% TX-100 were almost identical. Similar foundings were also true for other columns and other detergents, such as Brij 99.

Urease behaved abnormally on all the columns tested. It was greatly retarded, showing much longer retention time.

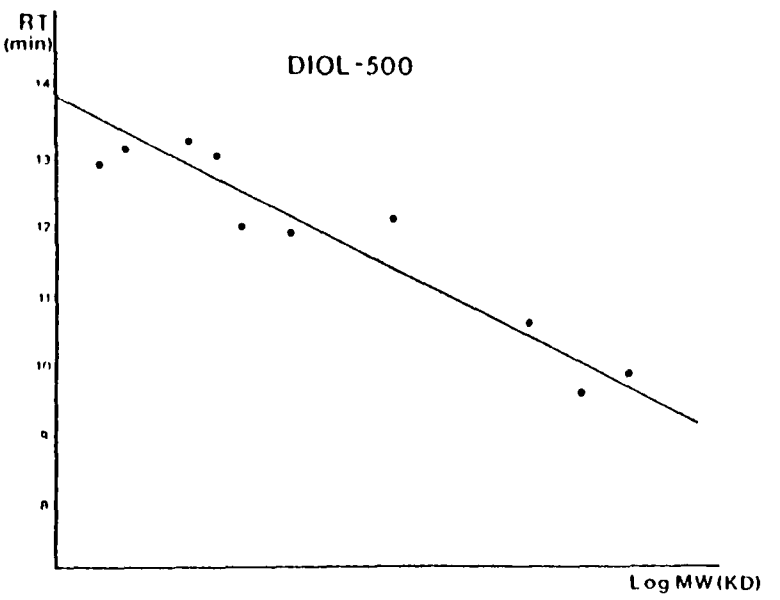
Fig 1. Calibration curves of HPLC size exclusion columns to show the resolution differences and the effect of Triton X-100 and ionic strength. (a) GF-250 (b) GF-450 (c) Diol-500 (d) the comparison of three columns by overlaying (a), (b) and (c) (e) the effect of TX-100 on the retention of proteins (f) the effect of ionic strength of the mobile phase.


RT
(min)
GF-250
Log MW(KD)

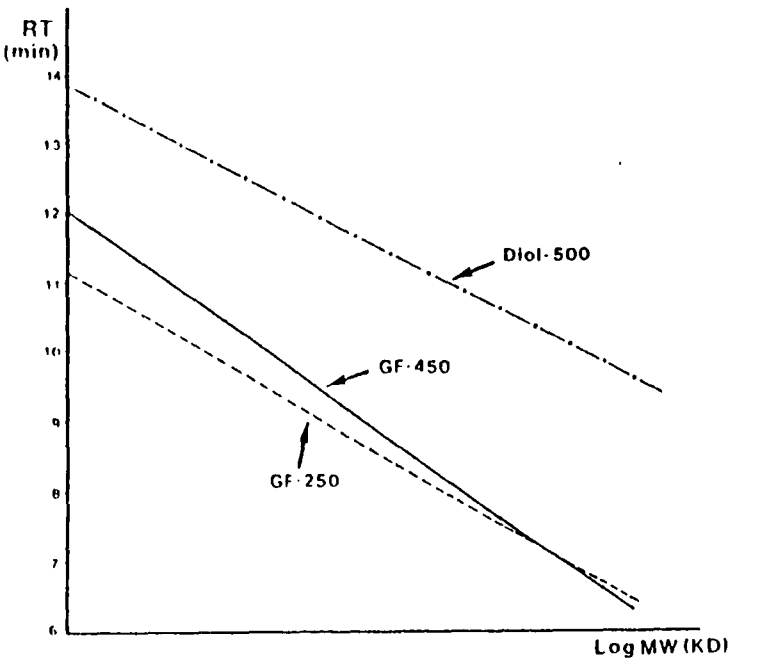
a


RT
(min)
GF-450
Log MW(KD)

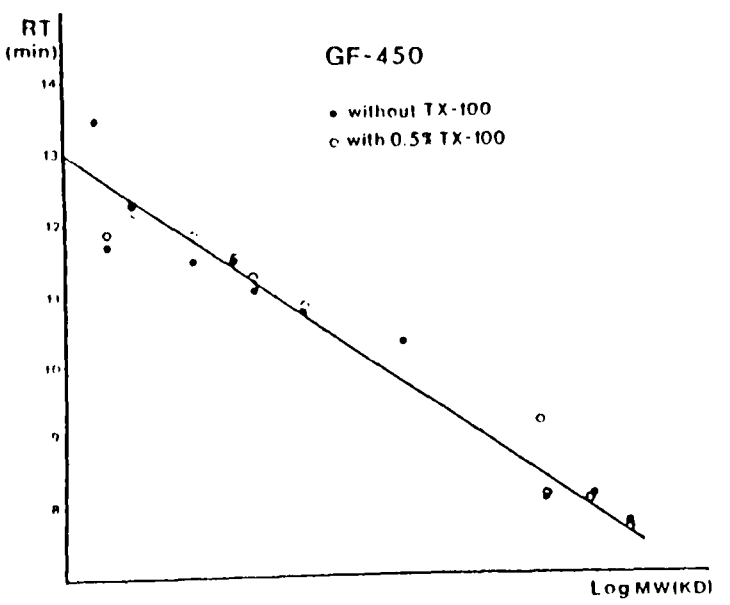
b


RT
(min)
DIOL-500
Log MW(KD)

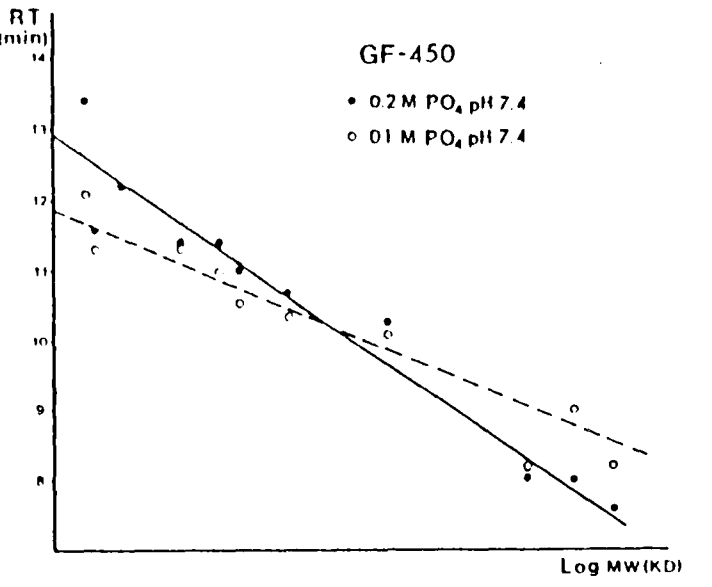
c


RT
(min)
Diol-500
GF-450
GF-250
Log MW (KD)

d


RT
(min)
GF-450
• without TX-100
○ with 0.5% TX-100
Log MW(KD)

e


RT
(min)
GF-450
• 0.2 M PO4 pH 7.4
○ 0.1 M PO4 pH 7.4
Log MW(KD)

f

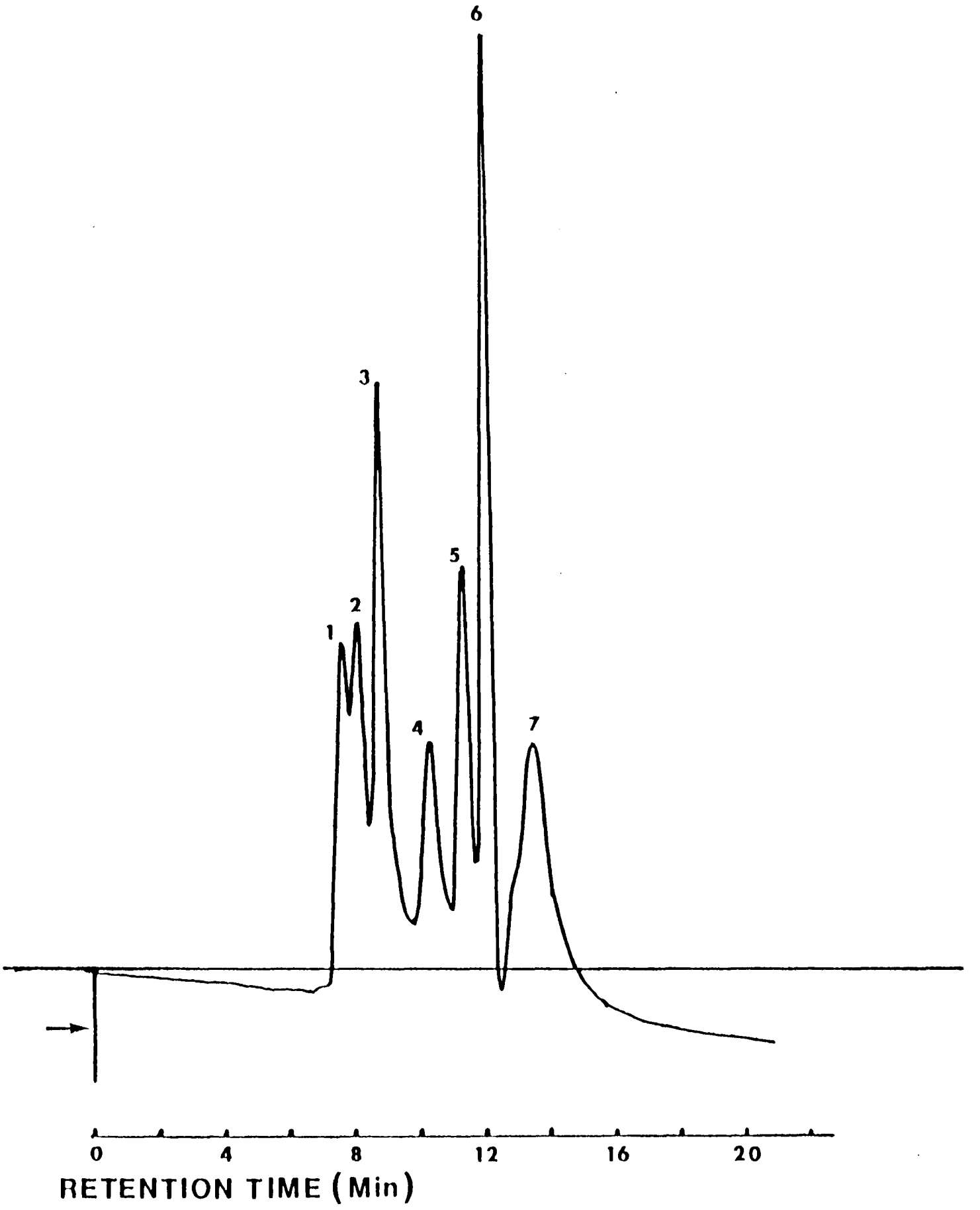


Fig 2

Fig 2. The graphic profile of HPLC-SE separation monitored by an UV detector, showing the resolution of a mixture of seven standard proteins by a GF-450. (1) IgM (2) thyoglobulin (3) spectrin (mono) (4) ß-lactoglobulin (5) carbonic anhydrase (6) α-lactalbumin (7) cytochrome C.

4. Discussion

The analysis of the plots of Log MWs *versus* retention times and the overall assessment favour the GF-450 for this study. Although GF-250 achieved similar separation at the molecular weight range of less than 150KD, bigger molecules, such as IgM and thyroglobulin are totally excluded. GF-450 column has shown sharp responses and high reproducibility within the molecular weight range of 20KD to 900KD. Several proteins showed anomalous retention on the column with respect to the linearity of the plots. Cytochrome C was abnormally retarded on both GF-250 and GF-450. This might be attributed to its basic and highly negative-charged nature. Ferritin was also, for some reason, retarded abnormally by GF-250 column. Bearing in mind that gel chromatography does not separate molecules strictly by their molecular weights but rather by an entropy-driven process, resulting from the differences in the hydrodynamic volume occupied by the substances, as determined by their radii of gyration (Yau et.al., 1979). Geometrical shapes of proteins and other factors such as ion-exchange and hydrophobic interactions (Regneier and Noel, 1976; Phannkock et.al., 1980; Schmit et.al., 1980; Roumeliotis and Unger, 1981) also affect the behaviour of the proteins in the column. Thus the relationship between Log MW and retention time is in fact only quasi-linear. These anomalous phenomena, therefore, are not totally unexpected.

Examination of the chromatographic behaviour of proteins as a function of ionic strength indicates that the elution of proteins is influenced to a different extent. IgM and Thyroglobulin were retarded while spectrin was excluded. The results presented here are concordant with the previous investigation (Stout and DeStefano, 1985) although the operating conditions are different. It is known that retention of proteins is affected by the ionic strength in a random fashion, without any correlation between the retention volume (or time) and physical properties of proteins as found by Engelhardt and Mathes (Engelhardt and Mathes, 1981), Roumeliotis and Unger's (Roumeliotis and Unger, 1979, 1981).

The operational paremeters established in this study render GF-450 column the best separation of the proteins across a wide range of molecular weights which should cover the membrane protein investigation. Triton X-100, within the examined concentration range up to 1%, has no measurable effect on the retention of the standard proteins.

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Phannkoch, E., Lu, K.C. and Regnier, F.E. (1980) J. Chromatogr. Sci. 18, 430.

Regnier, F.E. and Noel, R. (1976) J. Chromatogr. Sci. 14, 316.

Roumeliotis, P. and Unger, K.K. (1979) J. Chromatogr. 185, 445.

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CHAPTER 5. HPLC ANALYSIS OF CELL EXTRACTS

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CHAPTER 5. HPLC ANALYSIS OF CELL EXTRACTS

1. Introduction

The investigation of membrane proteins undertakes two different steps in methodology: the isolation and purification of the interested proteins from an usually complicated mixture and characterization of these separated components.

Conventional techniques for analysis of complicated protein mixtures present two major disavantages: (1) interactions except by covalent forces between polypeptides are prone to be disrupted (2) the separation usually involves a multi-step process which hinders high reproducibility and comparative study of samples. Another limitation of the conventional methods is that they are unable to construct an overall image of membrane protein composition although IEF-SDS PAGE does but glycoproteins are frequently poorly focused, giving rise to poorly defined dots which limits the resolution of the method.

Size exclusion chromatography, on the other hand, though having relatively lower resolution, is capable of retaining weak interactions while separating proteins according to their molecular sizes. The fractionation of the separated proteins should be able to reflect the nature state of the protein complexing and interactions, as well as the constituents. When individual fractions are analysed on SDS-PAGE to develop a second dimension, the elucidation of disulfide bondings of proteins can be achieved by selecting the electrophoretical conditions, either with reduced or non-reduced samples. The two-dimensional matrix of HPLC-SDS PAGE gives a comprehensive profile of the membrane protein composition (Wu and Maddy, 1989). The profiles are highly reproducible and comparable between different samples. As size exclusion packings do not bind sample molecules and all molecules are eluted before the total permeation, therefore, quantitative analysis becomes feasible. The potential and more advantages of this technique are gradually realized when its application is extended.

2. Materials and Methods

Cells were surface-iodinated by lactoperoxidase catalysis or tritiated as described in Chapter 2. Proteins were extracted into 0.5% Triton X-100 medium containing 2 mM EDTA, 1 mM PMSF and 0.5 TIU/ml aprotinin. Antibody treatment of either detergent extracts or intact cells was as described in Chapter 2. The Triton X-100 extracts were spun at 1000 rpm for 4 min prior to chromatography to pellet any possible precipitates. Routinely, 100 ul extract was applied to the column, which was equivalent to about $2.5x10^7$ cells.

A precolumn (length: 20 mm) packed with Diol 150 (Lichosphere, DuPont) was placed between the pumping system and the main column to trap impurities or particulates in the aqueous mobile phase and samples. Proteins were monitored at 243 um. All the control samples were fractionated and collected by a programmed time mode as follow:

Wait: 7.0 min
Time: 0.2 min
Collect: 24 fractions

With these parameters, the largest molecules normally appeared in about fraction No. 5 or 6 and the very front fractions reserve the flexibility of collecting molecular complexes for most occasions. However, in some cases a shorter waiting time was adopted as described in the text. 0.2 min collecting time yielded approximately 200 ul fraction at the flow rate of 1 ml/min. An equal volume of SDS sampling buffer was added to each fraction and the mixture was incubated in 50^o C water bath for 15 min. Such fractions could be left in 4^o C cold room for several weeks without noticeable proteolysis but were normally analysed within 48 hours.

Usually, 175 ul of each fraction was subjected to SDS-PAGE analysis on a 6-11.5% polyacrylamide gradient gel under reduction or non-reduction conditions--with or without 50 mM dithiothreitol (DTT) (Chapter 2). Proteins were stained by silver as outlined in Chapter 2 and the gels were vacuum-dried between two sheets of

cellophane and exposed on Kodak X-ray films for various periods of 4-20 days. For tritiated samples, a tungsten intensifying screen was used.

Cell extracts of three different concentrations of Triton X-100, from 0.1% and 0.5% up to 1%, were examined to elucidate the effects of the Triton X-100 concentration on the protein retention times. The detergent content in the phosphate mobile phase was equal to that in the sample. The other conditions were the same as the standard conditions.

The working temperature of the HPLC system was ambient, normally within 20-25^{0} C. For the same batches of samples, running pressures should remain stable. Elevated pressure usually indicates a blockage of the guard column and requires replacement.

Two-column combination was performed occasionally to improve resolution. Two combinations were tested: a GF-250 connected to a GF-450 or 2 GF-450s connected in tandem. All the working parameters were set the same as those for one column but with doubled waiting time, ie. 14 min.

In order to investigate the intermolecular disulfide bondings of the membrane proteins. HPLC of Triton X-100 extracts reduced before chromatography was performed with a mobile phase either with or without 50 mM DTT. After the fractionation, the samples, either with or without further DTT reduction for both cases, were run on SDS gels.

3. Results

3.1. Typical Patterns of CLL Lymphocytes and Designation of the Components

The two dimensional autoradiographic profiles of the lymphocytes present complex but well resolved patterns, particularly at the higher molecular weight range at which most of the proteins were clearly defined by their distinct HPLC fractionation and SDS-PAGE locations, although some may overlap with each other their peak distributions usually effectively indicate the differences of their identities (Wu and Maddy, 1989). As the proteins of 50-300KD were the focus of our present research interests, therefore, usually the first 15 fractions were analysed while the later fractions were also investigated whenever necessary.

A typical HPLC-SDS PAGE pattern of CLL lymphocyte Triton X-100 extracts is shown in Fig 1. Fig 1 demonstrates the efficacy of the size exclusion process in separating protein mixtures. The overall distribution of the radio-labelled membrane proteins on the SDS-PAGE indicated that the fractionation was based on the apparent physical sizes of molecules or complexes. One of the earliest proteins eluted was a group of proteins with apparent molecular weights of 180-230 KD, which were later identified as CD45 antigen (old name the leucocyte common antigen, LCA). The 230KD band with a retention time of about 8.0 min and the 200KD band of 8.4 min, were progressively resolved and fractionated into different fractions according to their molecular masses. Frequently, a radioactive band of 100KD was also detected in fraction 4-5, suggesting a possible complex form of the band, which was demonstrated by non-reduced samples on SDS gels to have been derived from a larger complex (see below).

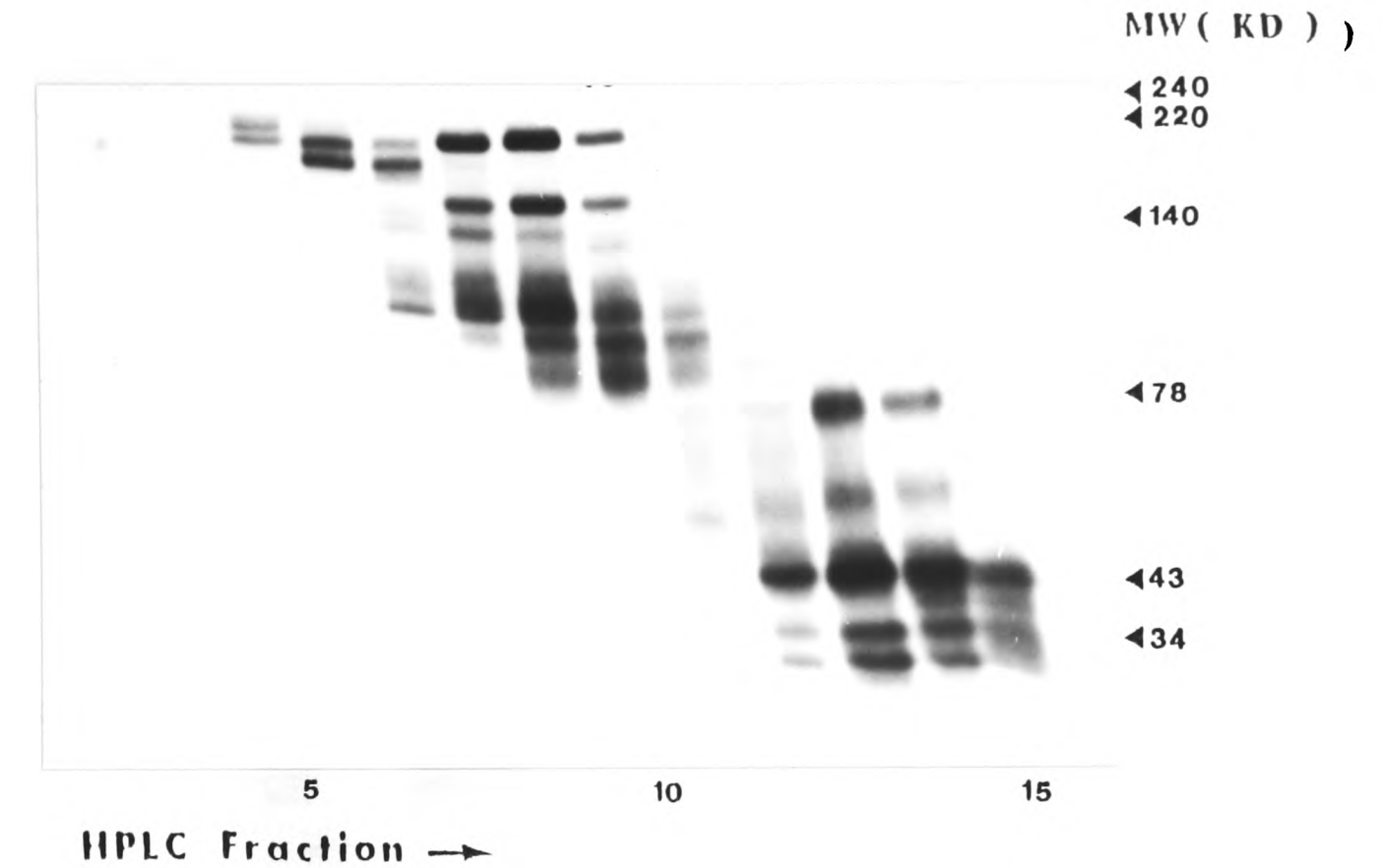
As the matrix of a whole cell extract is usually complicated, a matched diagram can help to elucidate the complexity (see following chapters, and Wu and Maddy, 1988 and 1989). A binomial labeling system was introduced to name the HPLC-SDS PAGE two-dimensional matrix of the autoradiography as shown by the schematic presentation of the fluorography. The first arabic figure stands for the sequential position of a protein from high molecular weight down to low molecular weight on SDS-PAGEs while the second indicates the HPLC fraction mumber. In order to avoid

confusion caused by naming some minor bands, which were gradually identified as the analysis extended, under the same system (see below), the fraction number is put into a bracket. Thus CD45 can be designated as band 1, the 230KD band of the antigen in fraction No. 5 can be labeled band 1(5) and the 200KD component band 1(7). The second arabic figure does not precisely define the exact fraction of a band because the majority of the bands occupy more than one fraction. Nine major bands have been defined in this way but minor bands will be quoted by using a dot following the first figure according to their relative positions, such as band 7.1 while band 7.1(15) indicates band 7.1 in fraction 15. This system will be quoted in the following chapters although standard nomenclatures will be used for proteins of known identities.

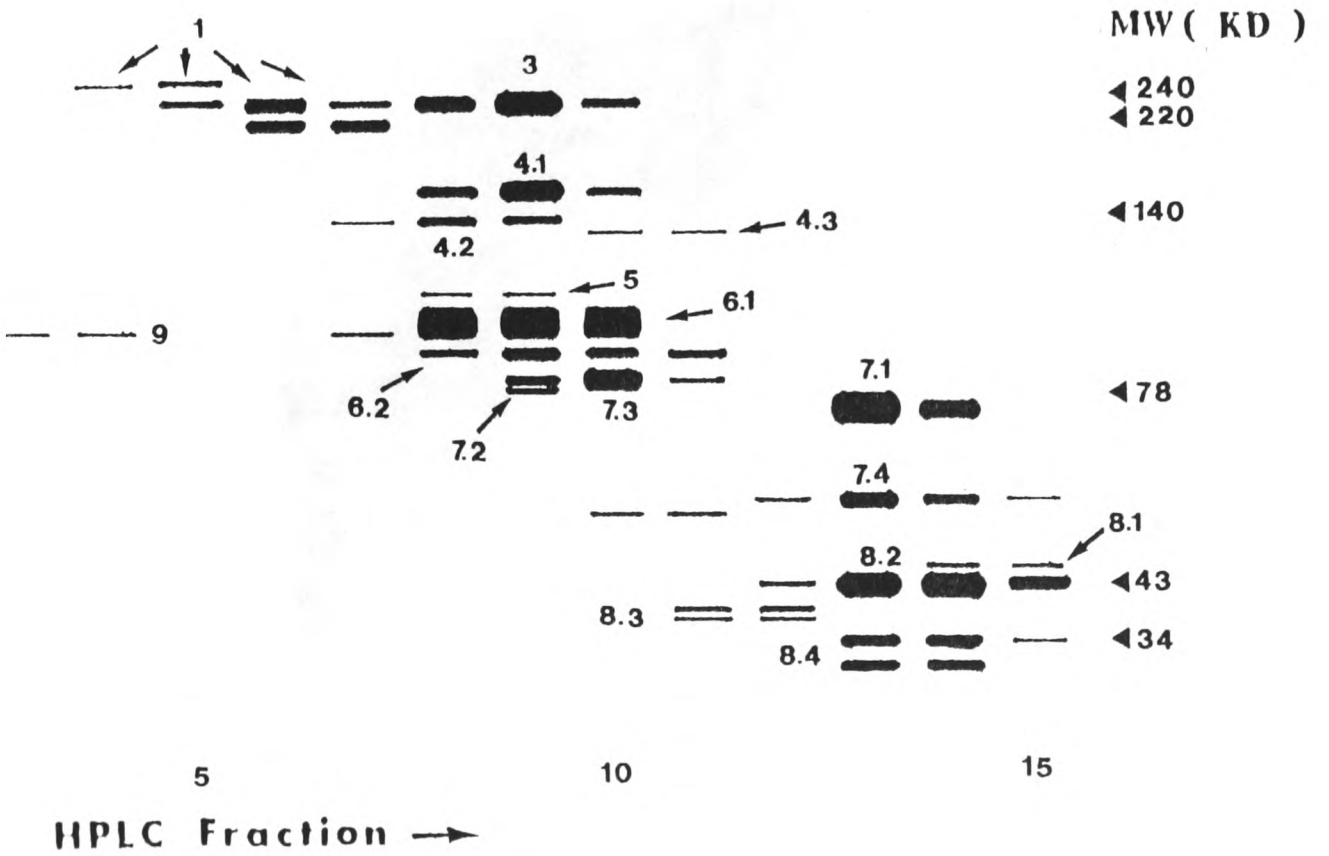
In fraction 9-12, a strongly iodinated band of 220KD was observed in all CLL lymphocytes, normal tonsilar B cells and PLL. This band is named band 3. Below band 3 are a pair of bands which were found in certain patients with variable levels of expression which will be discussed in **Chapter 7**. The lower band has been identified to be CD21 by a monoclonal antibody specifically against CD21 cluster (see **Chapter 6**). The higher band of the pair, having a molecular weight of 150KD, is designated as band 4.1 while CD21 is, alternatively, called band 4.2. It was found and confirmed, by non-reduced samples on SDS gels, that there is a sharp fine band, though having longer retardation on column, comigrating with CD21 on SDS PAGE. This band is numbered 4.3.

The area around 100KD is complex and similar in most of the cells examined. A strong diffuse band was present in all CLLs, PLL and normal tonsilar B cells. This band is named band 6.1. Other experiments showed that this band contains at least two polypeptides, one of them is gpL115 (CD43) (Remold-O'Donnell et.al., 1984), named here as band 6.2. Just below band 6.2 there is one, or maybe more than one, band(s) which has been demonstrated to be the surface Ig (IgD or IgM or both) heavy chain. Sometimes it becomes apparent that the upper band of the pair consists two bands with distinct retardations on the column. The band with slightly shorter HPLC retention was found to have different features from the H chain of the sIg and is assigned band 7.2 while sIg as band 7.3.

Fig 1. (a) A typical HPLC-SDS PAGE two-dimensional matrix (the first 15 fractions of the cllection) of ^{125}I radiolabelled B-CLL membrane proteins. (b) the schematic presentation of the fluorograph. In some patients, there is a high molecular weight component of 300KD above band 1, as shown in **Chapter 6**. The details of the matrix are described in the text.


MW (KD)
240
220
140
78
43
34
5
10
15
HPLC Fraction →

a


MW (KD)
240
220
140
78
43
34
1
3
4.1
4.2
4.3
5
6.1
9
6.2
7.2
7.3
7.1
7.4
8.1
8.2
8.3
8.4
5
10
15
HPLC Fraction →

b

Fig 1

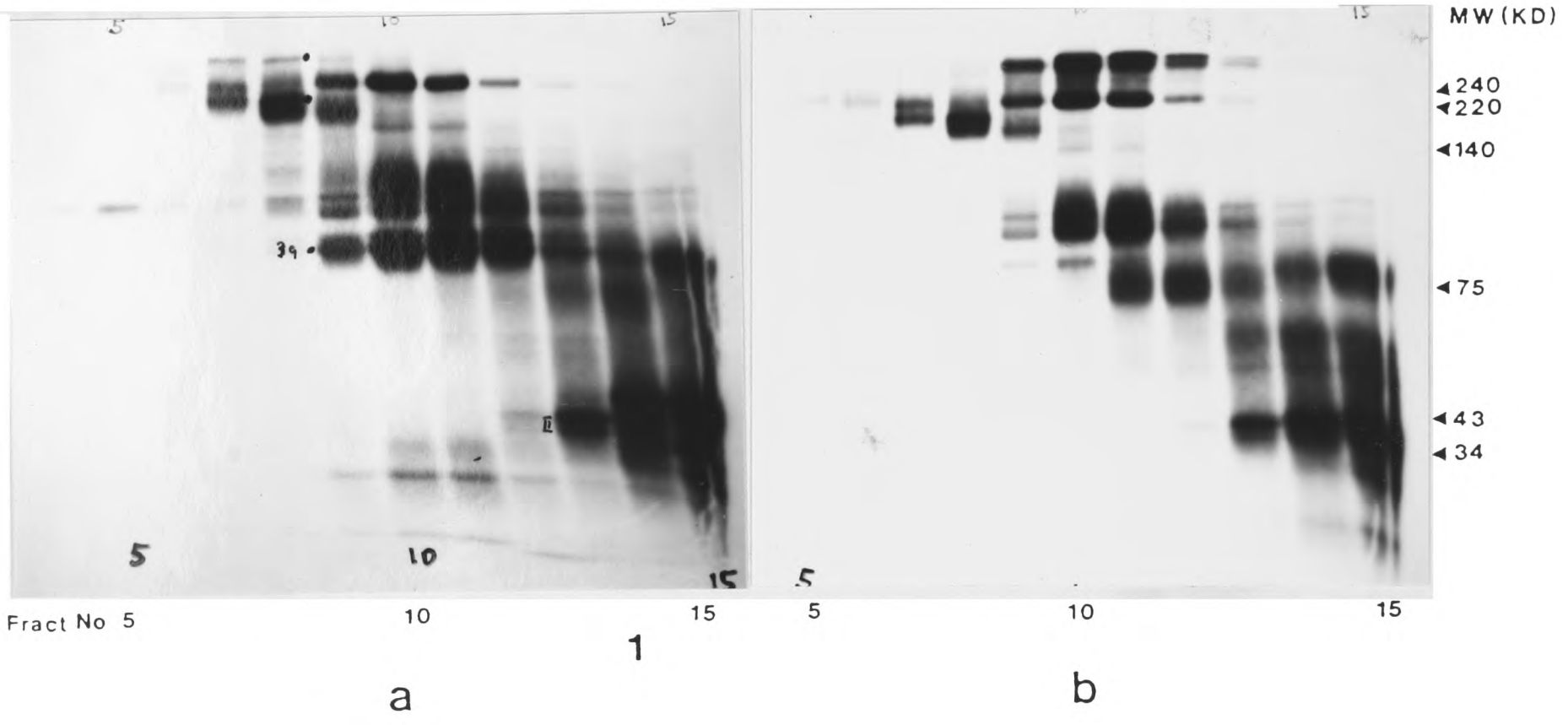


Fig 2

Fig 2. The comparison of membrane protein profiles upon reduction and non-reduction of the HPLC fractions before SDS-PAGE analysis. (a) reduced (b) not reduced. Details are described in the text.

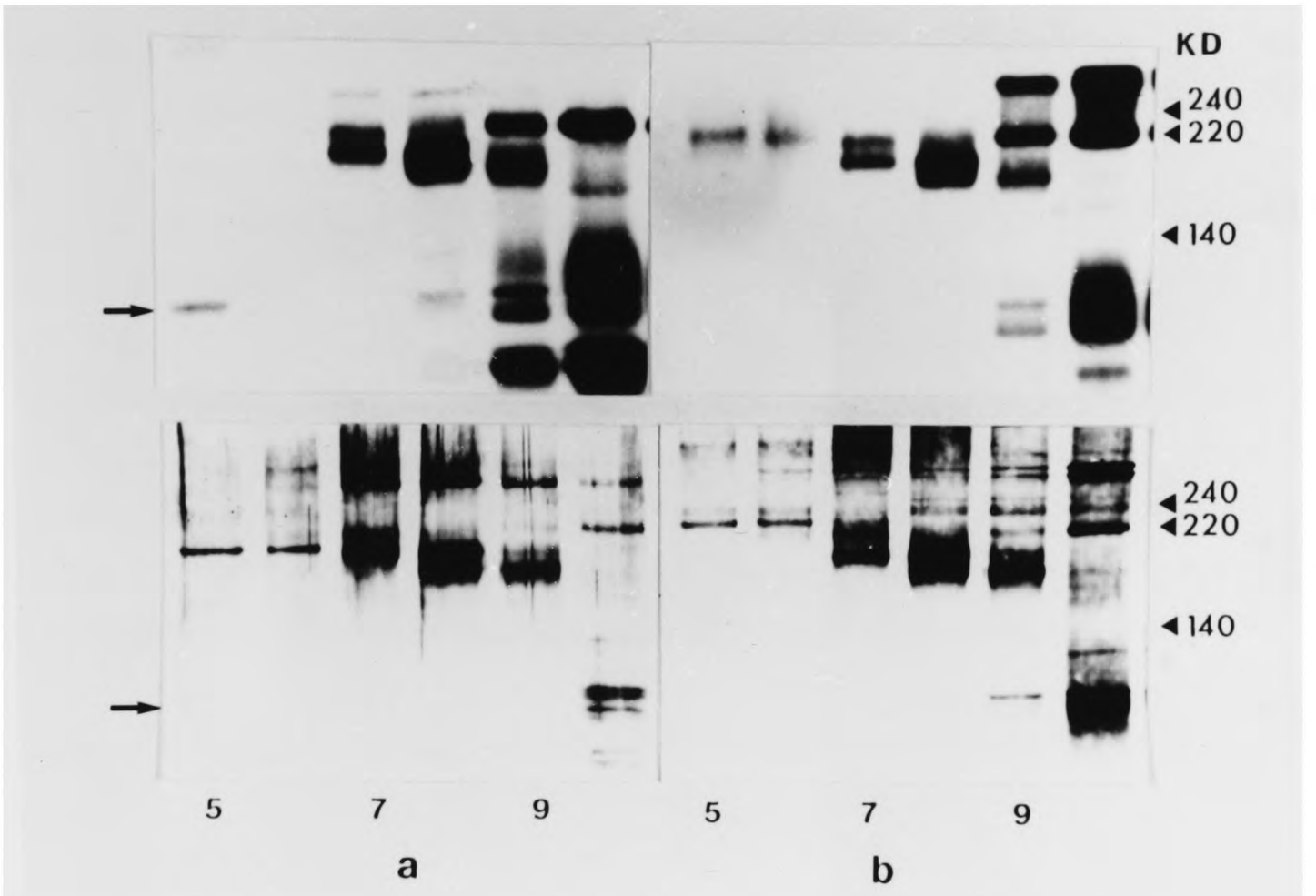


Fig 3

Fig 3. Demonstration of the covalent association between band 9 and an unnumbered component of 200KD. The 200KD component is not ^{125}I radiolabelled but stained by silver while band 9 iodinated but not silver-stained. (a) with reduction (b) without reduction. The upper panel is the fluorography and the lower panel is the silver-stained patterns. The figure shows that, without reduction, band 9 complexes with the silver-stained 200KD band, having a complexed MW of 220KD which can be revealed by both silver staining and fluorography.

In fraction 13-15, most of the bands were in the molecular weight range of 80-20KD. A strongly iodinated band was found in fraction 14-16 with an apparent molecular mass of 78KD which is designated as band 7.1. The next band of 45KD is the α chain of the Class I MHC antigen (see **Chapter 6**) numbered band 8.1 while the $ß_2$-microglobulin can be recovered in the later fractions with longer retention. Two 38KD bands were also found in the fraction 14-16, designated band 8.3. A pair of bands (band 8.4) have been identified as the α and ß chains of the Class II MHC antigen with apparent molecular weights of 35 and 28KD, respectively.

Fig 1b shows the whole designation of the iodinated surface polypeptide bands on the HPLC-SDS PAGE two-dimensional matrix. However, caution must be taken as the actual pattern can be more complicated and delicate, thus gives an oversimplified picture of the complexity of the surface membrane protein composition. Many other minor components can be detected but difficult to define and name.

3.2. Reduction, Non-reduction of the HPLC Fractions and Pre-reduction of Sample Extracts

SDS gel electrophoresis of the HPLC fractions without reduction yielded markedly different patterns. Fig 2 shows the comparison of the same fractions either reduced with 50 mM DTT or not reduced prior to the SDS-PAGE electrophoresis. The polypeptides affected by SDS-PAGE without the reduction of samples were:

(1). band 4.2, which has been identified to be CD21 by monoclonal antibody, reduced its molecular weight from 140KD to 120KD under non-reduction treatment of the fractions before SDS-PAGE.

(2). There are at least three bands in the band 7.2, 7.3 group, they have very similar retentions on the column and very close mobilities on SDS-PAGE gels. The upper band of 78KD and the lower band of 75KD, band 7.3(9-11), could both be recovered at 340KD and 320KD, respectively, without reduction before electrophoresis. With both the 78KD and 75KD components retarded to the top of the gel, a 22KD component disappeared simultaneously from the same fractions. The molecular masses of these bands and their behaviour upon reduction suggest that they are membrane

immunoglobulins, the higher molecular weight bands were the heavy chains of either sIgD (75KD) or sIgM (78KD) or both while the 22KD component was the light chain. In the meantime, Band 7.2 were demonstrated to be distinct components consisting of two bands of 75KD and 77KD, respectively, which were not affected by reduction before SDS-PAGE.

(3). In the same fractions of that of sIg (fraction 9-11), a new component was revealed in the position where the sIgD or sIgM heavy chains shifted up when the fractions not reduced. This diffuse band has an apparent molecular weight of 70-76KD on 6-11.5% SDS-PAGE (Fig 4).

(4). Running samples without reduction could reduce the molecular weight of the Class I MHC α chain by about 4KD. But no visible change was found on the ß chain.

(5). Class II MHC ß chain was greatly affected by non-reduction which brought its molecular weight down to about 25KD.

(6). Under the non-reduced conditions, band 9 of 100KD was retarded to the position of the second band of the CD45 antigen, having a molecular weight of 220KD, which suggests that other component(s) must be associated with the band *via* disulfide bonds. This point was proven by silver stained gels (Fig 3). The gel shows that the band 9 was not stained by silver while a 200KD component in the same fractions, not iodinatable, could be silver-stained. When non-reduced samples were analysed by SDS-PAGE, band 9, together with the 200KD component, was shifted up to 220KD which was detectable by both the silver-staining and the autoradiographs. In those patients who do not possess band 9, no such changes have been observed.

3.3 Two-column Combination

As expected, combining two columns should improve separation of the polypeptide mixture and help to understand the interactions between proteins. Two GF-450 columns resulted in higher resolution than GF450-GF250, particularly for the components of high molecular mass (Fig 5). The autoradiography of the 2 x GF-450

separation of the Triton X-100 extracts is shown in Fig 5. It is seen that the polypeptides of the CD45 family have been clearly separated from other components. Band 9, for some reasons, co-migrates with the earliest fractions of the CD45 polypeptides, ie. 230KD band, in a similar fashion as in one column chromatography. Thus, the separation of the band 9 from the CD45, proportionally, was much less than other polypeptides.

A sharp, fine band (band 4.3) co-migrating with CD21 (band 4.2) but separated from other components on SDS-PAGE became apparent under the two-column combination, demonstrating its distinct location with an apparent molecular weight of 135KD.

Band 7.2(8-9) (in one column system) was revealed in fracton 17-19 when the heavy chains of sIg were retarded and fractionated into fraction 20-24. This band was shown not to be affected by non-reduction on SDS-PAGE.

No separation was observed for the group of proteins of band 7.1, 7.4, Class I and Class II MHC. The relative retention times of this group of polypeptides were not affected except band 8.3(13-14) which was separated from the group.

It has been noticed that the retention times of the eluted polypeptides in two-column combination were less than doubled retention times of a single column.

3.4. Effect of Triton X-100 on Retention Time

Although the presence of Triton X-100 of up to 1% has no effect on the retardation of the standard proteins, different detergent concentrations resulted in different retention patterns of cell extracts. Fig 6 demonstrates the influence of three Triton X-100 concentrations from 0.1, 0.5 to 1%. There was not much change in those early fractions (big molecules) whilest the retardation of the small molecules was shortened by higher detergent. From the figure it is apparent that the Triton concentration had a bigger effect on the smaller molecules. During the process of chromatography under different detergent concentrations, no significant change of the operating pressure was ever observed.

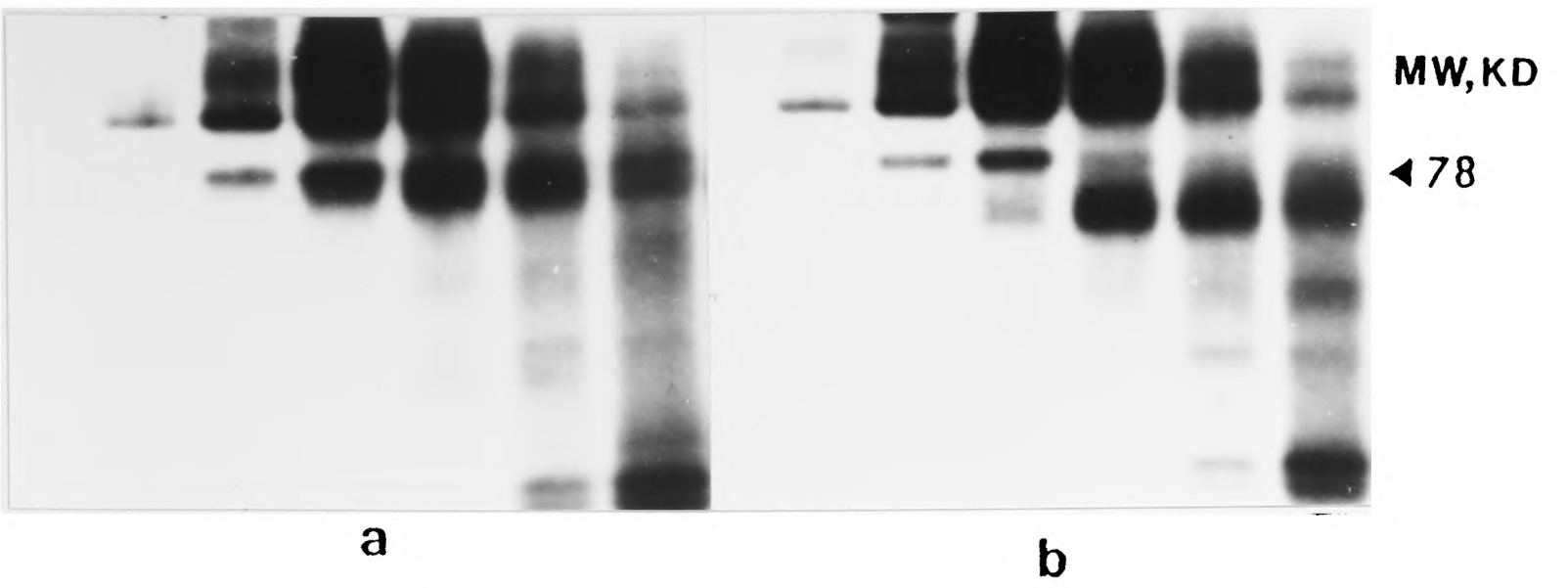


Fig 4

Fig 4. Demonstration of a 78KD and a 75KD components by SDS-PAGE analysis of non-reduced samples on a 6-11.5% polyacrylamide gradient gel. (a) reduced (control) (b) not reduced. Note the change of their molecular weights.

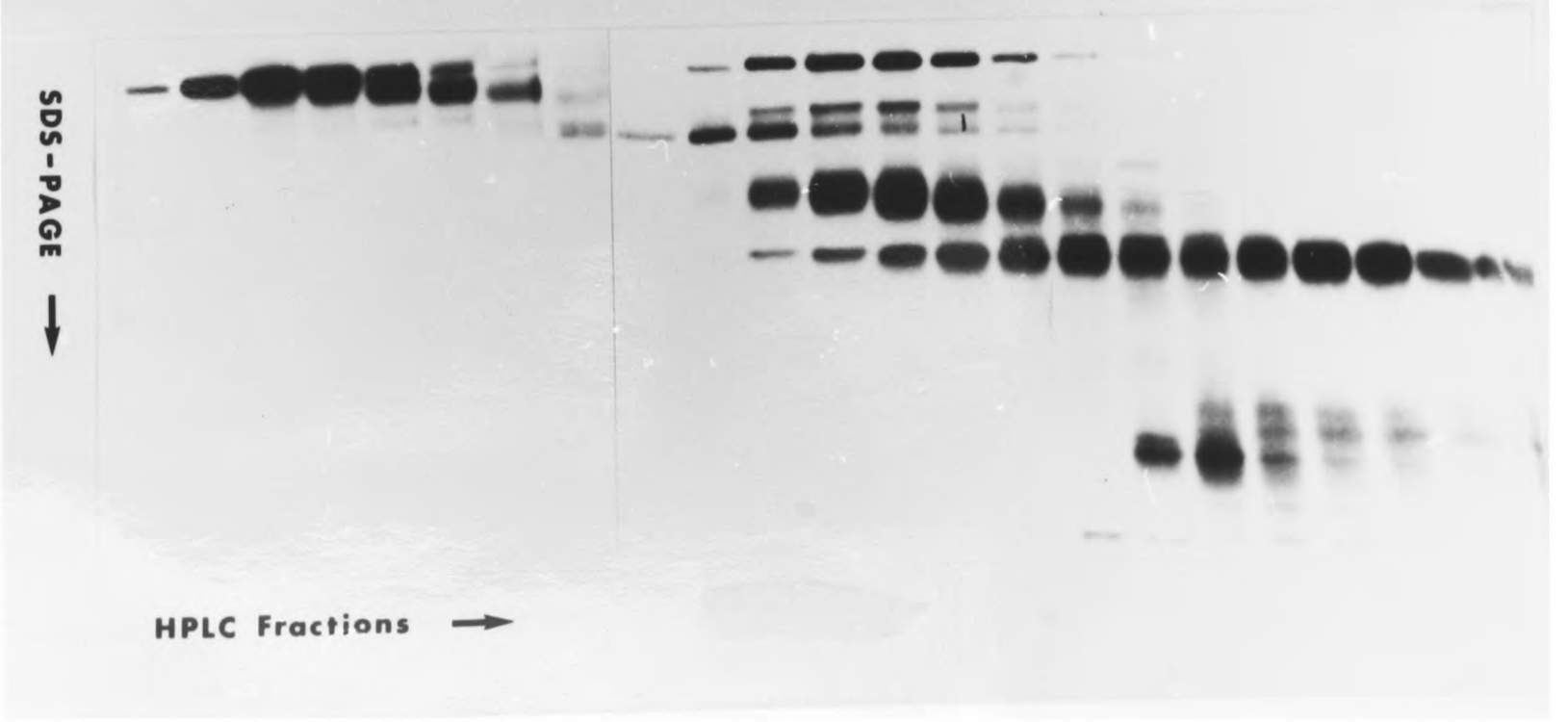


Fig 5

Fig 5. A HPLC-SDS PAGE profile of B-CLL extract fractionated by a two-column (2 x GF-450) combination, showing the complete separation of the CD45 antigen from other surface proteins and revealing the overlapping of some bands on an one-column system.

Fig 6. The effects of Triton X-100 concentration on the retention of membrane proteins. Proteins extracted with three different TX-100 concentrations were eluted with 0.2 M phosphate buffer pH 7.4, containing corresponding TX-100 concentrations (a) 0.1% (b) 0.5% (c) 1.0%. There is a general tendency for the retention times to be shortened, particularly for the molecules of lower molecular weights.

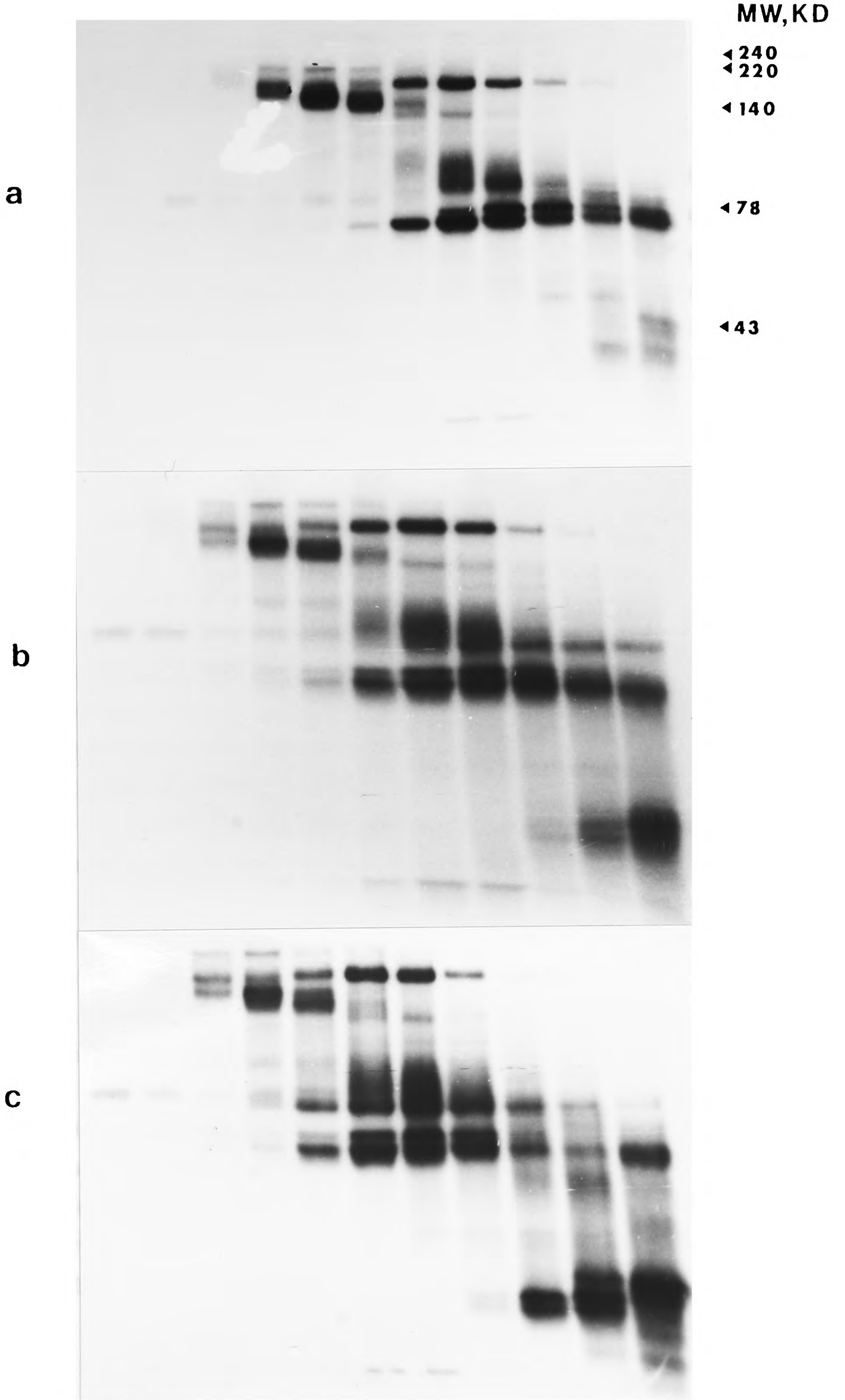


Fig 6

4. Discussion

4.1. Reduction and Non-reduction

The results presented above suggest that few CLL lymphocytic membrane polypeptides interact with each other *via* disulfide bondings. On the HPLC-SDS PAGE matrix, only two membrane proteins have been detected to associate in this way. Band 9 increased its apparent molecular weight from 100KD when reduced to 220KD when not reduced, indicating that the 220KD form is a disulfide-bound complex. Further experiment revealed that the 100KD iodinated polypeptide was actually associated with a 200KD component which is not surface iodinated but distinctively stained by silver (Fig 3). The fact indicates that (1) this 200KD component is probably located at the cytoplasm and (2) that the 100KD polypeptide may contain high intramolecular disulfide bonds because of its much greater change of the mobility on SDS-PAGE with or without reduction. Band 9 is consistently observed in certain patients, which helps to rule out the artifact possibility. Another surface molecule which has been proven, by reduction and non-reduction, to be intermolecularly bonded is sIg. Their apparent molecular weight without reduction appears to be that of a dimer. However, other researchers (Vitetta et.al., 1971) have identified only monomeric membrane-bound IgM or IgD. Their abnormal mobility on SDS-PAGE could be attributed to irrational behaviour of proteins on non-reduced SDS gels (Segrest and Jackson, 1972; Dunn and Maddy, 1976). But this point still reserves further investigation.

The failure of labeling of the Ig light chains of some patients by iodine although both silver stained patterns and FACS indicated the presence of the light chains is not understood. It could be that the light chain is shaded by steric effect and prevented from access of molecules like catalyst lactoperoxidase.

The HPLC patterns of Class I and Class II MHC under reduction and non-reduction are in agreement with the structural information on the Class I in mouse (Coligan et.al., 1981) and man (Orr et al, 1979) and on the Class II (HLA-DR) ß chain (Kratzin et. al., 1981; Kaufman & Strominger, 1982) and α chain (Kaufman & Strominger, 1982), respectively.

Analysis of HPLC fractions with reduction or non-reduction before SDS-PAGE is certainly a very simple but effective technique for understanding the membrane protein interactions, particularly those *via* disulfide bondings. It is also useful for the identification of some polypeptide bands overlapping with each other on SDS gel when fractions were reduced as exemplified by band 7.2. Moreover the difference of a band's mobility under reduced and non-reduced conditions could be taken as a rough indication of the extent of intrachain disulfide bonding of a polypeptide although caution must be taken as the numbers of amino acids between two cysteins can greatly affect the mobility.

Great dilution of DTT in protein extraction medium can result in reforming of disulfide bonds and impurities in the detergent could also be responsible for this phenomenon. Therefore, pre-reduced sample subjected to chromatography should be eluted in a medium containing equivalent amount of DTT.

4.2. Effect of Non-ionic Detergent on Retention

The effect of non-ionic detergent Triton X-100 on the membrane protein retardation during chromatography is different from the standard proteins described in **Chapter 4**. Generally speaking, all detergent-bound membrane proteins were eluted earlier with respect to the calibration curve constructed from the standard proteins. That membrane proteins possess certain portion of non-polar segments which could bind Triton X-100 may account for the phenomenon. The observation that small molecules were more affected by the presence of the detergent is well in line with this assumption. Another feature which is likely to contribute to the shorter retardation of the membrane proteins is their heavy glycosylation. The enormous protruding of sugar residues could greatly enlarge gyration radii of a protein, hence exaggerating the actual molecular size. A simple experiment can be designed to test this hypothesis by cleavage of the sugar structures.

In conjunction with the previous study, 0.5% Triton X-100 is recommended for routine use.

4.3. Two-column Combination

Theoretical plates are proportional to the effective length of a column (Yau et.al., 1979). Elongation by combining columns is an efficacious and economic way of achieving higher resolution as shown by two tandemly connected GF-450s. It is helpful for the separation of complex mixtures and the understanding of membrane protein interactions which are hard to achieve on one-column system. Because molecular interactions are usually not disrupted by the process of chromatography, unproportional retardation of the components by column combination may indicate such interactions.

In conclusion, one GF-450 column is able to separate membrane proteins effectively, the method is particularly useful for investigations which do not require total separation of individual proteins. The two-dimensional profiles provide a comprehensive view of the membrane protein constituents and their relationships as well as the relative levels of their expressions. The combination of HPLC and SDS gels exploits a new approach in protein separation and characterization.

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CHAPTER 6. IDENTIFICATION OF LYMPHOCYTIC SURFACE ANTIGENS BY HPLC-SDS PAGE

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CHAPTER 6. IDENTIFICATION OF LYMPHOCYTIC SURFACE ANTIGENS BY HPLC-SDS PAGE

1. Introduction

HPLC size exclusion chromatography, which separates the membrane components according to their molecular sizes, constructs the first dimension of the matrix, by the size distribution of the membrane proteins as either simple or complexed forms. The subsequent SDS-PAGE analysis forms the second dimension, showing the individual polypeptides of the disintegrated proteins and complexes, again in order of their sizes (Wu and Maddy, 1988).

Several proteins have been identified, either by their molecular weights on the matrix or by other characteristics revealed by certain analytical conditions as discussed in Chapter 5. However, a more definitive understanding of the patterns is possible by the use of appropriate antibodies. In principle, when an antigen is complexed with its antibody it should be eluted earlier than it is in its free state because of the enlarged molecular weight of the complex. A monoclonal antibody should only form a simple 1:1 binary complex with its antigen, therefore, the retention of the complex should be predictable. If a second antibody, either against the first antibody or different epitopes of the antigen, is used, an even larger complex will be formed and a shorter retention be expected (Wu and Maddy, 1989). In this study, the combined molecular weight of the complex (antigen-1st antibody-2nd antibody) would be no more than 600KD even for the largest antigen (see discussion below), well within the total exclusion limit of GF-450. Using the method, the identities of several components of the matrix have been elucidated and confirmed.

The protocol introduced here provides not only a method of antigen identification but also an approach for separating antigens.

2. Materials and Methods

CLL B lymphocytes were prepared as described in **Chapter 2**. The B cell viability and T cell contamination were monitored as in **Chapter 2**.

2.1. Antibody Treatment of Cell Extract

Monoclonal antibodies were added to 0.5% Triton X-100 extracts of surface-iodinated B-CLL cells. The quantity of the antibody was added to the saturation concentration based on the flow cytometric titration. Routinely 150 ul extract, derived from $2\text{-}3\text{x}10^7$ cells, was adequate for each treatment. The mixture was incubated on ice for 45 min before HPLC. A cell extract treated under parallel conditions without the addition of antibody was used as a control. For most of the antigen-antibody complexes, the standard HPLC operating conditions and collecting mode were found to be adequate.

2.2. Antibody Treatment of Intact Cells

Cells were processed and ^{125}I labelled as stated (**Chapter 2**). About 10^8 surface-radiolabelled cells were washed once with FACS medium (RPMI1640 containing 2% FCS and 0.1% NaN_3) and suspended in this medium at $4\text{-}5\text{x}10^7$/ml cells. Monoclonal antibodies were added to the suspension to the saturation concentration determined by flow cytometry. The suspension was then incubated on ice for 45 min with two inversions. Then the cells were pelleted, washed twice with RBC buffer pH 7.4 to remove unbound antibodies and extracted into 0.5% Triton X-100 medium.

2.3. Addition of a Second Step Antibody

10 ul rabbit anti-mouse Ig (3.7 ug) (Dakopatts, lot 056) was added to 100 ul Triton X-100 extract. The mixture was mixed gently and incubated on ice for 45 min. Direct Triton X-100 extract of cells treated with only the second antibody was employed as a control. In the HPLC investigation of the samples, the collecting parameters were as follow:

Delay: 4.0 min

Fraction Time: 0.2 min

Collect: 36 fractions

2.4. The following monoclonal antibodies were employed in this study (Table 1):

Table 1.

CD Cluster	Antibody	Specificity (KD)	Source
CD45RB	PD7/26	230,215,200	Dr.D.Y.Mason
CD45RA	F8-11-13	230,215	Dr.J.W.Fabre
CD45RO	UCHL-1	180	Dr.P.Beverley
CD44	F10-44-2	80	Dr.J.W.Fabre
CD43	gpL115	115	Dr.R.O'Donnell
CD21	RFB6	140	SAPU
CD22	RFB4	130, 140	SAPU
	EZB52	105	Zool.Ed.
	EZB54	125	Zool.Ed.
	W6/32	45, Class I MHC	Sera-Lab
	DA6/147	35, ClassII MHC	
	DA164		

3. Results

Twelve monoclonal antibodies have been tested in a panel of patients with B-CLL. Some antibodies were tested several times in the same or different individuals. The positive observations were usually further confirmed by at least one more test in different individuals. Eight out of twelve formed complexes identified by the HPLC. The reactivity of the antibodies is tabulated in Table 2.

Table 2.

Antibody	Reactivity	Antigen(Kd)
PD7/26 (CD45B)	+	230,215,200
F8-11-13 (CD45RA)	+	230 (215)
UCHL-1 (CD45RO)	-	180
F10-44-2	+	80
gpL115 (CD43)	+	115-120
RFB6 (CD21)	+	140
RFB4 (CD22)	-	130, 140
EZB52	-	
EZB54	+	125
W6/32	+	45,(12)
DA147	+	35,28
DA164	-	

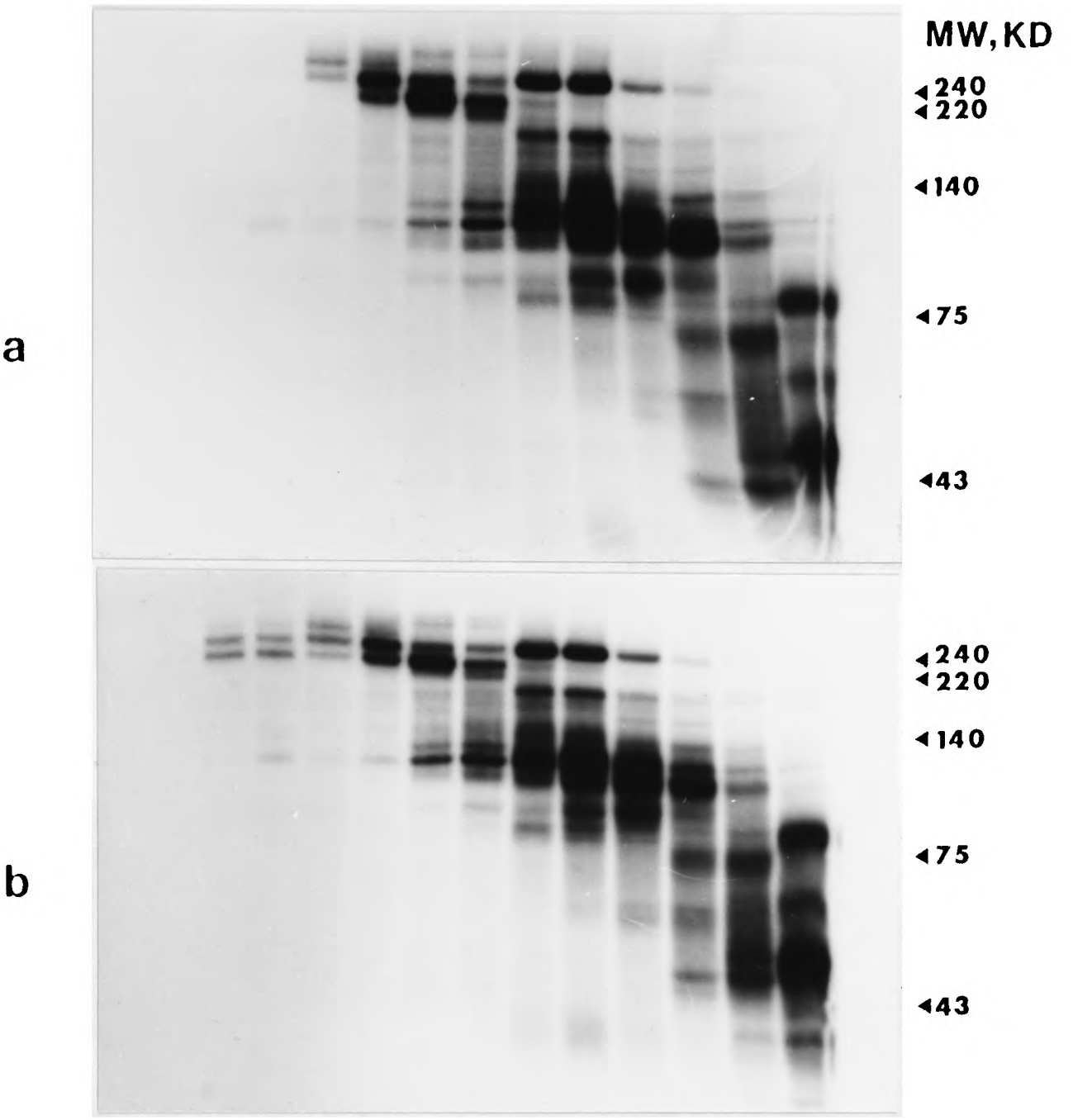


Fig 1

Fig 1. Demonstration of the CD45RB determinant shifted on HPLC by mAb PD7/26 binding. (a) control (b) the TX-100 extract treated with the mAb. Three components from the band 1 group were clearly moved forward for at least 2 fractions (arrowed).

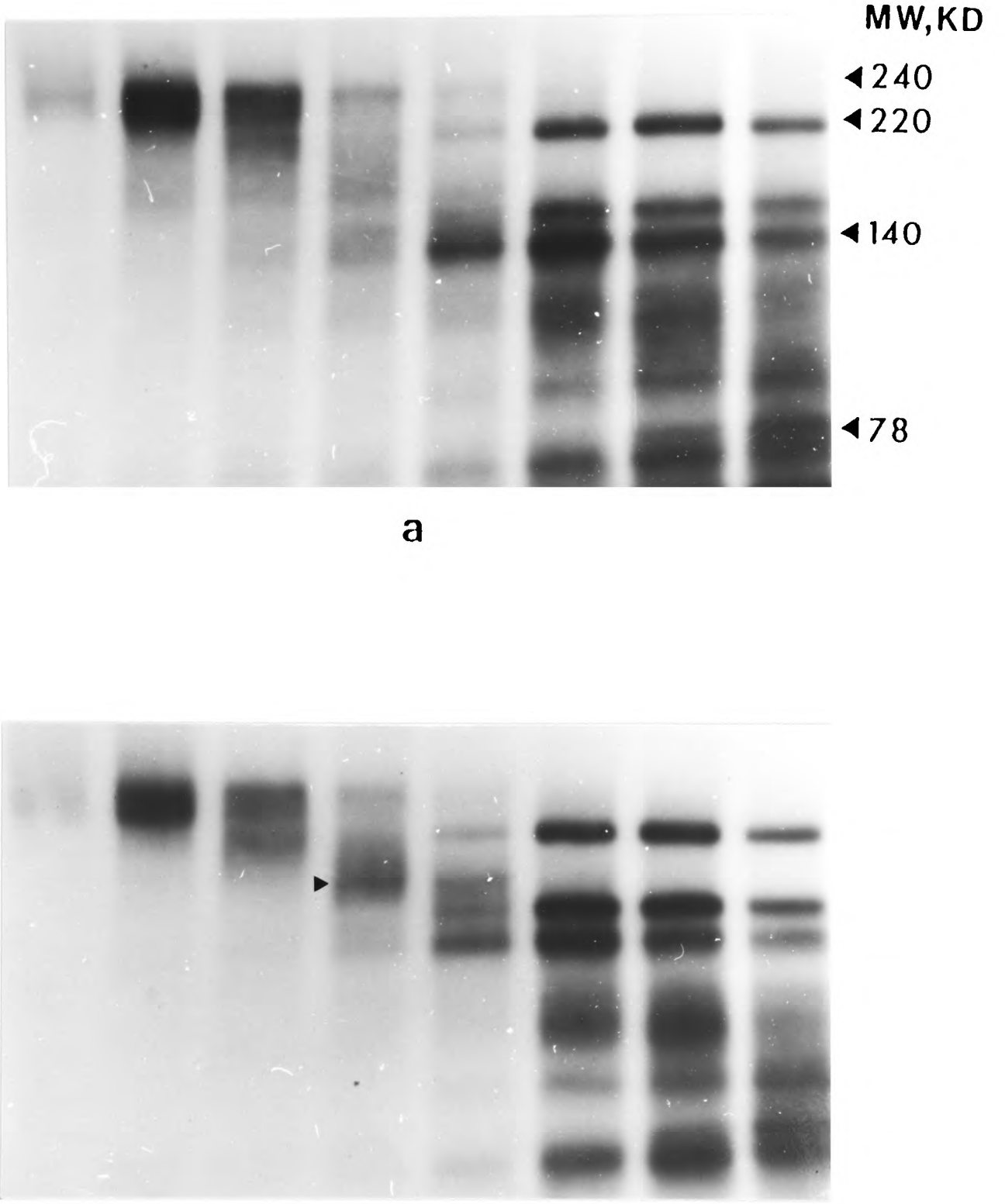
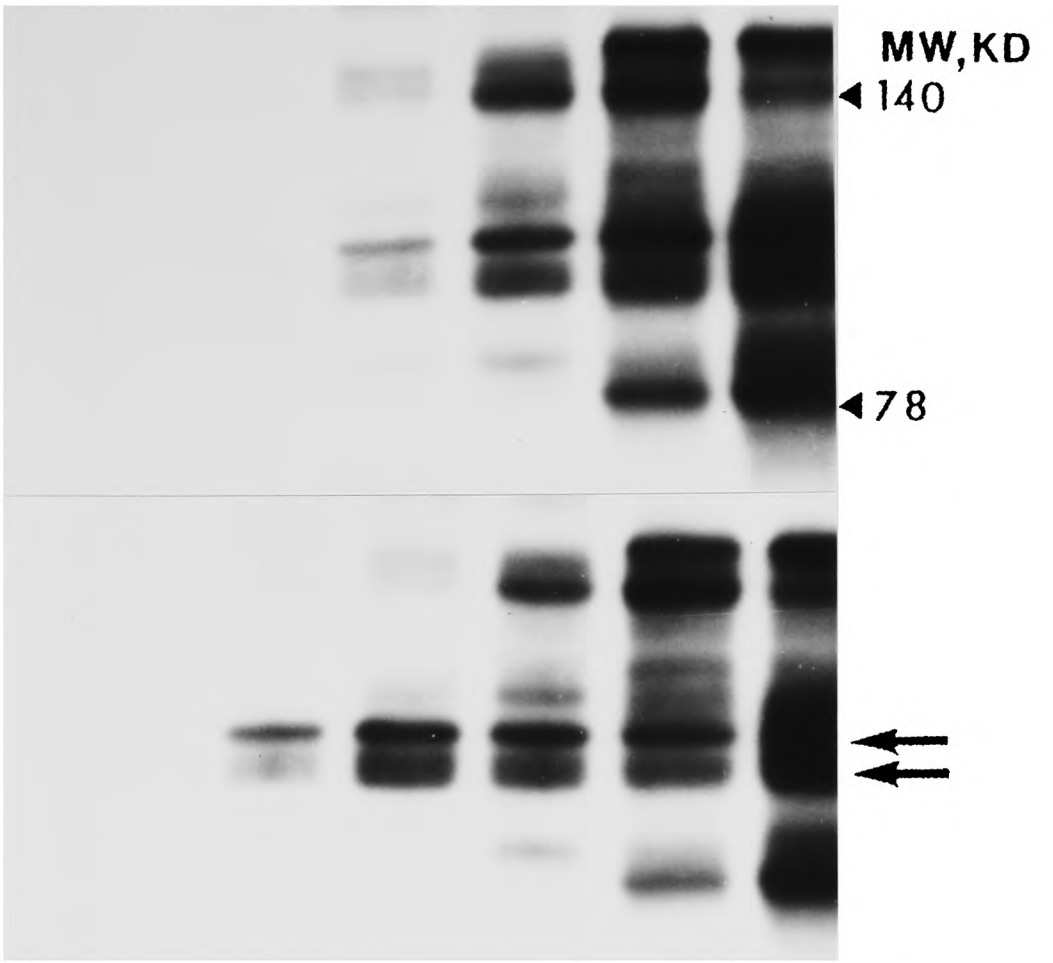


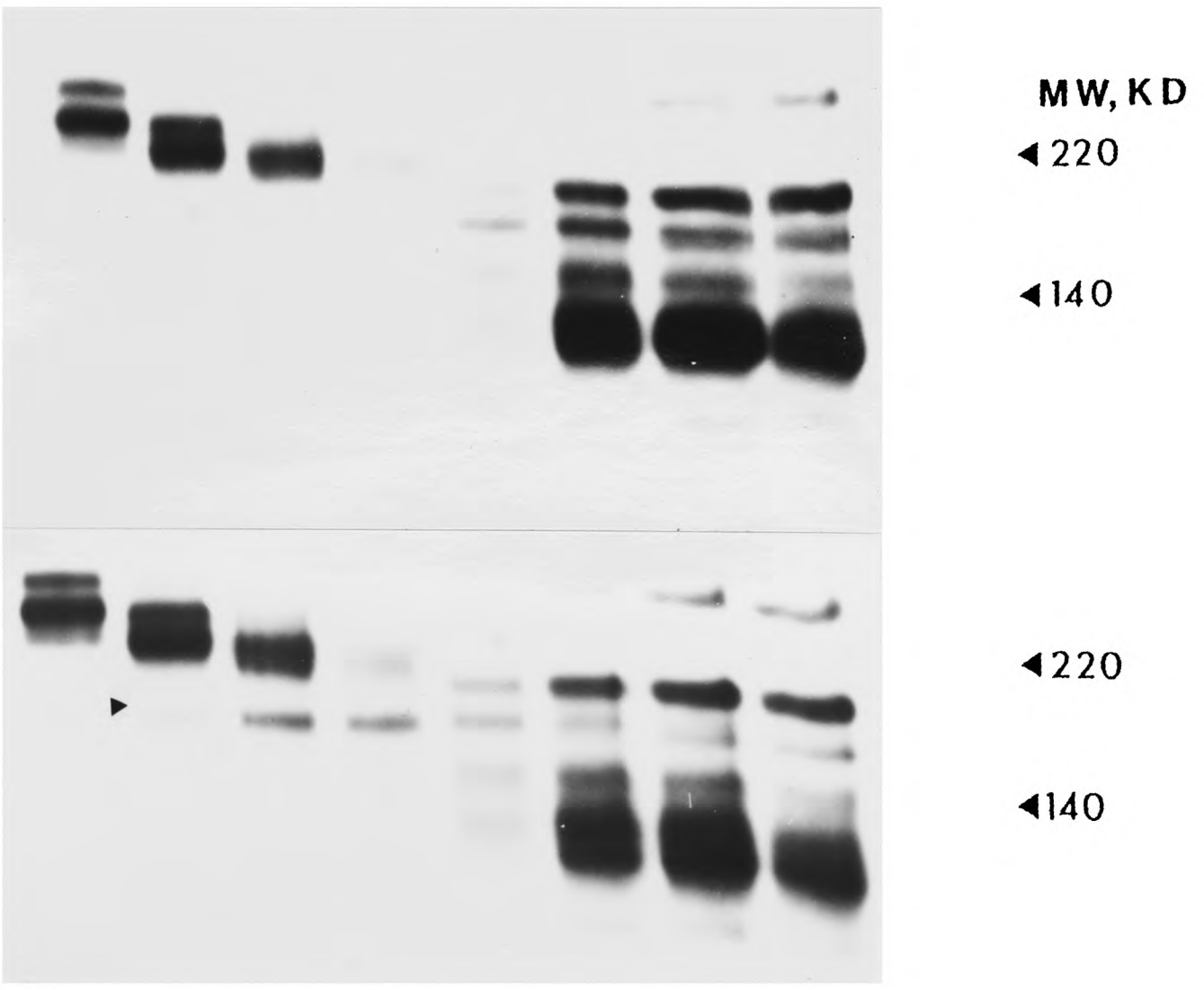
Fig 2

Fig 2. Demonstration of a 180KD band present in tonsilar T lymphocytes rather than B lymphocytes. (a) pure tonsilar B cells, showing a single predominant 230KD chain of CD45 antigen (b) tonsilar B+T mixture, showing the presence of a 180KD component (marked). The proteins were analysed by SDS-PAGE on a 6-11.5% polyacrylamide gradient gel. The fluorograph also shows the enhancement of band 4.1 intensity in B+T mixture.

Fig 3. Identification of CD43 (gpL115), CD21, MHC Class II and EZB54 antigens on HPLC-SDS PAGE matrix of B-CLL cells. (a) CD43 (gpL115), note that two components were shifted together by the antibody (b) CD21 (c) MHC Class II (d) EZB54, 125KD, the antigen can be found in fraction 17-18 when no antibody is added (e) the ^{3}H-labelled CD43 shifted by a monoclonal antibody specifically against CD43. The antigen is marked by an arrow. Note that only one component was shifted in contrast to the iodinated samples where two components were moved. The upper panel fluorographs are the controls. All tests were performed in TX-100 extracts and the samples reduced and separated on 6-11.5% polyacrylamide SDS gel.



a



b

Fig 3

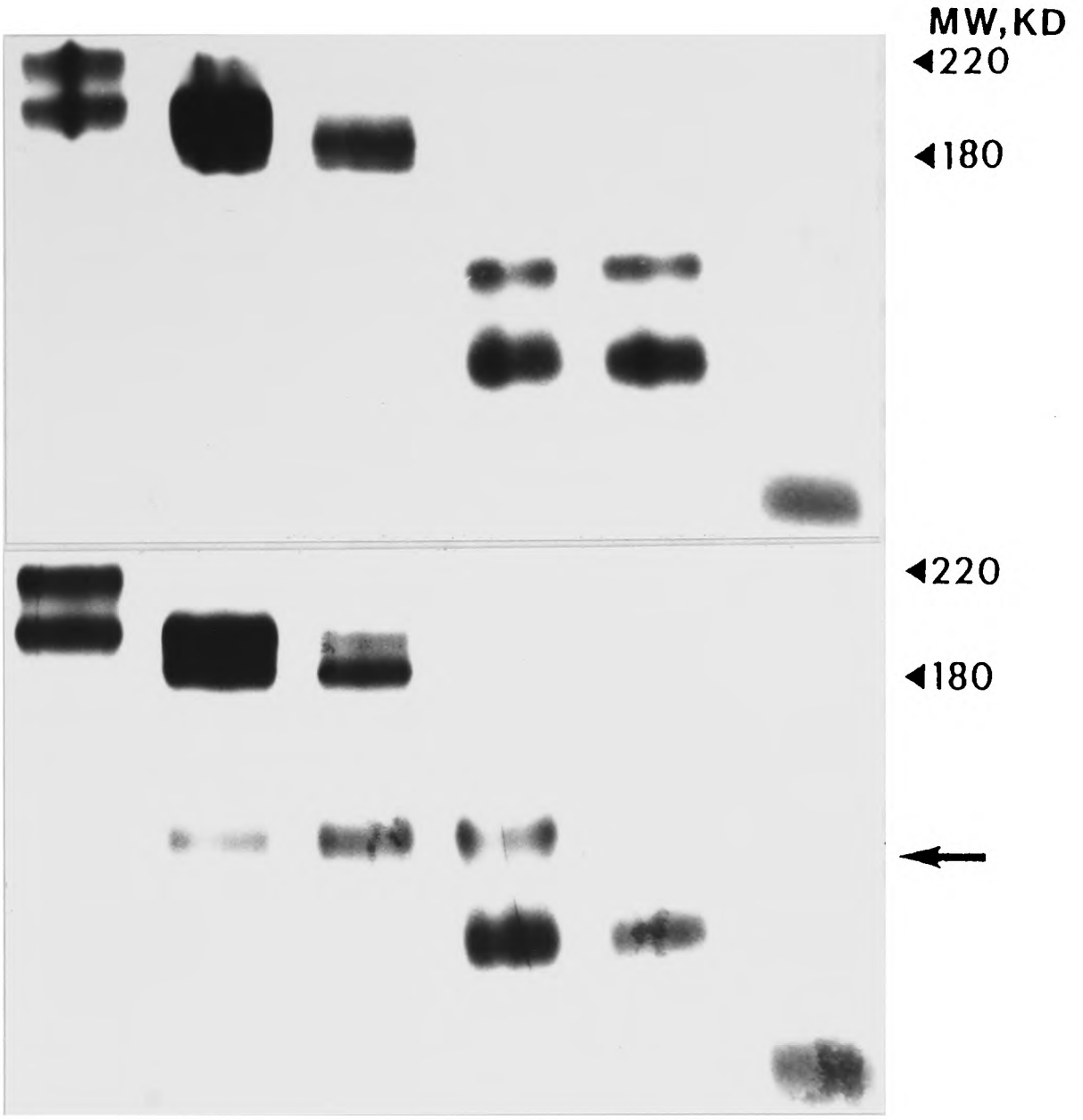


Fig 3e

PD7/26 reacted with three components of the CD45 family rather than four (Okamura et.al. 1982). Using PD7/26, three bands of the CD45 family could be moved forward 3 fractions and clearly separated from other surface proteins (Wu and Maddy, 1989). The apparent molecular weights of the three reactive bands were 230, 215 and 200KD in our 6-11.5% gel system (Wu and Maddy, 1989), respectively. The affinity seemed to be very similar for all three bands with respect to the intensity of the shifted bands (Fig 1b), which were proportional to their original intensity. The light chain of the antibody, which can be silver stained, was found in the same fractions of the shifted components on the SDS-PAGE gels of the reduced samples although CD45 antigen itself is poorly stained by silver. Carefully checking of the silver stained gels and the autoradiographs showed that no other bands moved concomittantly with the shifted CD45 antigen.

Monoclonal antibody F8-11-13 has been described in detail (Dalchau and Fabre, 1981), and reacts preferentially with a determinant on 2 higher forms of CD45 antigen encoded for by exon A. By using mAb F8-11-13, a single band at 230 KD was clearly shifted for two fractions, no other bands have been observed in the same fractions. However, prolonged exposure revealed a component of about 215KD with a small amount of radioactivity.

There was a diffuse band at 180KD , gradually eluted after the 200KD component of the CD45 family. This band could be UCHL1 with respect to its molecular weight although one test with UCHL1 antibody failed to confirm this. In order to elucidate whether this 180KD component is expressed by normal T cells, pure tonsilar B cells, B and T cell mixture were analysed on HPLC and it was found that normal B lymphocytes (E-rosetting negative) did not possess this 180KD component while the mixture of B and T cells showed a band of 180KD in addition to a 230KD and a 200KD bands (Fig 2). This 180KD component can be clearly revealed by neuraminidase-treated tritiated B-CLL samples on the HPLC.

F10-44-2 reacts with a component of 80KD (Dalchau et.al., 1980), now defined as CDw44 cluster which was co-eluted with and resolved by SDS-PAGE (gradient 6-11.5%). with slightly higher MW than the heavy chain of IgM. The antigen was

moved forward by the antibody about 2 fractions on HPLC. It was shown that a 80KD band of the same fractions could be affected by non-reduction prior to electrophoresis (see **Chapter 5**), reducing its molecular weight to about 70KD but previous report showed that the mobility of CDw44 antigen on SDS-PAGE was variable upon reduction and non-reduction (Stoll et.al., 1989). It is not certain whether this band and the F10-44-2 antigen are identical molecules. The HPLC-SDS PAGE profiles of the F10-44-2-treated samples demonstrated that (1) the band 7.1 was not CDw44 because it does not react with F10-44-2 (2) band 7.3 consists, at least, two polypeptides, one is the heavy chain of sIg and another CDw44 which overlaps with sIg.

Remold-O'Donnell et al. (1984) reported a monoclonal antibody gpL115, now assigned as CD43, to identify the Leucocyte Sialoglycoprotein (LSG). The antibody precipitated a glycoprotein with an apparent molecular weight of 115KD without neuraminidase treatment and 150KD with neuraminidase treatment. Our test with gpL115 antibody on the surface-iodinated cell extracts resulted in a two-fraction shift of a 115KD and a 100KD polypeptides which correspond to band 6.1 and 6.2, respectively, on HPLC-SDS PAGE matrix (Fig 3a). The 115KD component is likely to be the CD43 antigen as normal tonsilar B cells and PLL do not possess this band. The discret distribution of band 6.1 following gpL115 mAb binding suggests that band 6.1 may consist of more than one component. This component has been found present in the majority of the patients. CD43 antigen was also investigated on tritiated samples by HPLC-SDS PAGE and again shown to bind a molecule of 150KD following neuraminidase treatment (Fig **3e**).

The antibodies defining the CD21 cluster precipitate a protein of 140KD. The molecule is a single chain and reduces its apparent molecular weight under non-reduction on SDS-PAGE (Lida et.al., 1983). Band 4.2 has similar properties and its identity was confirmed by applying the mAb RFB6 (SAPU, code 054-201) in the HPLC chromatography. The antibody was able to move nearly all of the band 4.2 away from the fractions where the antigen eluted when no antibody was applied (Fig 3b). Band 4.3 was not affected by the antibody at all. Thus band 4.2 consists solely of CD21 and band 4.3 is not part of CD21 as was also concluded from the reduced- and non-reduced samples by SDS-PAGE analysis (**Chapter 5**).

Fig 4. Separation of MHC Class I molecules by applying a second antibody to the mAb-Ag complex. (a) control, the MHC Class I antigen is fractionated in the later fractions not included here (b) W6/32 to intact cells (c) mouse anti-rabbit IgG to the TX-100 extract of the W6/32-treated cells.

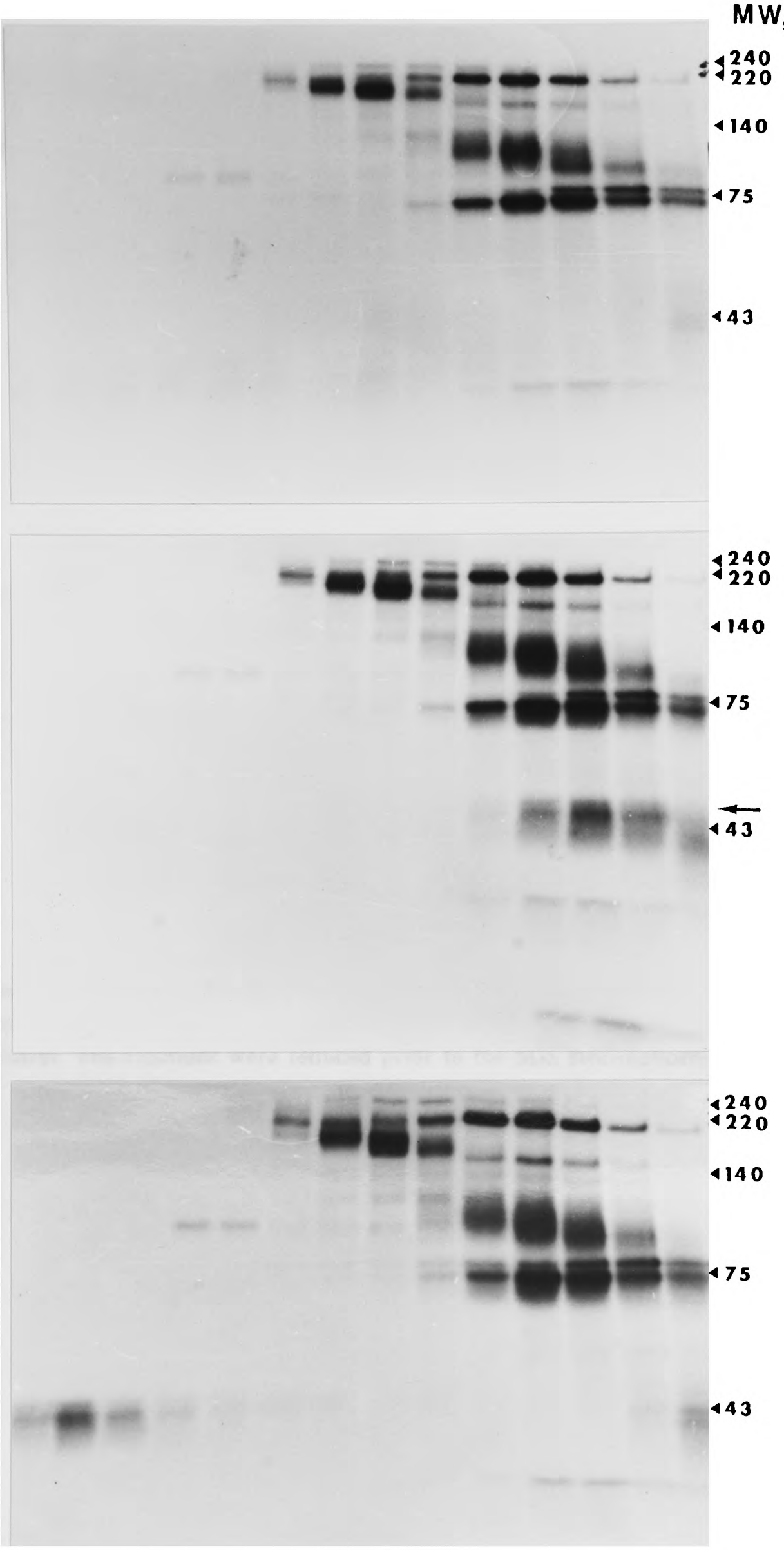


Fig 4

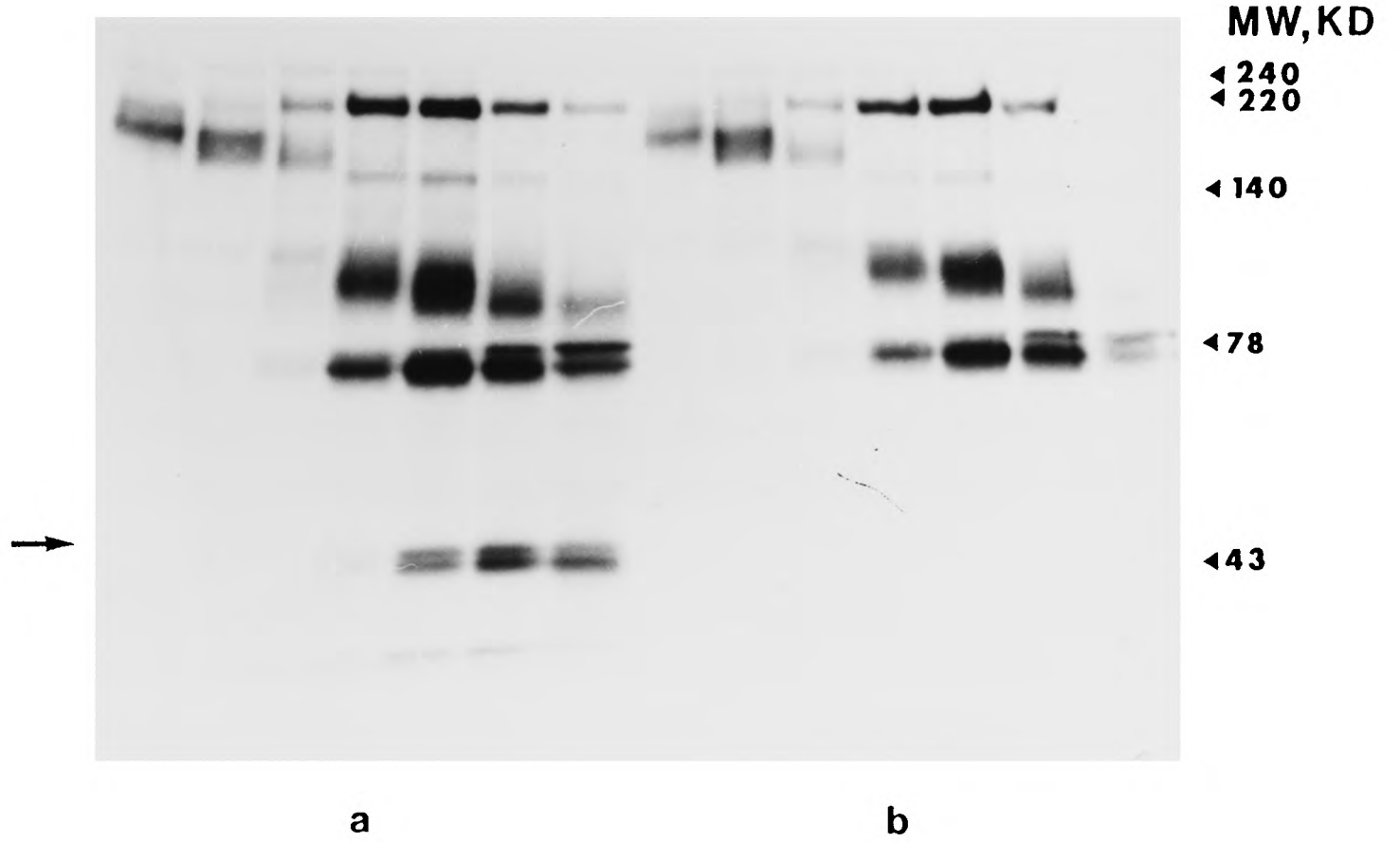


Fig 5

Fig 5. Detailed examination of the fractions containing the W6/32 mAb-Ag complexes. Two components were moved forward by adding the antibody. (a) W6/32 treated (b) control. The fractions were reduced prior to the SDS electrophoresis.

The Class I MHC antigen was detected by a monoclonal antibody W6/32 in both intact cells and Triton X-100 extract of the cells. W6/32 is an anti-Class I MHC monoclonal antibody (Parham et.al., 1979). One crucial difference of the shifted antigen was found between the treatment of intact cells and the Triton extract of the cells. With the intact cells, W6/32 binding resulted in reduced retention times of both α chain and $ß_2$-microglobulin. $ß_2$-microglobulin could be clearly observed in the fractions containing the α chain. However, this 12KD component was not co-shifted with the α chain if the cells were extracted before W6/32 added.

The W6/32-shifted band (Fig 5) was rather diffuse and the α chain appeared to be comigrating with another component with a very close mobility on SDS-PAGE. Several fractions containing Class I MHC-W6/32 complex were subjected to closer investigation to generate better defined images. It was shown clearly that W6/32 antibody actually moved another component at about 45KD, in addition to the α and ß chains of the antigen, It is not yet known which band is the α chain of Class I MHC. The apparent molecular mass of these two bands were 45KD and 43KD, respectively (Fig 6).

When a second antibody, an anti-mouse Ig, was incubated with W6/32-treated cell extract before chromatograhpy, the antigen, together with the unknown component, were completely separated from other surface antigens and recovered from fractions 2-4 (Fig 5c) (Wu and Maddy, 1989).

Monoclonal antibody DA6/147 reacts with the framework determinants of Class II MHC α chain (Guy et.al., 1981; Van Heyningen et.al., 1982). However, it was consistently found that both α and ß chains of the antigen were brought forward two fractions on HPLC by mAb DA6/147 (Fig 3c). Coincidently both chains were decreased in the fractions where they were normally recovered (Fig 3c, upper panel). By this method, two bands in band 8.4, a complex group of bands, have been clearly defined as the members of the HLA-DR family.

EZB54 is an IgG_3 raised in this department by fusion between NS-1 cells and CLL immunogenised mouse spleen cells. The determinant recognized by EZB54 exhibited a

restricted distribution--expressed only in certain normal and malignant cells of both lymphoid and myeloid origins. So far, repeated attempts by immuno-precipitation and Western blotting (Maddy, personal communication) to isolate the molecule have been unsuccessful. However, using HPLC the antibody was found to shift a component to fractions 13-14. This was a single band occupying a distinct position on 6-11.5% gradient SDS gels with an apparent molecular weight of 125KD under reduction (Fig 3d). It has not been possible to distinguish the antigen in its uncomplexed form from the bands in this region of the matrix, but chromatography of two-column combination revealed a band with a similar molecular weihgt in fraction 18-20.

Monoclonal antibody EZB52 (Brown et.al., 1985) is reactive with B-CLL and normal T cells as well as tonsillar B cells. The antibody precipitated a single band at 105KD. With two B-CLL samples tested using this antibody on HPLC, no observable movement could be found.

CLL B cells with band 4.3 were investigated with monoclonal antibody RFB4 (SAPU, code S053-201), which is specific for CD22. The antibody was tested in cell extract only and no shifting of the band was detected.

4. Discussion

The CD45 antigen consists of, mostly, four components with apparent molecular weights ranging from 180 to 240KD according to different authors, showing a marked heterogeneity in molecular mass, glycosylation and antigenicity. In our system their molecular weights are 230, 215, 200 and 180KD, respectively.

PD7/26 is the prototype antibody which defines the epitope shared by 230, 215 and 200KD chains. The results generated by HPLC are in consistent with previous work (Pulido et.al., 1988). The two-dimensional matrix of HPLC-SDS PAGE suggests that PD7/26 antibody does not bind preferentially to any of the three components.

F8-11-13 (Dalchau and Fabre, 1981) was one of the earliest antibodies defining the restricted human CD45RA. It precipitates the highest molecular weight members of the family. In our system, F8-11-13 can effectvely move the 230KD band of CD45 on HPLC but not the second chain of 215KD. Dalchau et.al. (1981,1986) and Brown et.al (1985) reported that F8-11-13 recognized two high molecular mass bands at 220 and 215KD, respectively. The difference could be attributed to the fact that two patients investigated in our system were both expressing predominantly the 230KD component, which yielded most dramatic shift of the antigen, while the autoradiography was based on the average exposure. A faint band was just detectable after a prolonged exposure but its identity has not been clarified because of the heavy shading of the overexposed 230KD band.

The 180KD glycoprotein of the CD45 antigen is recognized by a monoclonal antibody UCHL1 (Smith et.al., 1986). Research (Cebrian et.al., 1987) has shown that UCHL1 reacts with an unique antigenic epitope on the 180KD form of the CD45. Our failure to identify UCHL1 might be due to the use of tissue culture supernatant as a source antibody which has lower immunoglobulin concentration although is appropriate enough for FACS. As the UCHL1 antigen maintains its antigenicity after denaturation by SDS and the antigen is precipitable under the presence of 1% NP-40 (Terry et.al., 1988), it is unlikely that the unreactivity observed could be due to Triton X-100 destruction. We have found it somewhat difficult to use antibody tissue culture supernatant for the HPLC study with other

antibodies as well, such as EZB52. One explanation could be that there are not enough immunoglobulin molecules around to saturate cell surface or the antigens in the extracts to form detectable complexes. As the 180KD component on the matrix has not been identified with antibody, its being CD45RO is largely based on its molecular weight and the observation of tonsilar B and T cells.

Although only part of the CD45 is shifted by both PD7/26 and F8-11-13 it is unlikely that the unshifted bands are from some unknown proteins because (1) the variations of these three components recognized by PD7/26 in different patients suggest that these three bands are interrelated, in patients who express predominantly lower bands there is an insignificant amount of F8-11-13 (2) the results of mitogen-induced changes of the antigen expression (see **Chapter 8**) suggested the bands to be CD45 only. The incomplete shift may be attributed to incomplete binding of the antibodies.

The molecular weight of the antigen recognized by monoclonal antibodies of the CD44 panel has been demonstrated as 80-90KD (non-reduced) and 80-95KD (reduced) in different cells from the haemopoietic system (Spring et.al., 1988). It is possible that the 80KD component consists of several distinct molecules, including CDw44. A possible relation of CD44 antigen to the lymphocyte homing receptor was suggested (Omary et.al., 1988). Further experiment is desirable to distinguish and elabrate the molecules in this group since the surface Ig heavy chains also fall in this region which have long been a significant parameter of the cell physiology.

Both the reduced and non-reduced samples and the antibody-treated SDS-PAGE studies have proposed that band 4.2 consists solely of CD21 antigen and that band 4.3 is a distinct component. In our system, band 4.2 (CD21) has an apparent molecular weight of 140KD after reduction and 120KD when not reduced (**Chapter 5**). By employing HPLC separation, we are able to effectively reveal band 4.3 which is partially overlapped by CD21.

CD22 antigen is a pair of highly glycosylated proteins with apparent molecular weights of 130 and 140KD, respectively (Moldenhauer et.al., 1986; Dorken et.al., 1986). The antigen has a peculiar distribution during B cell ontogeny (Dorken et.al.,

1987) and it is found present in the cytoplasm of all B cells. 75% of CLLs are positive but always weakly. CD22 expression in the cytoplasm may account for the failure to detect this antigen on HPLC because cytoplasmic CD22 antigen could compete for the antibody, preventing the complex formation of the radioactively labelled membrane CD22 with the antibody. In addition, the low expression of this antigen on cell surface itself could also be a reason for the negative results although it could be detected by FACS. Moreover, no band was ever silver stained in the molecular weight range around 120-140KD, which means that CD22 perhaps can not be stained by silver and any possible shift of the cytoplasmic CD22 by the antibody would not be detectable. From this point, it is desirable to investigate the CD22 on intact cells by employing monoclonal antibodies on HPLC system.

From the HPLC results, it is demonstrated that either bands 6.1 or 6.2, or at least part of the bands, is CD43. Immunoprecipitation also resulted in two bands (Stefanova et.al., 1989). It is more obvious on neuraminidase-treated tritiated samples in which CD43 antigen was displayed in a distinct position of 150KD and shifted with the added antibody although a weaker band was found to overlap with it. The gpL115 (CD43) antigen can actually be better located on the tritiated gels where there are fewer bands as shown by Fig 6. The nature of the co-shifted band is still not clear and remains to be identified. The tritiated results also show that the gpL115 epitope activity is not affected or disrupted by cleavage of sialic acid residues, implying that the determinant is not located on the terminal sialic acid. By direct comparison of the autoradiographic patterns of the B cells of CLL patients, it was found that CD43 antigen was present in some patients but absent from others. However, no certain correlation has been found with respect to other documented surface antigens.

The nature of the band which was co-shifted with Class I MHC is unknown. Immunoprecipitation has been reported, at 45KD range, to generate only a single band (Parham et.al., 1979). Therefore, it is unlikely that the double band is a consequence of polymorphism. It seems that these two bands are associated as: (1) treatment of either cell extract or intact cells by W6/32 produced identical positive results and (2) both bands were shifted with indistinguishable retention times. It is

likely that the co-shifted component is in association with the α chain of the antigen rather than cross-react with the antibody.

The Class II MHC complex is composed of two non-covalently bonded polypeptide chains with an apparent molecular mass of 35KD for α chain and 28KD for ß chain, respectively. The association between the α and ß chains is found to be stable in non-ionic detergent as both components have the same retention times in the column and the combination of either two GF-450s and one GF-450 with one GF-250 failed to resolve the α and ß components, indicating that the similarity of their retardation is not caused by the limitation of column resolution. The integrity of the molecule after chromatography suggests that the analytical conditions established in this study are suitable for some weak interactions.

It is observed that the α component is more strongly iodinated than the ß component in some patients while the position is reversed in other cases. No correlation has been noted between the relative intensities of the α and ß chains and any other characteristics of the patients.

According to the previous study in this lab, the determinants recognized by monoclonal antibody EZB54 have a very restricted distribution, only expressed by some normal and malignant cell types of both lymphoid and myeloid origins. FACS shows that B-CLL cells are EZB54 positive. However, attempts to isolate the antigen by either Western blotting and immunoprecipitation have always met with failure. The success of identifying this antigen by HPLC suggests that (1) the antigen is a protein iodinatable by the method used in this study (2) neither the determinant of the antigen nor the binding of the antibody to the determinant are destroyed by solubilization as previously suggested (3) the failure of isolating the antigen by conventional methods may well be caused by the harsh conditions and low binding affinity.

This chapter describes the methodology of HPLC size exclusion, in combination with monoclonal antibodies, for the identification of surface antigens of lymphocytes. The technique has been proven to be a specific, consistent and one-step simple method. As size exclusion is a gentle physical process, one should expect that non-

covalent interactions between polypeptides, low affinity binding of antibody, and molecular integrity would survive under such a mild procedure. It has been observed that some silver stained bands, which were not surface-iodinatable, were co-migrating with some iodinated bands which were shifted after complexing with their antibodies. However, it is beyond the scope of this thesis to examine and discuss the silver stained HPLC-SDS PAGE profiles. But it would certainly be valuable to make a close investigation of these gels.

Although the amount of an antibody employed is based on flow cytometric titration for the maximum bindings, usually at the same dilution, the actual ratio of an antibody to the antigen may not be at its optimum because if a surface antigen is also expressed within the cytoplasm, after solubilisation of cells, the quantity of the antigens could be greatly increased in the medium. Therefore, caution must be taken in determining the antibody amount. Usually an 10-30% excess of antibodies is used in our routine studies. For some bands on SDS-PAGE, a shift does not necessarily exclude the presence of other bands with the same mobility in SDS-PAGE, hence optimum ratio of antibody to antigen could be essential.

Applying a second antibody to the first-antibody-antigen complex has been proven very effective to identify and to separate an antigen from a protein mixture, as demonstrated by mAb W6/32 in this study. The narrow distribution of the shifted multi-complex indicates that, under the conditions employed, the polyclonal antibody (the second antibody) did not yield a broad spectrum of cross linking at different levels, generating complexes with widely different molecular masses. It is preferable to apply the first antibody to intact cells as the excess antibody can be washed away, it is particularly advantageous when the second step involves polyclonal antibody (Wu and Maddy, 1989). In such case, it might be crucial to avoid using too large an excess of antibody and a too prolonged incubation time, otherwise, immuno-precipitation or a highly cross-linked surface protein network might occure. In addition, one should always use the minimum incubation time to avoid secondary effects of excessive incubation, neverthless reduction of cell viability, changing of the surface protein expression and of the physiological statue of interactions between receptors and the underlying cytoskeleton could become significant.

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CHAPTER 7. PHENOTYPE ANALYSIS OF LYMPHOCYTES BY HPLC-SDS PAGE

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CHAPTER 7. PHENOTYPE ANALYSIS OF LYMPHOCYTES BY HPLC-SDS PAGE

1. Introduction

The lymphocyte cell surface has the specialised function of recognizing external molecules. The consequences of this recognition are closely related to lymphocyte proliferation and differentiation to immunocompetent memory and effector cells. Mostly, this recognition is carried out by membrane surface glycoproteins.

Some surface glycoproteins are cell-type specific while others are common to more than one type of cell. In normal leucocytes, studies have shown that the expression of certain glycoproteins is associated with paritcular stages of cell development. As finer details of B-cell surface phenotypes are revealed, it is found that some surface markers are detectable only in some of the B cells, which helps to group B cells into distinct subsets with respect to the expression of these specific markers, i.e. $MRBC^+$ and $CD5^+$.

However, this correlated expression of surface proteins becomes disrupted in neoplastic cells. The neoplastic transformation of CLL cells involves a monoclonal expansion of the lymphocyte, accompanied by a heterogeneous expression of surface membrane proteins. Recently, new approaches have been developed to correctly define different B-cell populations and/or maturation stages and to compare B-cell malignancies to their putative normal counterparts (Gobbi et.al., 1983; Dighiero and Binet, 1987). But a direct comparison of surface phenotypes is complicated by the fact that CLL cells frequently express markers of both early and later B cell development, such as the expression of cytoplasmic Ig which is reminiscent of the pre-B stage of normal lymphocytosis and of CD23 which is normally expressed on activated cells. This disordered asynchronous expression of markers is also demonstrated in other types of leukaemias, e.g. PLL.

Attempts have been made to exploit the heterogeneity of CLLs, both in terms of their surface phenotypes and the clinical progression. Foon et.al. (1982) and Anderson et.al. (1984) examined the surface phenotypes of entire range of leukaemias and lymphomas and found that the different types of phenotype showed a continuous

variation and this variation can be correlated to the differentiation sequence of normal B lymphocytes. Systematic studies in this laboratory (Smith et.al., 1985; Brown et.al., 1985) have revealed that the expression of CD45 antigen in CLL exhibits a continuous spectrum, varying from typical B cell patterns to a T cell-like profile with intermediate forms between the two extremes. Later work in this lab established the correlation between sIg expression and that of CD45RA (Brown et.al., 1987). There have been a few reports on other differentiation markers, atttempting to depict interrelationships between different leukaemias.

Conventional methods for phenotypic investigation present difficulties in direct comparison of several markers in different patients and are also restricted to only known markers. This chapter describes the application of the HPLC technique for the analysis of neoplastic cell surface phenotypes and the construction of the membrane protein repertoire.

2. Materials and Methods

CLL lymphocytes were prepared as described in **Chapter 5**. Washed cells were surface-labelled by either iodination catalysed by lactoperoxidase or neuraminidase-galactose oxidase tritiation. Routinely, $2.5\text{-}5x10^7$ cells were adequate for one run on the column.

All samples were subjected to chromatography within 24 hours. Normally 24 fractions were collected, first 15 of them subjected to SDS gel electrophoresis either on 6-11.5% or 8-15% gradient polyacrylmide gels, depending on the molecules to be examined. Autoradiography was carried out as stated (**Chapter 5**). For the purpose of comparison, films were exposed to produce equivalent densities for different patients and different runs of the same patient.

Bands were designated as stated in **Chapter 5**.

The same panel of patients were also investigated by flow cytometry (by A.H. Maddy). The FACS data were compared with the HPLC observations and used as a comlementary source for the study.

3. Results

During the course of this study, 30 patients with B-CLL, one with PLL and one with T-CLL have been examined. The B-CLL patients were diagnosed at different stages of the disease progression. Comparison of the surface phenotypes was based on their HPLC-SDS PAGE autoradiographic patterns in conjuction with silver stained gels and FACS data. It was found that in all the patients band 3 was constant in intensity relative to other bands and showed no significant change. Studies in this lab have shown the variation of CD45 expression (Smith et.al., 1985) in B-CLL and its correlation with sIg (Brown et.al., 1987). In addition, the CD45 molecule was well separated by HPLC from other components and easily defined in the front fractions. Therefore both molecules were employed as bases for the comparison of autoradiographic intensity. The relative autoradiographic intensities of these related bands are tabulated in Table 1 of the appendix. The arabic numbers are the band numbers quoted from Fig 1 of Chapter 5. Band 3 intensity is employed as a criterion, which is rated five pluses. All ratings only mean relative value and were largely based on visual observations. However, the majority of these results are confirmed by at least two individual investigations of each patient and have demonstrated high consistency and reproducibility.

In the light of the intensity variation of three CD45 bands, namely 230KD, 215KD and 200KD, cell phenotypes are classified into two types, 1A and 1B.

3.1. 1A Type

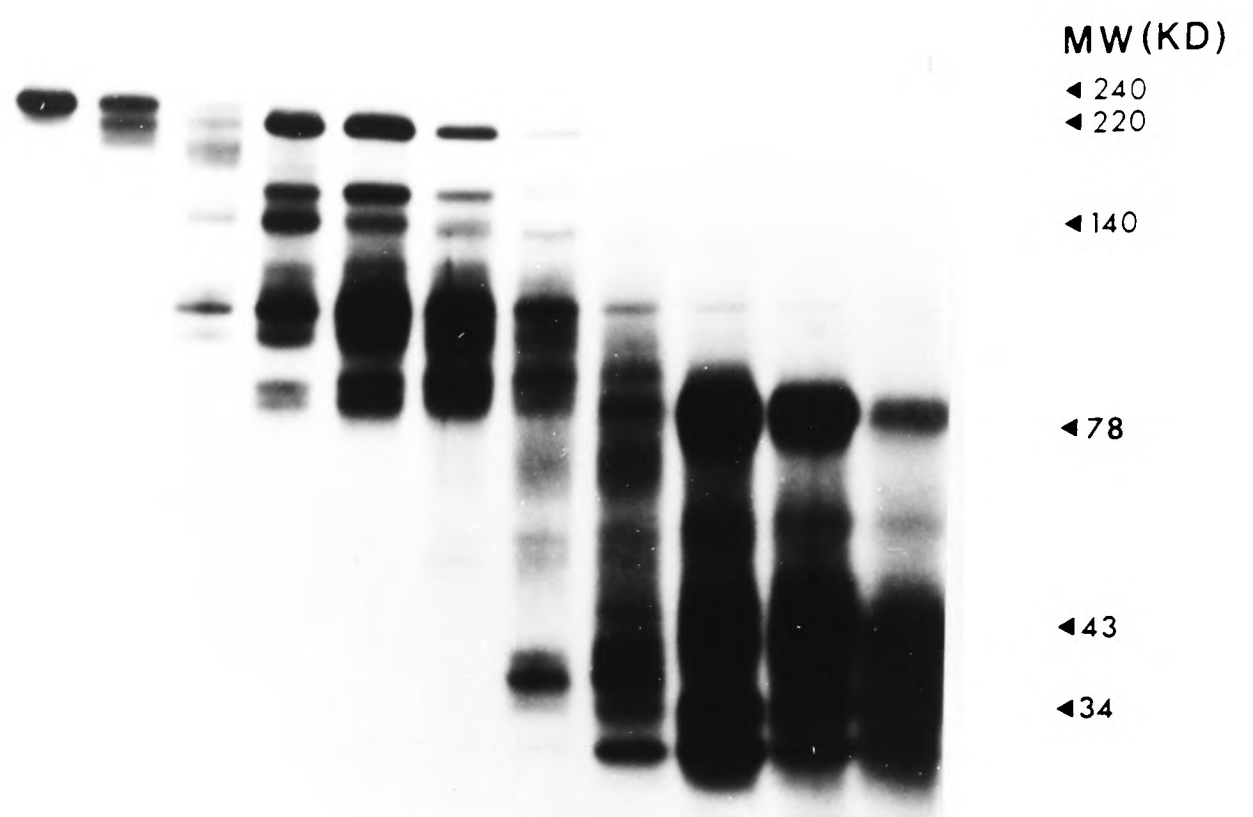
Thirteen patients in this group were investigated by HPLC, consisting of about 43% of the patients investigated. They are:

1A Type(i): McG, Lot (1987), Cur, Jac, Kay, Pau, Wal

1A Type(ii): Lyn, Bur, Cla(?), Sca, Gre, Ske, Gil.

The 1A form of CLL was correlated with the following phenotypic traits (Fig 1):

Fig 1. Typical 1A phenotype of B-CLL cells, with a schematic presentation showing different surface components and their relative intensity. The schematic intensity of the bands is not strictly proportional to the expression levels of the corresponding bands.


MW(KD)
240
220
140
78
43
34

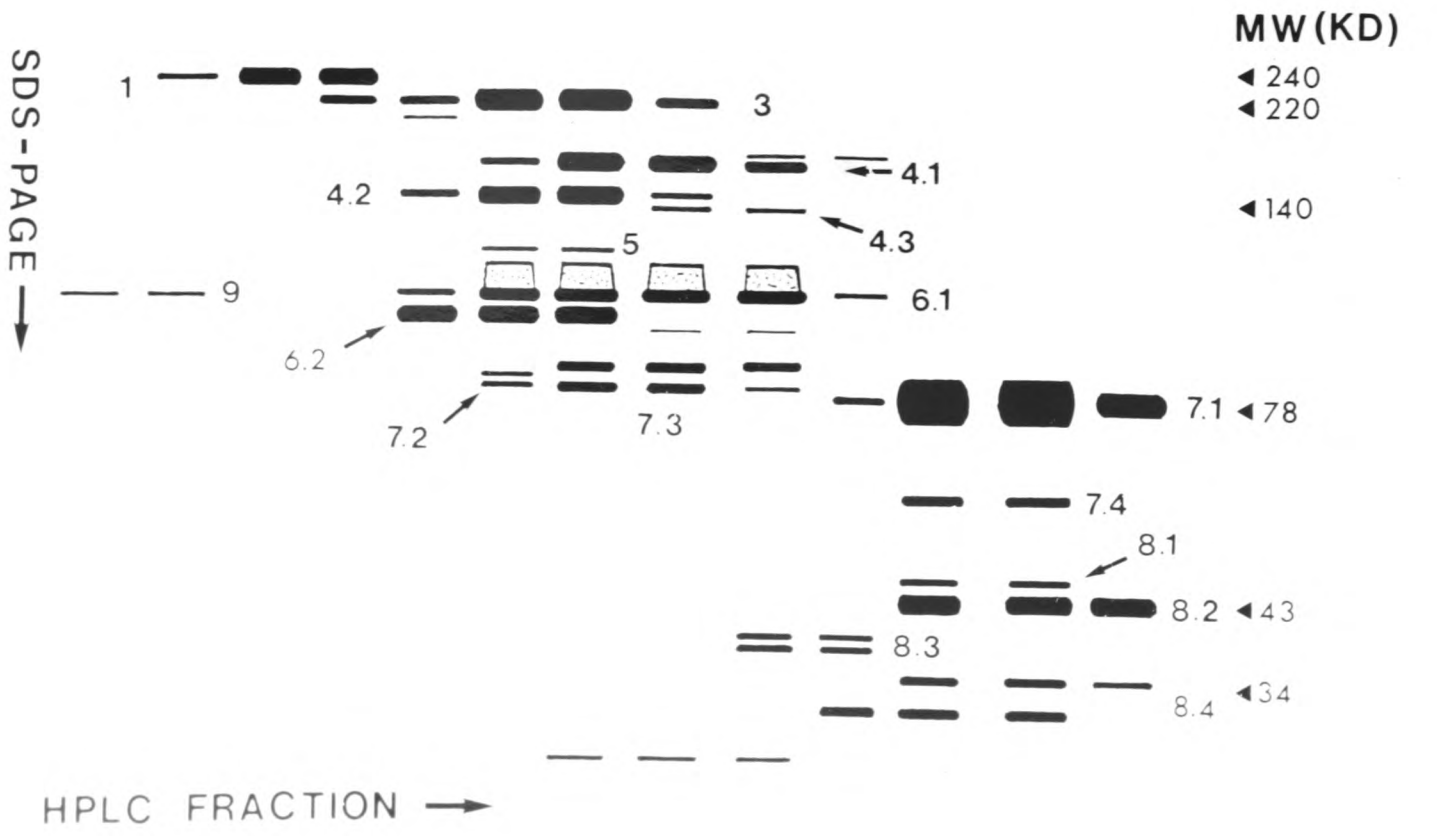
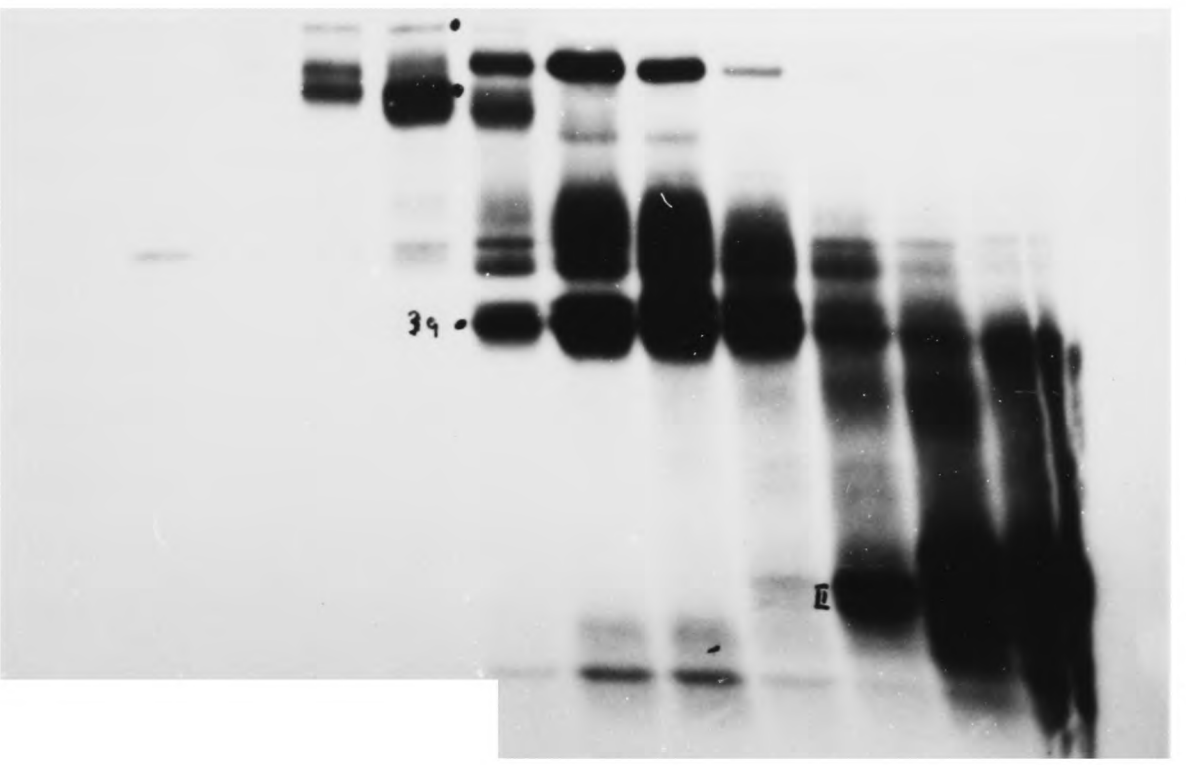
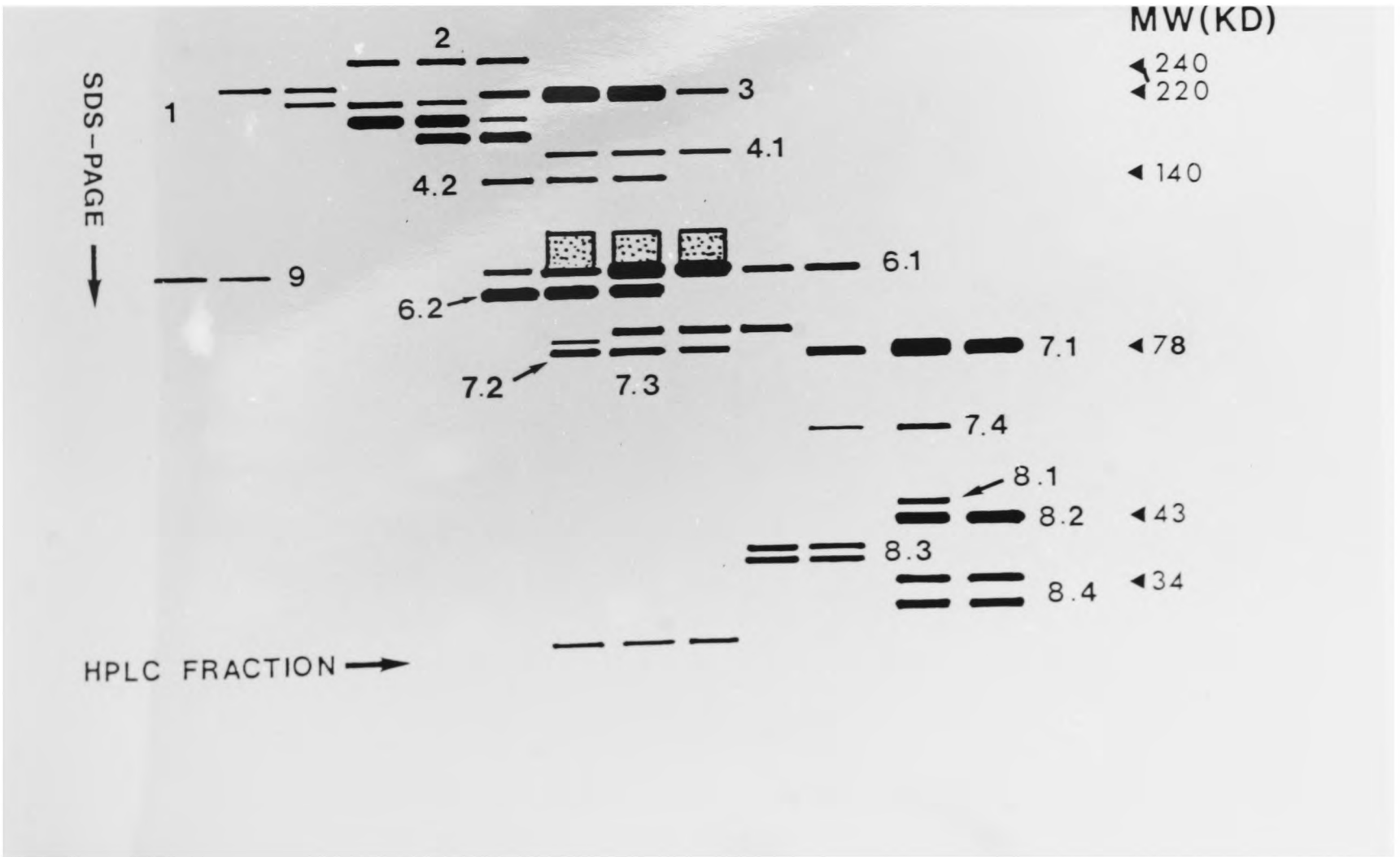

MW(KD)
240
220
140
78
43
34
SDS-PAGE
HPLC FRACTION
1
3
4.1
4.2
4.3
5
6.1
6.2
7.1
7.2
7.3
7.4
8.1
8.2
8.3
8.4
9

Fig 2. Typical IB phenotype of B-CLL. Note the presence of band 2, a 300KD component.


MW(KD)
240
220
140
78
43
34


MW(KD)
240
220
140
78
43
34
SDS-PAGE
HPLC FRACTION
1
2
3
4.1
4.2
9
6.1
6.2
7.1
7.2
7.3
7.4
8.1
8.2
8.3
8.4

3.1.1. This group is featured by a strong CD45RA (230KD and 215KD) with, sometime, weak 200KD forms of the CD45 family. Previous study by FACS (Smith et.al., 1985) has demonstrated that the level of UCHL1 expression is very low in this group. Similarly, HPLC-SDS PAGE only detected a very weak or no 180KD band in the vicinity of other CD45 bands. As the expression of 230KD varies greatly, it is, therefore, helpful to subdivide this group according to the relative intensities of 230KD and 215KD bands. 1A(i) type is characterized by the predominance of 230KD and weak 215KD forms of the CD45 antigen while 200KD band is consistently undetectable. By this criterion, seven patients were included in this catalogue. 1A(ii) type features relatively strong 215KD chain and significantly increased intensity of 200KD band of the CD45 although 230KD was still the predominant component. This classification of the autoradiographic patterns was found to be consistent with that of the FACS results by Smith et.al. (1985), for the same panel of patients. Other common features of 1A type CLL B lymphocytes, in addition to CD45 expression, are described as follows:

3.1.2. The 140KD band of CD21 was strongly expressed by this group of patients although weak expression was observed in 2 1A(ii) type patients. It was demonstrated that a high level of 230KD was consistently accompanied by a high level of CD21 expression, i.e. 1A(i) patients have higher CD21 than 1A(ii) patients. The intensity of autoradiography of this 140KD band was comparable to band 3, a 220KD component. Monoclonal antibody to CD21 could shift the 140KD band almost completely, which means that the band is contributed solely by CD21. In addition, CD21 is strongly positive in P-LL (Fig 3a) and normal tonsillar B cells but very weak in T-CLL (Fig 3b) with respect to both autoradiography and flow cytometry.

3.1.3. Band 4.1, a component of 150KD, was present in all patients of this group with high positivity. This 150KD molecule was positively correlated to 230KD band of CD45RA expression. The behaviour of this polypeptide under reduction and non-reduction has been described in **Chapter 5**. Band 4.1 was also observed to be strongly expressed by PLL, normal tonsillar B cells (Fig 3c) and malignant cell lines (see **Chapter 10**). This component was eluted to fraction 8-10, always one or two fractions later than CD21 although it has a higher apparent molecular weight. Normal

tonsilar T cells were found to express this component, probably strongly as shown in Fig 2 of Chapter 6.

3.1.4. Band 4.3, of an apparent molecular weight of 135KD in fraction 10-11, is present in all 1A type CLL B cells and with significant amounts in PLL. This component was found in the vicinity to band 4.2 on SDS-PAGE and partially overlapping band 4.2 but the band 4.3 was sharp and well defined. The band was unlikely to be artifactual since (1) the component was detected only in 1A type CLLs and consistently expressed by individuals within this category (2) its autoradiographic intensity was constant within individuals over the course of investigation and (3) more importantly, its expression was reduced by mitogenic activation of the cells (see Chapter 8). In addition, this component was unreactive with a monoclonal antibody to CD22 and its mobility was not affected by reduction-non reduction SDS-PAGE. These observations exclude its being CD22. The band, however, was detected on neither normal tonsillar B nor normal tonsillar B and T cell mixtures (Fig 2 in Chapter 6).

3.1.5. Band 7.1 is one of the major surface proteins with respect to its autoradiographic intensity. This 78KD polypeptide is present on all B-CLL, PLL and normal tonsillar B cells but B-CLL 1A and PLL were found among the strongest while tonsillar B cells, somewhat, weaker. 1A(i) type patients usually possess higher levels of band 7.1 than 1A(ii). The expression of this component is correlated positively with that of CD45RA.

3.1.6. A fairly faint band with an apparent molecular weight of 170KD was consistently found in fraction 6-8. This band appeared to co-elute with 230KD chain of CD45RA and to be associated with 1A(i) type only. Of course, it is possible that in other patients, this faint component is shaded by lower forms of CD45 bands. In PLL, this band was clearly defined and quite strongly expressed. However, it was undetectable on normal tonsillar B or B and T cell mixtures.

3.1.7. All 1A type B-CLL demonstrated relatively high surface immunoglobulin. In comparison, PLL showed even higher expression of sIgM. It seemed to be that the PLL case studied only expresses sIgM as shown by Fig 3a. Normal tonsillar B

lymphocytes were also strongly surface Ig positive but overall intensity was lower than PLL and it has been observed to express both sIgD and sIgM with higher IgD than IgM, so as indicated by FACS.

3.1.8. Two patients out of ten in this category were found to express a strong band of 60KD in fraction 12-14. Little is known about it.

3.2. 1B type

Eleven patients are grouped into this category, they are:

1B Type(i): Tho, Col, Sul, Web, Bla , Sow, For.
1B Type(ii): Tot, Par(87), And, Woo.

The rest of the tested patients could not be definitly grouped into either of these categories either becuase of (1) poor gel patterns (2) not typical 1A or 1B patterns, which, therefore, were called ' intermediate '.

3.2.1. The lower forms of the CD45 antigen are now predominant, usually with different combinations of band intensities. This type can also be subdivided into two subgroups by their CD45 expression. The 1B(i) type cells usually contain all four chains of the antigen, i.e. 230KD, 215KD, 200KD and 180KD (the UCHL1) while the 1B(ii) type lacks or expresses very weakly 230KD form, and much more prominant 200KD and the UCHL1 components. Extreme 1B(ii) type B-CLL cells showed complete absence of 230KD band of CD45 antigen and extraordinarily high UCHL1 expression as shown in Fig 2, a feature represented by T-CLL cells (Fig 3b). This 180KD component, eluted gradually following the other three chains of the CD45 antigen, was not observed on normal tonsillar B cells but apparently present on tonsillar B and T cell mixture (Fig 2 in **Chapter 6**). Other features of this type include:

3.2.2. All 1B cells express an unique component, band 2, a sharp band with an apparent molecular weight of about 300KD, being usually found in fraction 8-10 (Fig 2). By its molecular mass, this component appeared to be anormalously retarded

by the column, it should be excluded from the column earlier than the CD45 components. Its mobility on SDS gels appears not to be affected by reduction-non reduction prior to electrophoresis. The level of this component varies considerably in different individuals. Generally, 1B(ii) exhibits higher band 2 than 1B(i). Those patients with strong 200KD or 180KD of CD45 showed strong expressions of the component. The expression of this molecule varied upon cell activation. 1A type B-CLL lymphocytes acquired this component while 1B type showed elevated expression following phorbol ester treatment (see **Chapter 8**). Band 2 might have positive correlation with UCHL1 expression but it is still imponderable. An attempt to raise monoclonal antibodies to it is being undertaken.

3.2.3. Generally, band 4.1 expression was weak, with considerably lower levels than in 1A cases. The positive correlation between band 4.1 and CD45RA (230KD) has been further substantiated by B-CLLs in this group, the band 4.1 declined in parallel with the reduction of the CD45RA expression. This 150KD component was found very weak or undetectable in 1B(ii) type cells.

3.2.4. CD21 expression varied in different patients of this group. Largely its expression is positively correlated with CD45RA expression although exceptions were observed. However, it has been noticed that in those with a fair amount of UCHL1 expression CD21 is always low. One 1B(i) type patient was shown with exceptionally high CD21 with other features being typical of 1B(i).

3.2.5. Band 4.3 was rarely observed in most of the patients in this category. It was only weakly expressed when present.

3.2.6. Band 7.1 is weaker in 1B type than in 1A type and shows the similar relationship with CD45RA as described for 1A type cells.

3.2.7. In all cells of this group, surface immunoglobulin is decreased significantly, as detected by either autoradiography or FACS, in comparison with those in 1A type.

In addition to B-CLL lymphocytes, one prolymphocytic leukaemia and one T cell CLL have been investigated in this study. PLL has very similar patterns to the

extreme 1A type, but with much higher surface immunoglobulin, IgM, as shown by both HPLC-SDS PAGE and flow cytometry.

Normal tonsillar B cells are also found to be similar to 1A type cells with respect to band 4.1, CD21, CD45RA and band 7.1 (Fig 3c). However, two marked differences have been observed. (1) there is a band co-migrating with 230KD chain of CD45 with an apparent molecular weight of 250KD. This component is not band 2 because of its fractionation and the molecular weight (2) normal tonsillar B cells do not express band 4.3.

T-CLL exhibits features of 1B type CLL cells with respect to the absence of 230KD of CD45RA, a strong 200KD chain of CD45 and the UCHL1, the expression of band 2 and absence of CD21. However, band 4.1 is abnormal. It is highly expressed but appears to have higher molecular weight than in B-CLL cases. It appears to be about 170KD. This band might not be band 4.1, and needs further identification.

3.3. *in vivo* Changes of CLL Phenotypic Presentations

Surface phenotypes of patients normally remain stable over the course of the disease but two unusual cases have been found to change those surface phenotypes progressively which distinguish 1A type from 1B type. The findings provide back-up evidence for the typing system as well as the correlations among these markers.

Example one, Lot

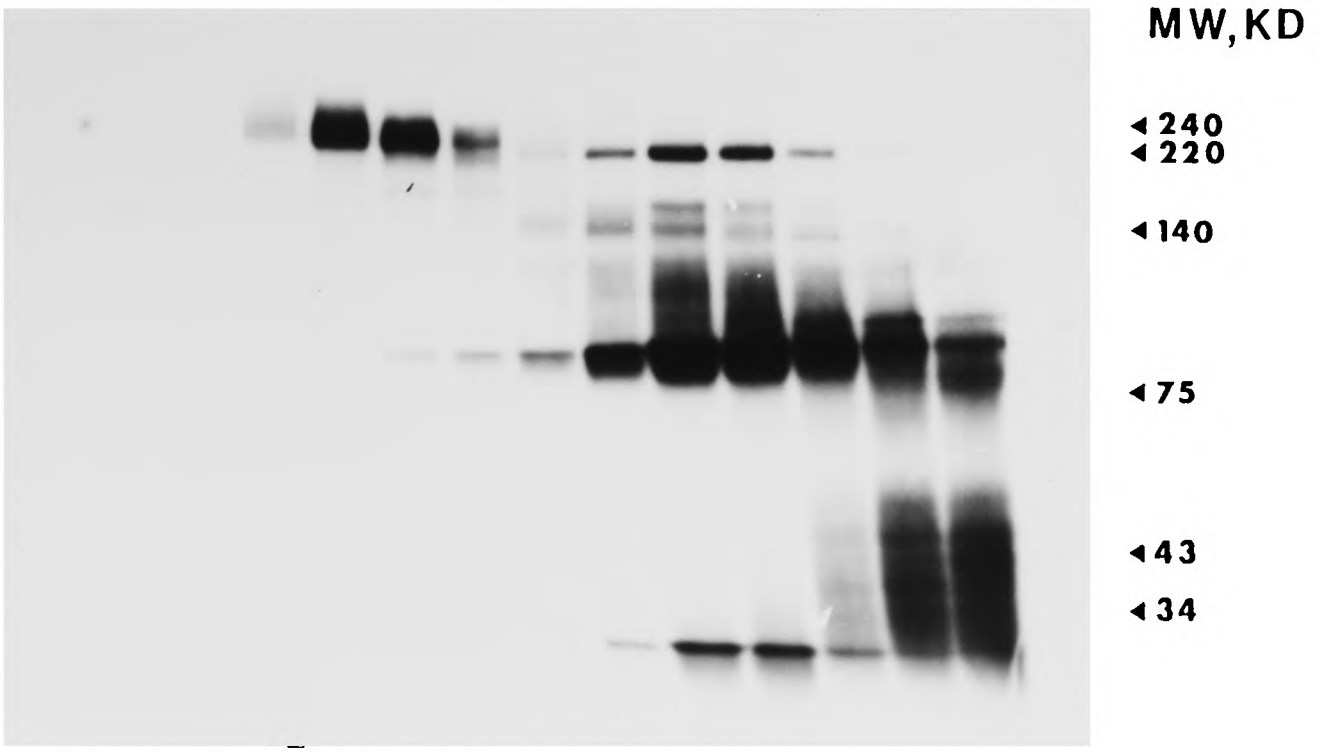
Investigation of this patient extended over more than two years. Distinct changes were found during this period (Fig 4). In 1987, when the first investigation was conducted, the patient was a typical 1A type, having only 230KD band of the CD45 antigen as revealed by autoradiography of HPLC-SDS PAGE (Fig 4a). By 1989 the 230KD component had decreased while the 215KD appeared and the 200KD band was the strongest among three as shown by Fig 4b. Band 2, absent in 1987 samples, was expressed in 1989 as the CD45RA declined. In addition to these shifts, both CD21 and band 4.1 markedly decreased in intensity. The band 4.1 was stronger than

CD21 in 1987, but was weaker in 1988 and 1989, indicating its close correlation with the CD45RA expression. The band 4.3 disappeared as the patient developed from 1A type to 1B type phenotype as elaborated by Fig 4b. In 1988, the phenotype was found at an intermediate stage (data not shown), where 215KD and 200KD bands were emerging although other changes were still unnoticeable. It was noticed that the lymphocytosis of the patient increased in severety over this period.

Example two, Par.

Patient Par was initially shown to be typical of 1B characteristics (Fig 5a). Investigated by HPLC in 1987, the patient showed a very low 230KD expression of the CD45 antigen, positive band 2, weak band 4.1 and negative 4.3 (Fig 5a, I and II). One year later, in a routine investigation, it was discovered that the phenotype developed towards 1A type. The 1988 autoradiograph (Fig 5b) indicated that the CD45RA gained an equal intensity as the 200KD chain, being distinct from those of 1987. Moreover, band 2 no longer existed while band 4.3 could be clearly defined. The most dramatical change was band 4.1, which became one of the strongest bands, comparable to band 3 in the term of autoradiographic intensities.

Above changes were also confirmed by FACS study but the autoradiographs showed much more marked changes.



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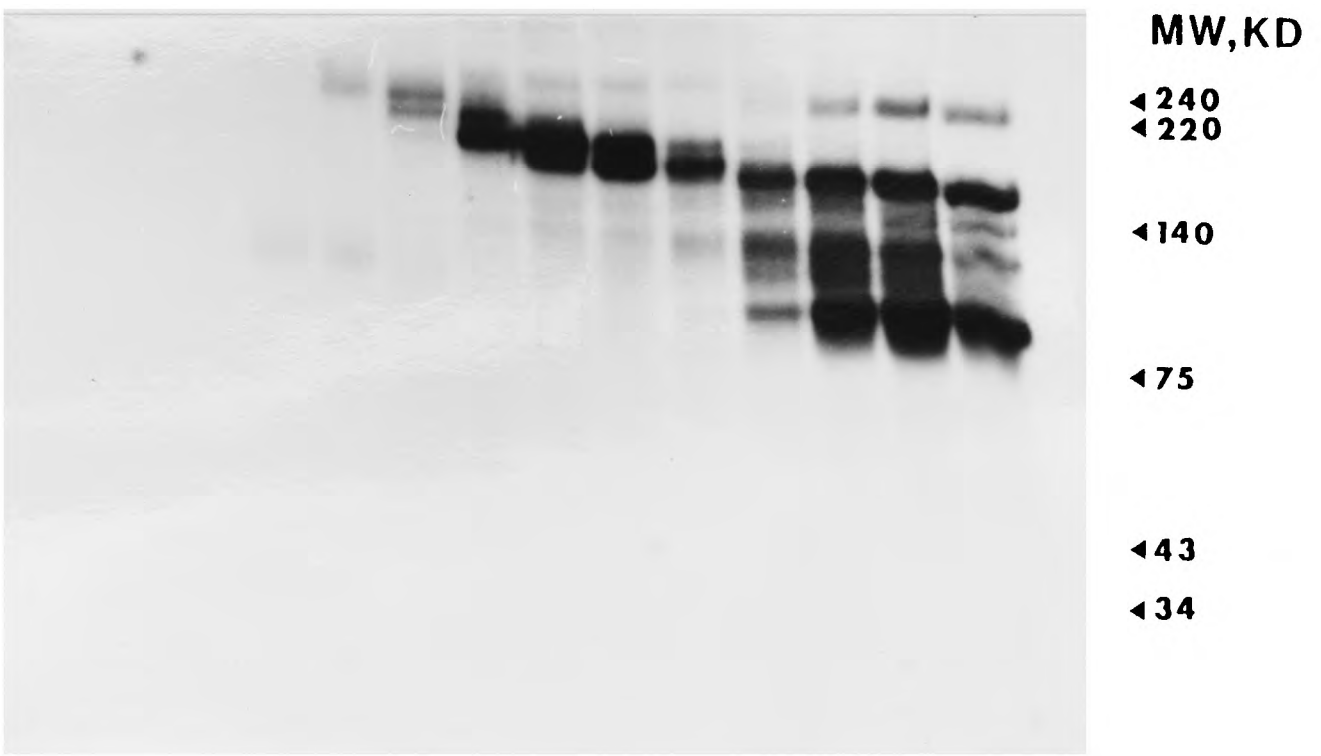
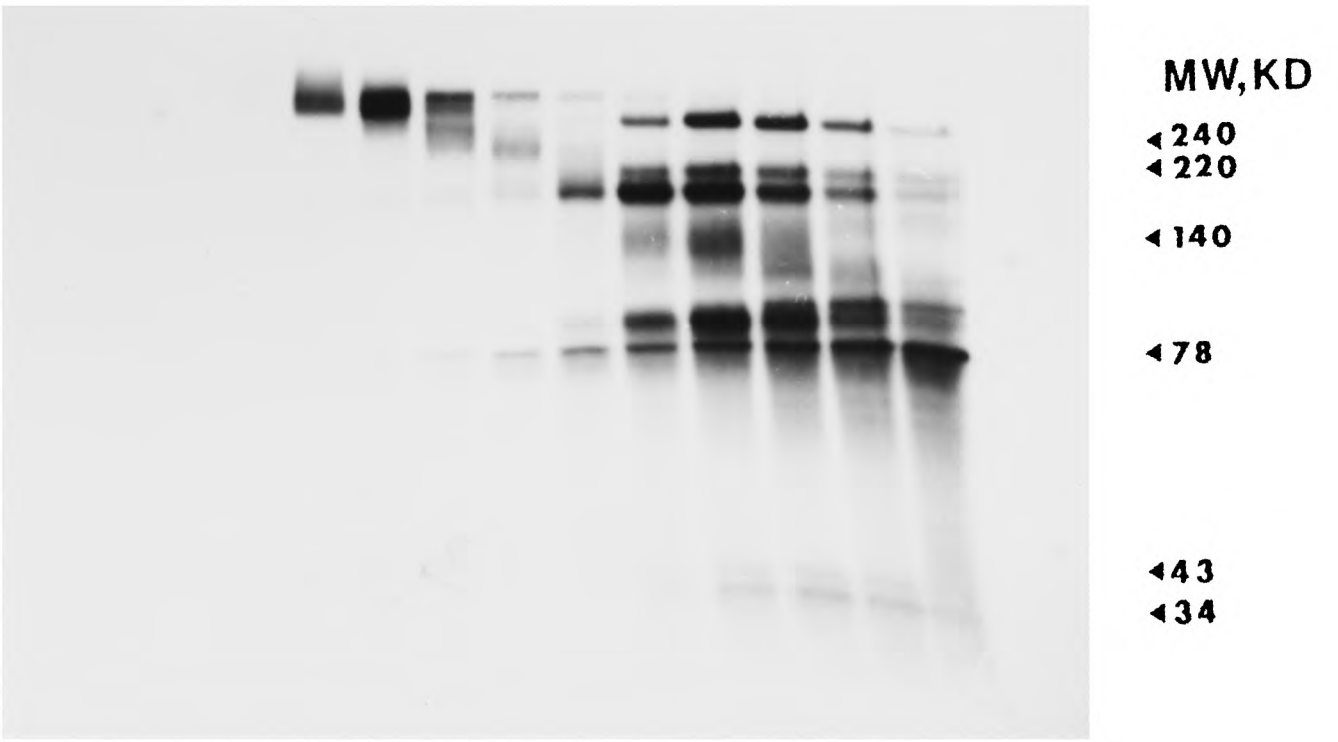


b

Fig 3



C

Fig 3

Fig 3. The fluorographic profile of a two-dimensional HPLC-SDS PAGE matrix of some other lymphocytic leukaemias. (a) PLL, showing its similarities with B-CLL 1A with respect to the expression level of CD45RA, CD21, band 4.1 and 4.3 as well as high surface immunoglobulin (b) T-CLL, showing its similarities with B-CLL 1B (c) normal tonsilar B cells.

Fig 4. Demonstration of the *in vivo* phenotypic shift from 1A to 1B in patient Lot. (a) the profile in 1987, a typical 1A profile, featuring high CD45RA, CD21, band 4.1 and band 4.3. (b) the profile in 1989. Note the progressively decreasing level of 230KD, band 4.1, CD21 and increasing of 215KD and 200KD of CD45 and the acquisition of band 2.

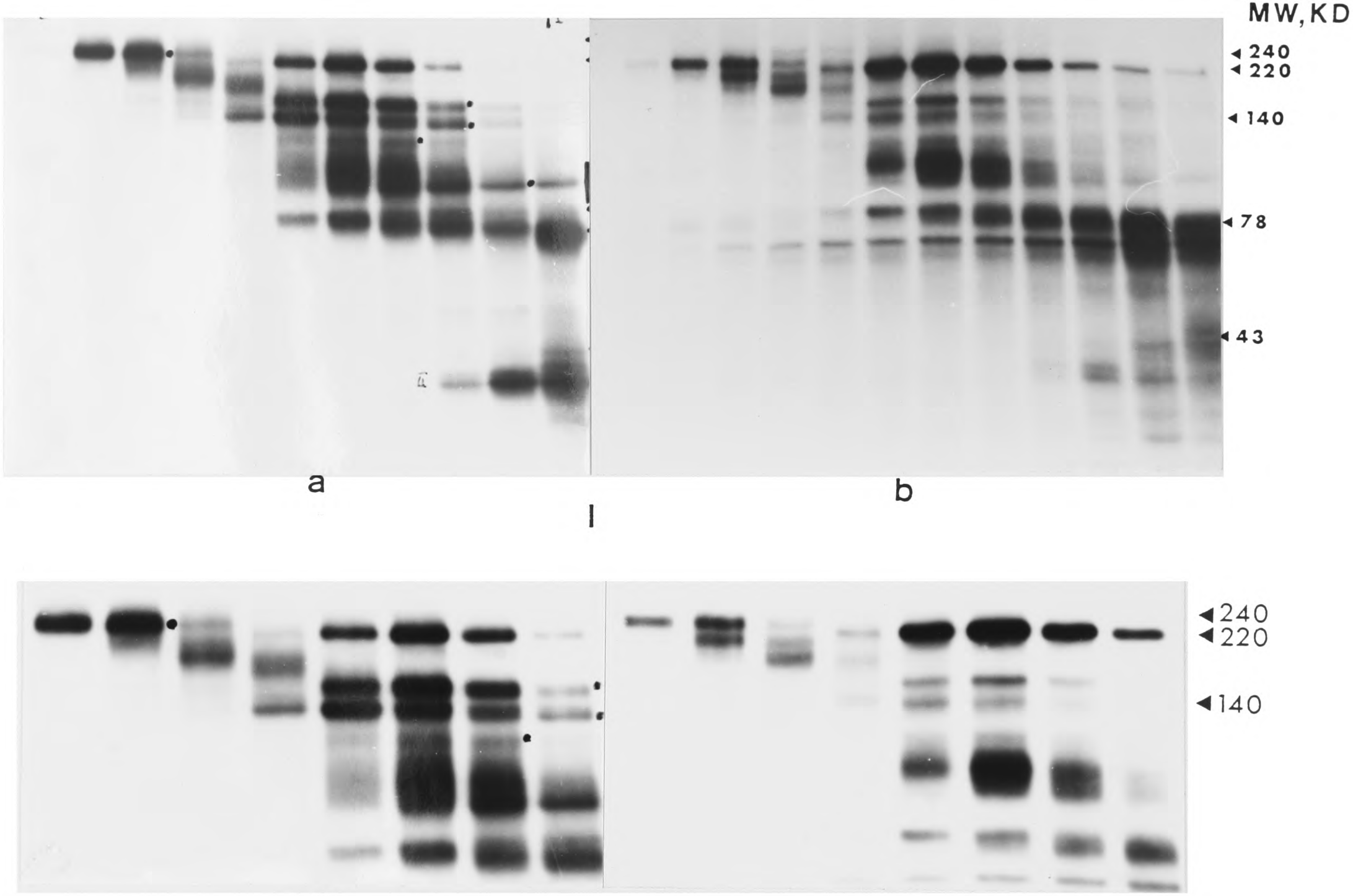


Fig 4

Fig 5. Demonstration of the *in vivo* phenotypic shift from 1B to 1A in patient Par. (a) the profile in 1987, a typical 1B profile (b) profile in 1988. Note the progressively changing levels of 230KD, 215KD and 200KD of CD45, band 4.1, the acquisition of band 4.3 and the loss of band 2.

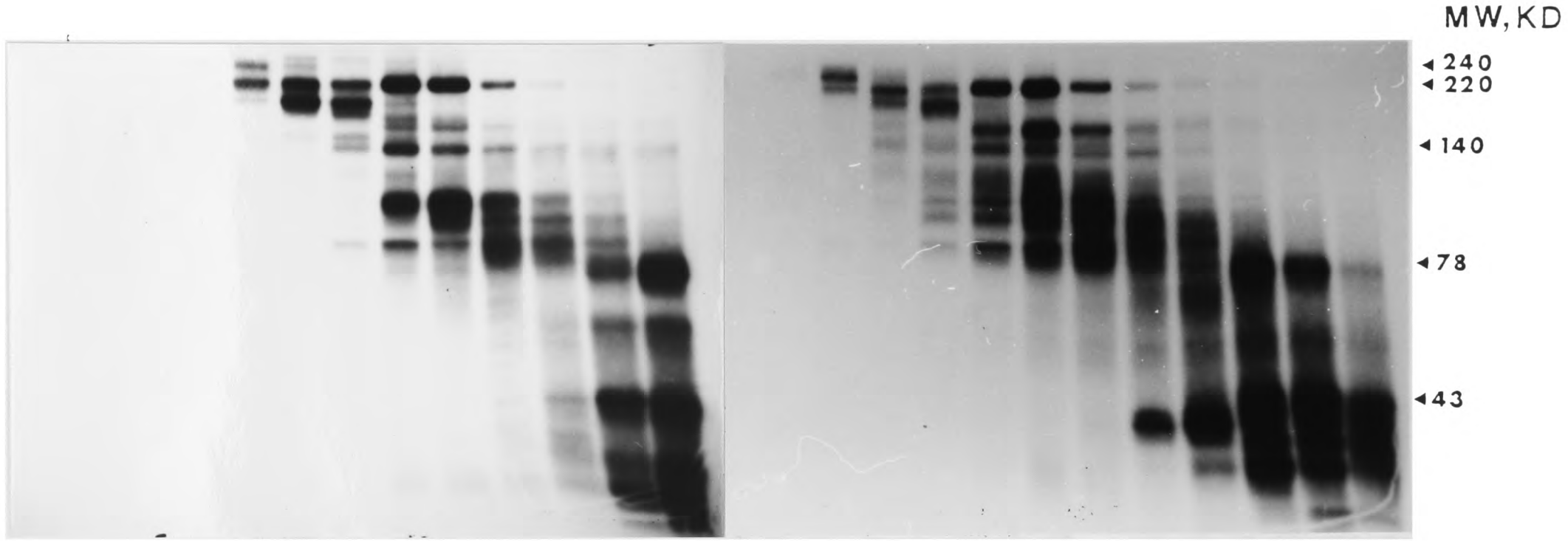


Fig 5

4. Discussion

The study in this chapter examined the phenotypes of the B lymphocytes from patients with chronic lymphocytic leukaemia. The phenotypes vary among patients and show a spectrum of continuous changes across the patient panel. HPLC has confirmed the earlier findings in the variation of CD45 expression in the same panel of B-CLL patients and its correlation with sIg expression (Brown et.al., 1987). In addition, four new surface components have been described with respect to their relationships with CD45 expression and more delicate associations of surface markers with CLL heterogeneity have been revealed.

The CD45 expression on B-CLL cells shows marked heterogeneity, ranging from the typical of B cells to the profile featured by normal peripheral T cells. The B lymphocytes of CLL appear to frequently express the lower molecular mass forms of the CD45 antigen with a concomittant reduction of CD45RA. Such a reciprocal relationship has been demonstrated by flow cytometry (Smith et.al., 1985; Maddy et.al., 1989). The continuously progressive expression of the four chains of CD45 antigen within the CLL panel suggests a relationship between the antigen expression and the physiological conditions of the cells. Considering the differential expression of the CD45RA, various cells tested can be ranked in the order of PLL, tonsillar B lymphocytes, 1A CLL, 1B CLL and T-CLL in the order of decreasing CD45RA expression. Therefore the $230KD^{+}$ and $230KD^{-}$ cells are at the both extremes along the spectrum of CD45 expression with cells bearing different combinations of the four chains in the middle. This finding is consistent with flow cytometric data by Smith et.al. (1985). The significance of this delineation is that the variation of CD45 expression is continuous and in an interrelated differential way and may indicate the differences of maturity as the expression of individual members of the CD45 antigen is controlled not only in a cell-type-specific fashion (Scheid and Triglia, 1979), but also by the maturational states of the cells (LeFrancois and Bevan, 1984; Katz et.al., 1985)

Band 2 has been described for the first time in lymphocyte. In order to ascertain whether band 2 is originated from T cell contamination in B-CLL or whether normal B cells bear this component, normal tonsillar B cells, and a B and T cell mixture (T

cells > 25%) were analysed by chromatography. The autoradiographs showed that this component was neither present in the purified B cells nor in the B+T cell mixture, suggesting that band 2 is not expressed in either normal tonsillar B or T lymphocytes. On the other hand, pure tonsillar B cells displayed a dominant 230KD band while the B+T cell mixture generated a pattern with four chains of the CD45 among which two distinct bands of 230KD and 180KD were discretly defined, the 180KD is likely to be CD45RO contributed by the T cells while pure B cells consistently lack this component.

Band 2 could be biologically interesting as its expression is closely associated with $CD45RA^{-}UCHL1^{+}$ cells, (i.e. T-cell features) and its exclusive absence from 1A type CLLs raised an interesting question about its expression. The finding of band 2 on T-CLL cells further indicates its tendentious association with cells bearing more T cell features. The enhancement of band 2 on mitogen-activated CLL B cells suggests that it might be linked with a more advanced stage of differentiation. The significance of the absence of this component in either normal tonsillar B or T cells is that this component might be specifically associated with CLL malignancy or certain stages of the malignancy. The acquisition of the component as in the Lot case described above is such an indication of staging. The reciprocal change of CD45RA and band 2 has been noticed to be concomittant with the clinic frequency of the patients as exemplified by two clinical examples abovementioned, i.e. as the phenotype was approaching 1B the clinic frequency went up, *vice versa*. But this relationship needs to be further investigated.

The expression of band 2 exclusively in 1B type patients suggests that this band is not from T cell contamination of B-CLL. T cell contamination in the B-CLL cell preparation used was routinely below 5%. Moreover, the correlated expression of band 2 with the change-over of CD45RA to 200KD-predominant in Lot. and *vice versa* in Par, further excludes the possibility of being T cell contamination and supports its validity as a marker for 1B type CLL. Actually the proportion of T cells in B-CLL decreases as the lymphocytosis increases. Therefore, it is unlikely that the enhancement of band 2 is resulted from T cells in malignant B lymphocytes. These data strongly imply that the band is in association with malignant state of B lymphocytes in B-CLL patients. But it obviously requires further study to clarify

whether the acquisition or enhanced expression of this band is associated with a certain stage of lymphocyte maturation and the prognosis of the disease.

Though little is known about band 2, its unique expression is of great interest: prolymphocytic leukaemia is negative, all CD45RA-predominant B-CLL is negative while 200KD$^-$ and 180KD-predominant B-CLL is positive, particularly those CD45RA$^-$. T-CLL is strongly positive. Evidence that band 2 may be a maturational marker of B-CLL is provided by *in vitro* activation of B-CLL lymphocytes by TPA (manuscript in preparation). CD45RA$^+$UCHL1$^-$band2$^-$ B-CLL cells were converted to CD45RA$^-$UCHL1$^+$band2$^+$ after 4-day TPA incubation. The effect of TPA on lymphocytes has been extensively described (Nadler et.al., 1981; Lida et.al., 1983; De La Hera et.al., 1988) but the acquisition of a 300KD (band 2) polypeptide upon such stimulation has not been previously reported. In other cases, the enhanced expression of band 2 in CD45RB$^+$UCHL1$^+$band 2$^+$ patients by TPA also indicates maturational differences among CLL clones. A detailed discussion is given in **Chapter 8** and the general discussion.

The variation in the expression of band 4.1 is strongly correlated with that of CD45RA although a weak expression can be detected on CD45RA$^-$ cells. De La Hera et. al. (1988) immunoprecipited a 150KD polypeptide from CD5$^+$ B-CLL cells by using HC1/1 monoclonal antibody specific for CD11c. In term of the apparent molecular weight, it seems to suggest that band 4.1 could be CD11c, but the expression of band 4.1 and CD11c with respect to cell types is distinct (De La Hera et.al.,1988). In addition, the expression of band 4.1 was reduced drastically upon TPA activation (see **Chapter 8**), in contrast to recent studies that CD11c$^-$ B-CLLs acquired the antigen after *in vitro* activation (De La Hera et.al., 1988). These data indicate that CD11c and band 4.1 are distinct proteins. The nature of this protein deserves further study.

The high expression of this 150KD protein in prolymphocytic leukaemia together with other features further substantiates the positioning of PLL along the 1A side of CLL, well in concert with its CD45RA expression and the lack of band 2.

In this study, CD21 is found in positive correlation with CD45RA, although it is expressed in some 1B type (230KD weak). However, comparing with other surface markers, such as band 4.1, CD21 lacks consistent correlation with CD45RA cross the whole patient panel. It is likely that this molecule involves central role of the complement system and acts as a receptor for the pivotal component, C3d (Weis et.al., 1984; Mold et.al., 1986) and the Epstein-Barr virus (Fingeroth et.al., 1984; Frade et.al., 1985; Nemerow et.al., 1985), and its expression is relatively stable within a narrow span of differentiation, unlike the CD45 antigen in CLL, whose expression, although also confined to a rather narrow stage, is in a differential fashion along B cell maturation. Campana et.al. (1985) and Bofill et.al. (1985) found that in subpopulations of normal resting B cells, peripheral blood, bone marrow and secondary lymphoid organs, CD21 was negative on the majority of IgM^+/IgD^- cells and positive in the majority of IgM^+/IgD^+ cells. The finding of this study, however, is not in favour of this observation as shown in Table 1. The discrepancy might be due to differences of cell types or more likely of the techniques.

The positive correlation between band 4.3 and CD45RA has been established. By SDS gel electrophoresis of non-reduced samples, band 4.3 can be readily defined. The observation in **Chapter 6** has demonstrated that band 4.3 is not CD22. At present little is known about this component but its marked expression in 1A type patients and reduced expression upon *in vitro* mitogenic activation (see **Chapter 8**) are suggestive of its possible association with cell development.

Band 7.1 shows a decreasing trend along the $CD45RA^+$ to $CD45RA^-$ sequence in autoradiographic intensity. This 78KD protein is ubiquitously expressed in most of B-CLL cells, tonsillar B cells, PLL, and probably some Namalwa cell lines (see **Chapter 9**), (but not in Jurkat line), with considerable variation of the expression level. A protein with a similar apparent molecular weight can be detected on neuraminidase-treated, sodium borohydride tritiated samples. Monoclonal antibodies G28-8 and G28-10 (Ledbetter et.al., 1987) react with and precipitate a 85KD and a 80KD peptides from tonsillar lymphocytes. The antigens have been clustered as CD39. CD39 was found restricted to B cells in peripheral blood and tonsils, about 60% B-CLL and 100% B cell lymphoblastoid lines (Ledbetter et.al., 1987). The behaviour of band 7.1 under reduction and non-reduction prior to electrophoresis is also similar to CD39 (

Ledbetter et.al., 1987). The similarities of the abovementioned characteristics between these two molecules are reminiscent of that the component may be CD39 but FACS observation (by Maddy, personal communication) did not find any correlation between CD39 and CD45RA expression in our panel, which implicates that band 7.1 is probably not CD39.

The reduced expression of band 7.1 upon cell activation (see **Chapter 8**) may suggest that this molecule functions in the regulation of B cell activation.

Band 9 is an interesting component in terms of its short retention time (8:00 min) and its consistent elution in the vicinity of CD45RA, implicating its possible association with some other molecules. Only some patients possess this band and no certain relationship has been found with either cell types or other surface protiens. The knowledge about this component is very poor. In **Chapter 5**, this band is discussed in detail.

In summary, the phenotypes of B-CLL, T-CLL and PLL as well as normal tonsillar B cells have been demonstrated inter-related with respect to their surface protein expression. On the basis of the expression of their membrane proteins, such as CD45RA, band 4.1, band 4.2, 4.3, CD21 and band 2, both in qualitative and quantitative terms, different types of cells can be arranged at such an order from PLL, normal tonsillar B cells, 1A B-CLL and 1B B-CLL to T-CLL. The significance of this finding is discussed in **Chapter 11**.

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CHAPTER 8. HPLC ANALYSIS OF TPA-INDUCED DIFFERENTIATION OF CLL LYMPHOCYTES

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CHAPTER 8. HPLC ANALYSIS OF TPA-INDUCED DIFFERENTIATION OF CLL LYMPHOCYTES

1. Introduction

Development of cells in the normal haematopoiesis is regulated by specific growth and differentiation factors and involves, in a programmed fashion, cell lineage specific phenotypic changes. These events are called differentiation and proliferation. Differentiation is a process that cells progress towards more advanced stages with specified functions which are essential for their physiological committment, whereas proliferation allows for the necessary self renewal or the expansion of the cell population. Immature cells have the highest potency of proliferation and the proliferation process comes to cease when a cell of a given differentiation lineage has reached the terminal differentiation stage. The two events appear to be coupled processes. However, in some circumstances, such as lymphoid cells and neoplastic cells, the cells appear to have either proliferation or differentiation without the concomitance of the other process.

For long, this uncoupling had been assumed irreversible until Sachs (1980) demonstrated *in vitro* in a mouse leukaemia that differentiation is inducible. It is now generally believed that differentiation in human leukaemias and lymphomas may be induced with their cellular changes towards the terminally differentiated features. According to studies in the HL-60 promyelocytic leukaemia (Huberman and Callaham, 1979; Nilsson and Totterman, 1984) and the U-937 histocytic lymphoma (Nilsson et.al., 1981; Nilsson and Totterman, 1984) cell lines, the phenotypic changes upon induced differentiation seem to be equivalent to that of normal cells.

Although increased Ig production has been found in the case of B-cell lines upon induction of differentiation, malignant human lymphoid cells show resistance to such induction. However, even the picture of the induced differentiation of B-cell lines is less than clear as only a few differentiation markers have been documented and the increase of Ig production could be simply due to a modulation of the capacity of Ig secretion (Rosen and Klein, 1983; Nilsson and Totterman, 1984). However, studies in some T cell leukaemias have demonstrated that the activated T cells undertake

phenotypic changes and develop a decreased proliferative capacity, which are also observed in normally differentiating T-cells.

Attempts to induce proliferation and differentiation in CLL cells by conventional T- and B-cell mitogens had largely failed. Later, *phytohemaglutinin* was found to result in an elevated secretion of a single isotype light Ig chain in some CLL populations (Maino et.al., 1977). Robert (1979) and Juliusson et.al. (1983) were able to induce non-Ig secretory CLL to proliferate and secret Ig using T-cell-dependent (PHA, LPS et.al.) and -independent (EBV) B-cell mitogens. The previous observations that mitogen and allogeneic helper cells/factors induced plasmacytoid differentiation in CLL cells (Fu et.al., 1979) were further extended by Kishimoto's work (Saiki et.al. 1980) and they showed that PWM and allogeneic T-cells induced Ig secretion, proliferation and class-switching in a secretory population.

CLLs of B-cell type have become a focus for the investigation of B-cell differentiation since B lymphocytes of this leukaemia are found to be readily inducible, by a phorbol ester, 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (Totterman et. al., 1980), towards maturation and to develop features of lymphoblasts-plasmablasts but without significant proliferation. The mechanism of TPA activation differs from those other stimuli by the fact that TPA does not interact with a cell surface receptor but directly activates protein kinase C, mimicking the physiological activator diacylglycerol (Niedel et.al., 1983; Leach et.al., 1983). Nilsson et.al. (1985) proposed a mechanism suggesting the involvement of small subsets of autologous T cells during TPA activation. Upon activation, B-CLL cells undergo distinct phenotypic changes in their surface antigens. A wide scale of investigation of the phenotypic changes during TPA stimulation by Caligaris-Cappio (1984) confirmed the previous results on sIg and MRBC, and showed that B-CLL cells became CD22 (RFA-4) and TRAP positive after being activated by TPA. B-CLL cells are almost totally TRAP negative with a very small population being CD22 (RFA-4) positive. CD22 is expressed by both B-PLL (Gobbi et.al., 1983) and, to a less extent, HCL cells. TRAP is virtually not expressed by PLL while high TRAP is a feature of HCL.

It is believed that TPA drives B-CLL cells, which are regarded as being blocked at certain stages of the B lineage differentiation pathway, to differentiate towards a more advanced level but it is still controversial whether the induced B-CLL cells approach PLL-like characteristics or resemble HCL cells. TPA induction of PLL-like characteristics is featured by the loss of MRBC-rosetting and the gaining of CD22 and FMC-7 (Gobbi et.al., 1983; Catovsky et.al., 1981) whereas the loss of sIg and the expression of Cyt IgM (no Cyt IgD) and of TRAP are more compatible with an early plasmacytic differentiation (Preud'Homme, 1977). The induction of hairy features in B-CLL by TPA has also been extensively discussed (Caligaris-Cappio et.al., 1984; 1985; Ziegler-Heitblock et.al., 1986). However, the conclusions are not compelling and often jeopardized by controdictory results of different surface markers examined (Caligaris-Cappio et.al., 1984). Evidence has suggested that CLL, PLL and HCL could be derived from different lineages (Yukihiro et.al., 1989). Before scrutiny of the individual differentiation markers in normal B cell maturation is accomplished, a wider investigation of surface markers may provide a more comprehensible approach for the understanding of the changes in their expression upon *in vitro* mitogenic induction.

The relationships between several surface markers established in **Chapter 7** provide a strong indication of the heterogeneity of B-CLL lymphocytes as represented by the interrelated phenotypic changes. The possible correlation of this phenotypic variation and the maturational position of the CLL cells is strongly inferred by some well-documented markers (such as sIg, CD45 etc.) (Maddy et.al., 1989). TPA stimulation of B-CLL cells would be able to further demonstrate, or even verify, these correlations. The variation of phenotypes induced by TPA might confirm a differentiation progression reflected by the phenotypic presentations of the cells.

To explore this possibility, the study of the TPA-induced differentiation was conducted by HPLC-SDS PAGE, in conjunction with FACS technique, which is proven to be a new approach for such an investigation.

2. Materials and Methods

Preparation of B lymphocytes was as described in **Chapter 2**. The cells were suspended at $1x10^6$ per ml in RPMI1640 supplemented with 10% heat inactivated FCS, 2 mM glutamine, 1 mM pyruvate, 50 units/ml penicillin, 50 ug/ml streptomycin, 5 ug/ml amphotericin, TPA at $1.6x10^{-7}$ M and A23187 at $7x10^{-7}$ M. The mixture was incubated at 37^{o} C for up to 6 days.

The TPA-treated cells were then iodinated and extracted by Triton X-100 as in **Chapter 2**. The extracts were subjected to HPLC-SDS PAGE analysis.

Cells incubated under the same conditions without TPA were used as controls.

3. Results

In this study, the expression of surface markers was investigated on 6 B-CLL cases of both 1A and 1B types (see **Chapter 7**). The cell responsiveness to TPA treatment was found to be maximal in 4 days by either flow cytometry (carried out by Maddy et.al.) or HPLC-SDS PAGE analysis. Longer incubation resulted in lower viability. Therefore, 4-day incubation was chosen for this study. 5 out of 6 patients responded well with respect to their CD45 antigen expression and other surface markers as described below. The following changes of cell surface proteins were observed after TPA treatment.

(1) There was a significant increase in the Class II MHC expression after TPA stimulation, as shown in Fig 1. 4-day TPA treatment elevated the expression of both α and ß chains of the antigen but the extent varied in different patients. Among these tested cells, one patient was found to exhibit an apparent inversion from ß-predominant to α-chain predominant after TPA incubation. No correlation, however, has been found between the Class II MHC expression and other surface antigen expression, which is consistent with the FACS analysis and the results of others (Guy et.al., 1986).

(2) The TPA treatment dramatically reduced the expression of CD45RA as shown in Fig 1 and 2, which was a 1A type patient with a dominant expression of the 230KD and 215KD chains of CD45 antigen. In other patients, of 1B type, the 215KD band of the antigen was reduced markedly with a probably lesser reduction of the 200KD component. It was noticed that the whole autoradiographic intensity of CD45 molecules decreased markedly upon TPA stimulaton, which was in contrast to the flow cytometric findings that the antibody binding of CD45 antigen remained unchanged during TPA treatment because the expression of the 200KD and 180KD (CD45RO) were enhanced, supplementing the decrease of the higher bands. But it was hard to document the alteration of UCHL1 on SDS-PAGE although a diffuse band of 180KD was detected after TPA treatment.

(3) The expression of Band 2, a 300KD component, was increased in 1B patients while in 1A patients, who lack this band naturally, gained it after 4-day TPA

treatment. The change is shown in Fig 2. The levels of the band 2 expression after TPA treatment were universely correlated with the initial CD45RA levels of the cells.

(4) Upon TPA induction, all patients consistently lost band 4.1 and band 4.3, lost or reduced CD21 markedly (band 4.2) (Fig 2). With the disappearance of CD21, a diffuse band was revealed with an apparent molecular weight of 130KD in fraction 12-14. The nature of this band and the effect of TPA on its expression are unknown at the present.

(5) All patients tested with TPA express both u and δ heavy chains on their surface as demonstrated by flow cytometry (Maddy, personal communication). The autoradiography showed a reduced expression of the sIg heavy and light chains. Research demonstrated that the percentage of sIg positive cells dropped greatly after TPA treatment longer than 3 days (Gordon et.al., 1984). This is in agreement with our findings.

(6) After surface Ig diminished, a polypeptide of 80KD was detected with an enhanced expression in fraction 10-11 (Fig 3). This component is very likely to be induced by TPA because the non-reduced SDS gels showed no trace of bands at that position of the gels when the sIg remained as an intact complex.

(7) TPA also reduced band 7.1 drastically, band 7.4 and band 7.2 to an undetectable level. The 78KD band 7.1 component had a marked response to the TPA treatment.

(8) Above the band 7.1, a component of 90KD was induced by the TPA treatment (Fig 3b). This band was not present in any untreated cells tested in this study.

(9) Upon the TPA induction, a 43KD band was increased significantly though not markedly. This band recovered from fracton 15-17 was not detectable in tonsilar B cells but is expressed in CLL B cells (Fig 3).

(10) Among the documented bands on the HPLC-SDS PAGE matrix, the most predominant component which was apparently unaffected by the treatment is band 3, a 220KD band (Fig 1 and 2). In all samples tested it neither increased nor decreased

significantly under the assay employed. Therefore, this band was used as a frame of reference to monitor the changes of other components.

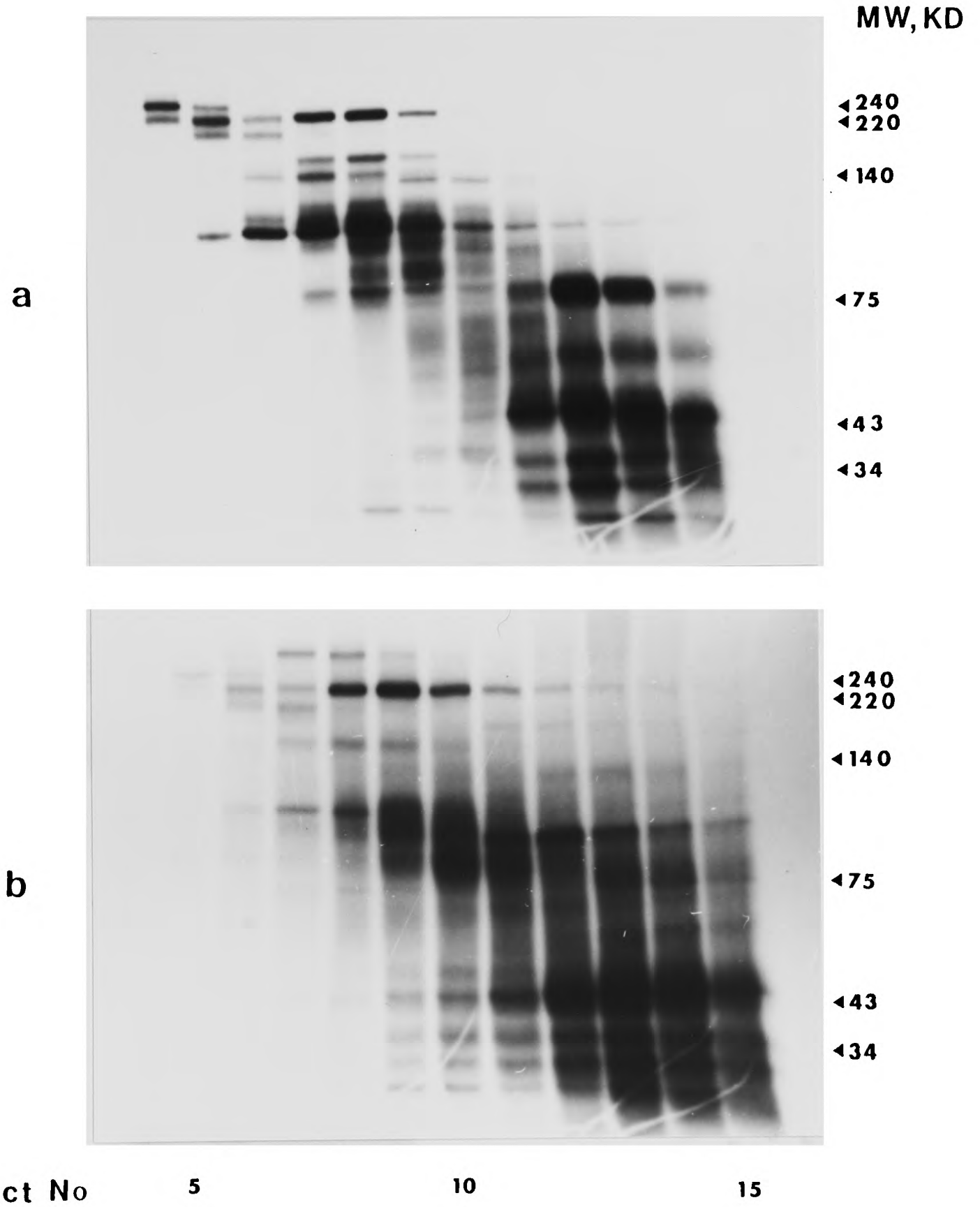

Fig 1

Fig 1. The phenotypic changes of B-CLL induced by the TPA activation. The CD45RA expression is significantly reduced, together with disappearance of band 4.1, 4.2 and 4.3 while Band 2 is induced. (a) control (b) 4-day TPA treatment. Human erythrocyte membrane proteins are used as molecular weight markers.

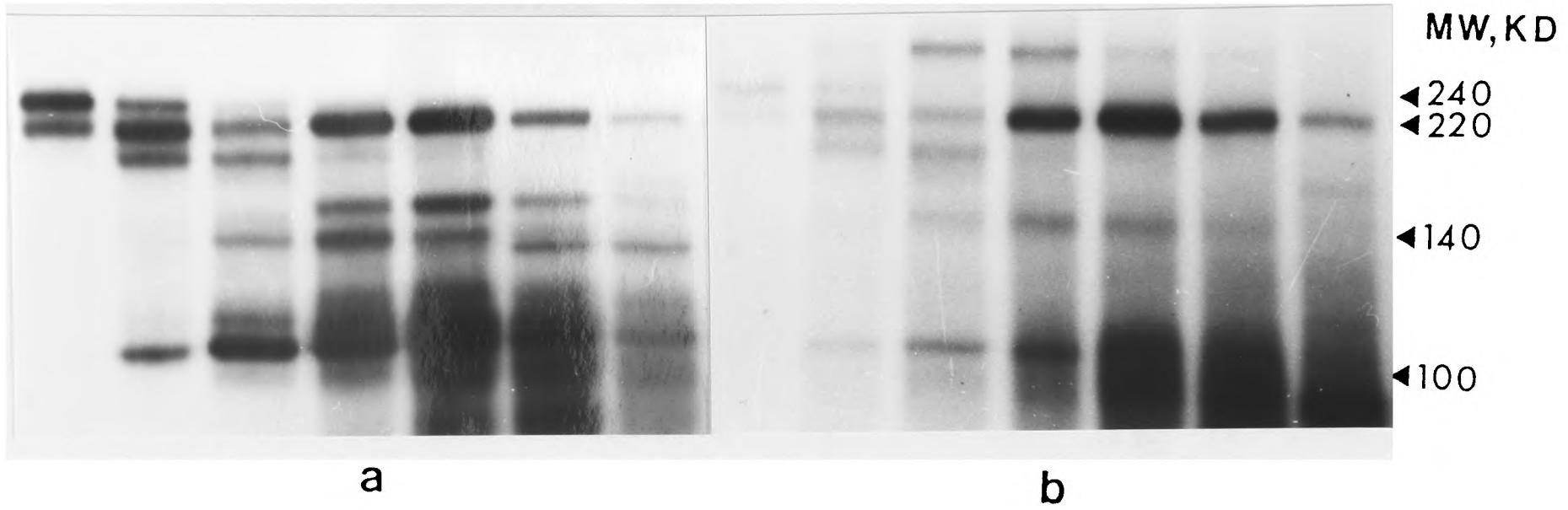

Fig 2

Fig 2. The details from Fig 1 to illustrate the induction of band 2 in the TPA-treated B-CLL cells. (a) control (b) 4-day TPA treatment. Band 3 is used as a framework of reference for the comparison.

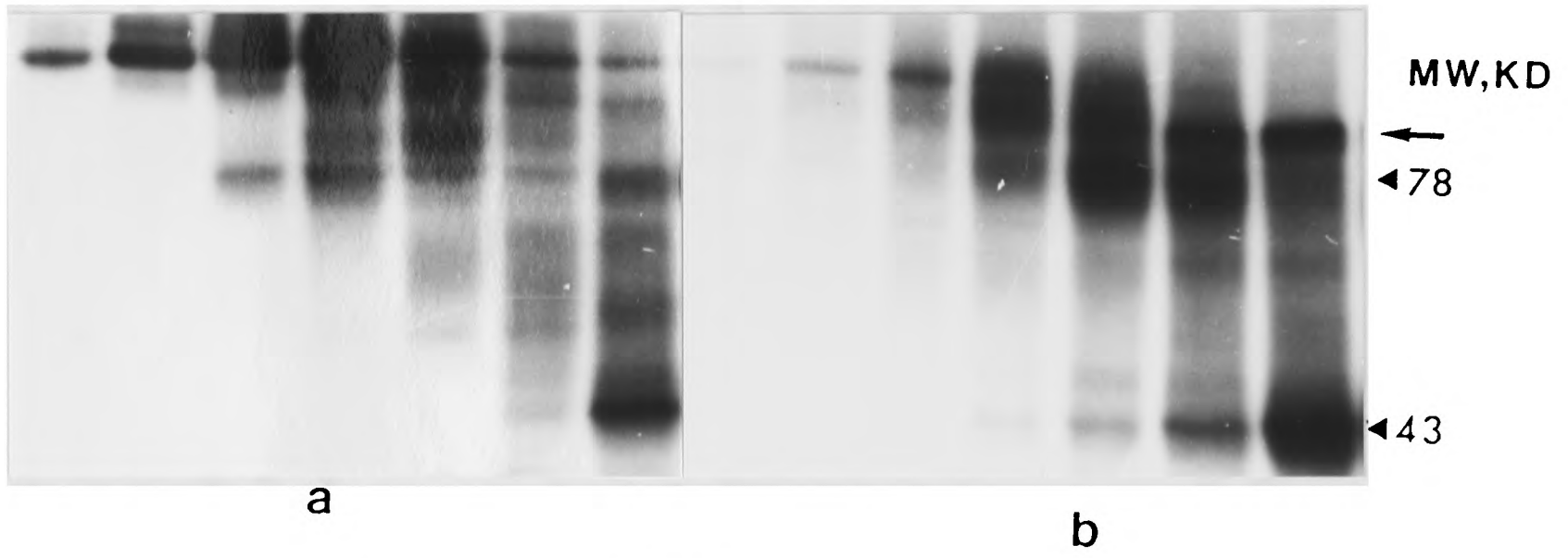

Fig 3

Fig 3. The induction of a 76KD and a 85KD components after TPA activation. (a) control (b) 4-day TPA treatment. The 85KD component is indicated by the arrow. In addition, the increase of a 43KD band is observable here.

4. Discussion

B cell differentiation comprises a series of processes during which lymphoid stem cells mature into plasma cells, with correlated changes in the expression of certain surface antigens. The heterogeneity of leukaemic cells can be interpreted as being arrested at different points of maturation along lymphocytic lineages. The cells can be induced to continue to differentiate when provided with appropriate signals.

The TPA-induced phenotypic changes observed in this study support the suggestion that the heterogeneous phenotypes of B-CLL cells could be compressed towards a more homogeneous state (Maddy et.al., 1989). Upon the TPA activation, cells from 1A type patients were induced to differentiate towards the 1B type extreme, showing decreased expression of CD45RA (Maddy et.al., 1989), CD21, surface Ig and band 4.1, with a concomittant induction of the 180KD chain of CD45 antigen as detected by UCHL1 binding (Maddy et.al., 1989), and band 2. 1B type patients showed the similar changes of their phenotypes but to a lesser extent. However, the increased or the decreased expression of the surface proteins of both 1A- and 1B-type cells appears to have actually surpassed the 1B type extreme as implied by the comparison between the TPA-induced patterns and the end 1B patterns. The acquisition of band 2 in 1A and the enhancement in 1B cells suggest its expression is associated with later stages of B-CLL cell differentiation pathway. (It seems that the differentiation diversity of B-CLL cells represents only a narrow span of this pathway while the expression of band 2 extends to perhaps more mature plasmacytoid cells as band 2, after TPA, is stronger than any 1B CLLs tested).

The overall radioactive intensity of the CD45 antigen is reduced drastically by TPA induction but the FACS study shows no significant fall of the antibody binding. This discrepancy may be due to the change of tyrosine contents when CD45RA expression inverses to the expression of lower forms of the antigen. It is shown, by protein sequencing, that exons A, B and C (see **Chapter 1**) contain 19.7%, 10.6% and 16.7%, respectively, together consisting of 47% of the total tyrosine of the CD45 external domain. Therefore, the CD45RA/CD45RO inversion will cause a significant reduction of tyrosine residues available for iodination although the antigenic epitopes may remain constant.

It is the first time that a 300KD component has been reported in B-CLL. The correlation between the band 2 expression and CD45RA/CD45RO inversion is further confirmed by the cell activation study and its association with a later stage of cell differentiation is strongly implicated. This 300KD component appears to start emerging when 230KD of CD45 decreases below the level of 200KD chain and its expression covers the whole 1B range and likely much later stages of the cells, or, at least, the stage reached by TPA-induced differentiation. The absence of this component in both normal tonsilar B and T cell gives rise to an interesting suggestion that this component is specific to malignancies. The expression of band 2 in T-CLL but not in normal T cell may also suggest that the component is associated with neoplastic state rather than cell type. The question is whether this band is specific to CLL or is also found in other forms of leukaemias? If the band is expressed by malignant cells only, what is the significance? Before these questions can be answered, it is desirable to develop a specific antibody to this molecule.

The marked decrease of sIg expression (detected by HPLC-SDS PAGE) consistent with previous reports (Carlsson et.al.; 1988; Tokumine et.al., 1989), together with the accumulation of cytoplasmic immunoglobulin (Gordon et.al., 1984; Tokumine et.al., 1989), is a strong indication of progression towards the terminal differentiation in the B-CLL populations. TPA was shown to induce CLL B cells to secrete IgM (Gordon et.al., 1984) and even a class switch (IgM > IgG) has been observed in certain CLL clones although in normal cells no overt isotype switch from surface IgM to IgG has ever been detected.

Although the vast majority of resting B cells express both CD20 and CD21, the antigens can be expressed at quite different levels. Anderson and co-workers (1985) found that human splenic ($CD20^{+}CD21^{+}$) cells responded to anti-Ig or anti-Ig and T-cell factors but not to T-cell factors alone. By contrast, however, the ($CD20^{+}CD21^{-}$) cells, while not proliferating in response to anti-Ig with or without T-cell factors, did respond and secret Ig, following co-culture with $CD4^{+}$ T cells in the absence of mitogen. The finding suggests that ($CD20^{+}CD21^{-}$) subset may represent a more mature or differentiated population and that CD21 (B2) antigen is associated with a less mature state.

TPA study also confirms the correlation between CD21 and the CD45RA expression establised in **Chapter 7**, showing that CD21 antigen is lost when 230KD decreases, which is in consistent with the FACS analysis of the same panel of patients (Maddy et.al., 1989). The observation that in 1B CLLs some cells express sIg but lack CD21 leads us to suggest that CD21 is probably lost before the loss of sIg. CD21 is lost somewhat earlier than CD20 following activation and correlates with the loss of surface IgD (Stashenko et.al., 1981) which is consistent with our findings that the TPA induction leads to the loss of both CD21 and sIg expression.

In this study, several other distinct surface proteins have been found to respond to TPA activation, which have not been reported elsewhere. In addition to band 2, band 4.1, band 4.3, band 7.1 and a 43KD and a 80KD components exhibit changed expression after TPA treatment. Band 4.1, band 4.3 and band 7.1 are of particular interest because their correlations with CD45RA are confirmed by this observation and in line with the conclusion drawn in **Chapter 7**. Although little is known about these proteins their correlation with cell differentiation appears to be strongly implicated by the evidence presented by both resting and activated (TPA induced) B-CLL cells. The significance of such a correlation will be interpreted in the General Discussion.

These lines of evidence confirm the correlations of surface proteins in **Chapter 7** and suggest that the differences of surface protein expression may result from the heterogeneity of CLL cells and that TPA does drive CLL cells to differentiate towards a more advanced stage. There seems to be an end stage at which the TPA-induced differentiation stops, as reflected by the fact that the phenotypic heterogeneity of CLLs is diminished by the treatment and similar phenotypic profiles are resulted from cells with different phenotypes.

In addition, the experimental protocol may suggest a new approach for tracing the differential expression of surface proteins during cell development. For example, leukaemic cells can be induced to differentiate to various levels of maturity by culturing with TPA in a series of different periods. The expression of the surface

markers should change in a sequential way, therefore, the expression span of some markers may be understood.

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CHAPTER 9. ANTIBODY-INDUCED CAPPING

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CHAPTER 9. ANTIBODY-INDUCED CAPPING

1. Introduction

The capping phenomenon was first observed by Taylor et.al. (1971). When lymphocytes are treated with cross-linking ligands (such as anti-sIg and Con A), the receptors on the plasma membrane, under certain conditions, undergo drastic changes in cell topology, forming clustered particles termed 'patching' or converging to a single pole of a cell called 'capping'. Like patching, capping is usually observed only with cross-linking ligands although there are several reports that certain non-physiological conditions could induce cap formation (Yahara and Kakimoto-Sameshima, 1977); unlike patching, capping is an energy-dependent process which requires energy (Taylor et.al., 1971). As the capping and co-capping phenomena involve molecular interactions, HPLC, therefore, should be a favourable method for the analysis of the molecular components present in a cap since as long as their association is resistent to Triton solubilisation the extent of the cross-linking will be reflected by the decreased retention times, thus, individual components are identifiable.

For certain receptors, ligands trigger highly programmed processes of receptor redistribution, ameboid movement and endocytosis which underpin the effector functions of adhesion, chemotaxis and secretory activity. The perturbations indicate a major regulatory influence of membrane receptors on the underlying cytoskeleton and conversely the control of cytoskeleton on the receptors. Studies in B-lymphocyte surface Ig cap formation, which has been investigated in detail, have demonstrated the corresponding involvement of microfilaments (Karnovsky et.al., 1972; De Petris, S., 1975) and the coordinated accumulation of myosin (Gabbiani et.al., 1977; Bourguignon et.al., 1978), and a-actinin (Geiger and Singer, 1979; Hoessli et.al., 1980) as well as microtubule redistribution (Gabbiani et.al., 1977; Bourguignon et.al., 1978; Yahara and Kakimoto-Sameshima, 1978). More direct biochemical experimentation has indicated the ligand-induced association of surface Ig to actin (Flanagan and Koch, 1978) and to the non-ionic detergent-insoluble cytoskeleton (Braun and Unanue, 1978). The cap formation for other membrane receptors has been assessed but the knowledge is very limited and indirect (Laub et.al., 1980).

Capping in the CLL lymphocyte has been found sluggish or not to happen at all (Cohen, H.J., 1975; Jarvis et.al., 1976) although the B-CLL cells can progress to patch formation (Karnovsky et.al., 1972; de Petris, S., 1975). Lateral movement of membrane proteins is reported to be restricted on leukaemic cells even without the presence of external ligands. It had been demonstrated that leukaemic cells and normal lymphocytes bound equal amounts of the anti-serum (Liebes et.al., 1978), therefore, the impaired capping ability of malignant cells should not be attributed to decreased binding of the cross-linking ligands but suggested defects of their cytoskeletal functions. Defects of cytoskeletal components have been reported in B-CLL and other neoplastic cells. F-actin microfilaments in B-CLL cells are shown to be abnormal and organized in a peculiar way (Caligaris-Cappio et.al., 1986), and the adherent behaviour of these cells is different from normal cells, in vitro B-CLL cells adhere to substrates by dot-shaped close contacts refered to as podosomes rather than by conventional focal contacts known as adhesion plaques (David-Pfeuty and Singer, 1980; Marchisio et.al., 1984; Tarone et.al., 1985). B-CLL cells also exhibit some other unusual features in the organisation and function of their cytoskeleton (Dighiero et.al., 1978; Liebes et.al., 1983; Stark et.al., 1984).

Both actin and tubulin, as a percentage of total protein, are decreased in CLL lymphocytes (Stark et.al., 1982; Atkins and Anderson, 1982). A novel observation (Bachvaroff et.al., 1980) was reported that both actin and tubulins were present on the surface of human lymphocytes undergoing blastogenic transformation, while normal resting lymphocytes do not possess surface actin and tubulins. Another striking feature of CLL cells is that during cap formation they lack organized bundles of intermediate filaments whereas normal lymphocytes show an intimate association between cap formation and the polymerization of the filaments (Zucker-Franklin et.al., 1979). A relevant finding may be of particular interest that Sezary cells, which are considered to be leukaemic T lymphocyte and possess an abundance of intermediate filaments (Zucker-Franklin et.al., 1974), are capable of capping as normal lymphocytes.

Luciana Bergui et.al. (Luciana Bergui et.al., 1988) reported induction of CLL B lymphocyte cap formation with monoclonal antibodies against CD5 and CD21 under a

prolonged incubation. The protocol of the present investigation is largely based on that method with some small modifications. The mild conditions used for HPLC should allow the cap with its associated cytoskeletal elements to be isolated intact. This study showed that monoclonal antibody PD7/26 (Chapter 6) and EZB17 (Brown et.al., 1985) could induce B-CLL to cap and several surface proteins co-capped with the antigen. It is, to my knowledge, the first report concerning CD45 capping and co-capping in B-CLL lymphocytes.

2. Materials and Methods

$8x10^7$ B cells from patients with chronic lymphocytic leukeamia (CLL), washed twice with ice-cold RPMI1640 containing 10% FCS, were suspended in 1 ml RPMI1640-10% FCS medium. 100 ul monoclonal antibody PD7/26 was added to the cell suspension and the mixture was incubated at the room temperature for 15 min. Unbound antibody was removed with two washings of RBC buffer pH 7.4 containing 50 U/ml penicillin and 50 ug/ml streptomycin and the cells were suspended in 1 ml RPMI1640-10% FCS medium and 25 ul (50 times dilution) FITC-conjugated anti-mouse $IgG(ab)_2$ added as a second step. The cell suspension was cultured in 37^o C with continuous rotation. At different time points from 10, 12, 15 and 18 up to 22 hours, the capping was monitored by immunofluorescent microscopy (Zeiss, Photomicroscope III). The viability of the cells was checked by acridine orange/ethidium bromide staining.

The capped cells were washed twice with WBC buffer pH 7.4 and then surface ^{125}I labelled by the method described in **Chapter 2** but with washings reduced to only once.

The conditions for HPLC chromatography and SDS-PAGE were identical to **Chapter 5** except that the collection parameters were set at: wait: 4 min and 40 fractions were collected.

Cells, treated with FITC-conjugated anti-mouse $IgG(ab)_2$ but without the first antibody, were used as a control.

3. Results

3.1. Incubation Time and Capping

The criteria for patching and capping were according to Taylor et.al. (1971), briefly, if the fluorescent ring on the cell surface clustered into discreet dots but occupying more than 50% of the circumference of the cell, it was refered to as 'patching' and if the fluorescence was concentrated at one pole of a cell, forming a bright 'cap' with less than 50% occupation of the cell surface, the cell was considered as 'capped'.

At up to 10 hours of incubation, most of the cells showed patched structures, i.e. fluorescent dots were obvious, but spreading along the circumferences of the cells. Few cells were observed with capped fluorescent poles, but some cells showed transitional states from discreet fluorescent dots to converging accumulation of fluorescence at one or two poles of the cells. 15 hours incubation showed a significant increase of capped cells. At 17 hours more than 80% cells were capped and the remainder were patched. Longer incubation did not increase the proportion of the capped cells. Actually, a decreased percentage of the capped cells was observed at 22 hours and long incubation tended to result in low viability and fragile cells. Therefore, 17 hours incubation was chosen for this study.

Control cells, incubated under the same conditions, were rarely capped and few were patched. Morphologically, they were round-shaped and no protrusion or microvillar structures were observed, in contrast to the capped cells which protruded bright poles with microvillar structures on the majority of the cells although they remained small cells.

A high percentage of PD7/26 or EZB17 treated-cells cultured with the second antibody while in the presence of 10 mM sodium azide were patched but virtually no cells were capped. The cells were morphologically similar to those of the control.

The viability of the cells was found to be usually not less than 70%. The capped cells tended to form clumps during the culture but they could be easily dispersed. It

was also noticed that the control cells appeared to give better recovery after surface labeling.

3.2 HPLC and SDS-PAGE of the Capped Materials

Fig 1b shows the HPLC-SDS PAGE two-dimensional profiles of the CD45RB antigen capped by monoclonal antibody PD7/26. Although more than 80% cells were capped only a fraction of the CD45 radioactivity was shifted. The majority of the radioactivity of CD45 antigen remained in the fraction 5-7. In addition to the shifted CD45 molecules, there were several other iodinated components co-shifted with the antigen. The bands with dramatically shortened retardation appeared to consist of two groups by their peak distributions (Fig 1b): one group, from fraction 16 to 19 (wait: 4 min), contained the shifted PD7/26 antigens and three other major bands of 78KD, 60KD and 50KD, respectively; another group, with a shorter retention time, composed of components of 78KD, 60KD and 50KD but without the shifted PD7/26 antigen. The two groups also showed distinct differences in other minor components described bellow. Fig 1 shows that the PD7/26 antigen was shifted to fraction 2-4, with a reduction of the retention time of 0.6 min. But the amount of the moved antigen was, relatively, much less than that of the other components which were co-capped by PD7/26 mAb and that of the CD45 antigen shifted under non-capping conditions (see **Chapter 6**). In the capped samples, the 78KD component was found in the fractions with a broad retention time from 5 to 9 min although the peak fractionation was at 7.2-7.8 min. A similar pattern of the distribution was also found for both 60KD and 50KD bands. The 78KD component was predominant. Comparison of the profiles of capped cells with that of the control (Fig 1a) revealed that the 78KD component may be band 7.1, or at least part of it since the fraction 15-18 of the PD7/26-capped cells contained much less band 7.1 than that of the control. The band was moved forward more than 10 fractions, ie. a reduction of its retention time by more than 2 min. Meanwhile, the heavy chain of the surface Ig was found to have vanished from the fraction 12-14, implicating its co-capping with the cross-linked CD45RB antigens (PD7/26). There seemed to be two 78KD components retarded to different extents by the column, fractionated into group 1 and group 2, respectively. They can be distinguished by their peak distributions. It is not yet

known which is the heavy chain of surface Ig or whether the surface Ig is present in both groups.

Close examination found that the 60KD component was probably from band 7.4, which is weakly expressed in most of the patients (see **Chapters 5** and 7). However, we have been unable to locate the 50KD component, which was co-capped with the PD7/26 antigen, on the control gels. In some patients, a band, usually weak, was detected in fraction 13-18 with a similar mobility on SDS-PAGE. But this band showed enhanced expression after cells were incubated under the capping conditions. The enhancement of band 7.1 expression has been ascertained (see **Chapter 8**), as also shown by the control (Fig 1a) for capping, which was under the same treatment except the addition of the first antibody.

In addition, there were some other minor components co-capping with the CD45 antigen. One was the α chain of the Class II MHC and another was a component having a slightly different molecular mass as that of the class II MHC α chain. Both were fractionated, with same retardation, in fraction 1-4. In fractions of retention time of 6.6-6.8 min, two bands were discovered, one having an apparent molecular weight of about 160KD and another 130KD. While in fractions 5 and 6, a 40KD sharp band was detected.

Initially, it was suspected that the pre-column and the inlet filters of the column would capture those extensively cross-linked materials if the capped structures were big enough. Therefore, the pre-column packing and filters were extracted with the SDS sampling buffer (see **Chapter 2**) and the extracts subjected to SDS-PAGE and autoradiography. The result ruled out such a possibility although insignificant amounts of 70KD, 60KD and 50KD bands could just be detected after prolonged exposure. However, a low molecular weight band of 10-15KD was found in the pre-column.

The PD7/26-treated cells, with the presence of sodium azide, yielded similar profiles as a normal PD7/26-treated sample except a small amount of 60KD component and the class II MHC were found in the same fractons as the shifted CD45RB.

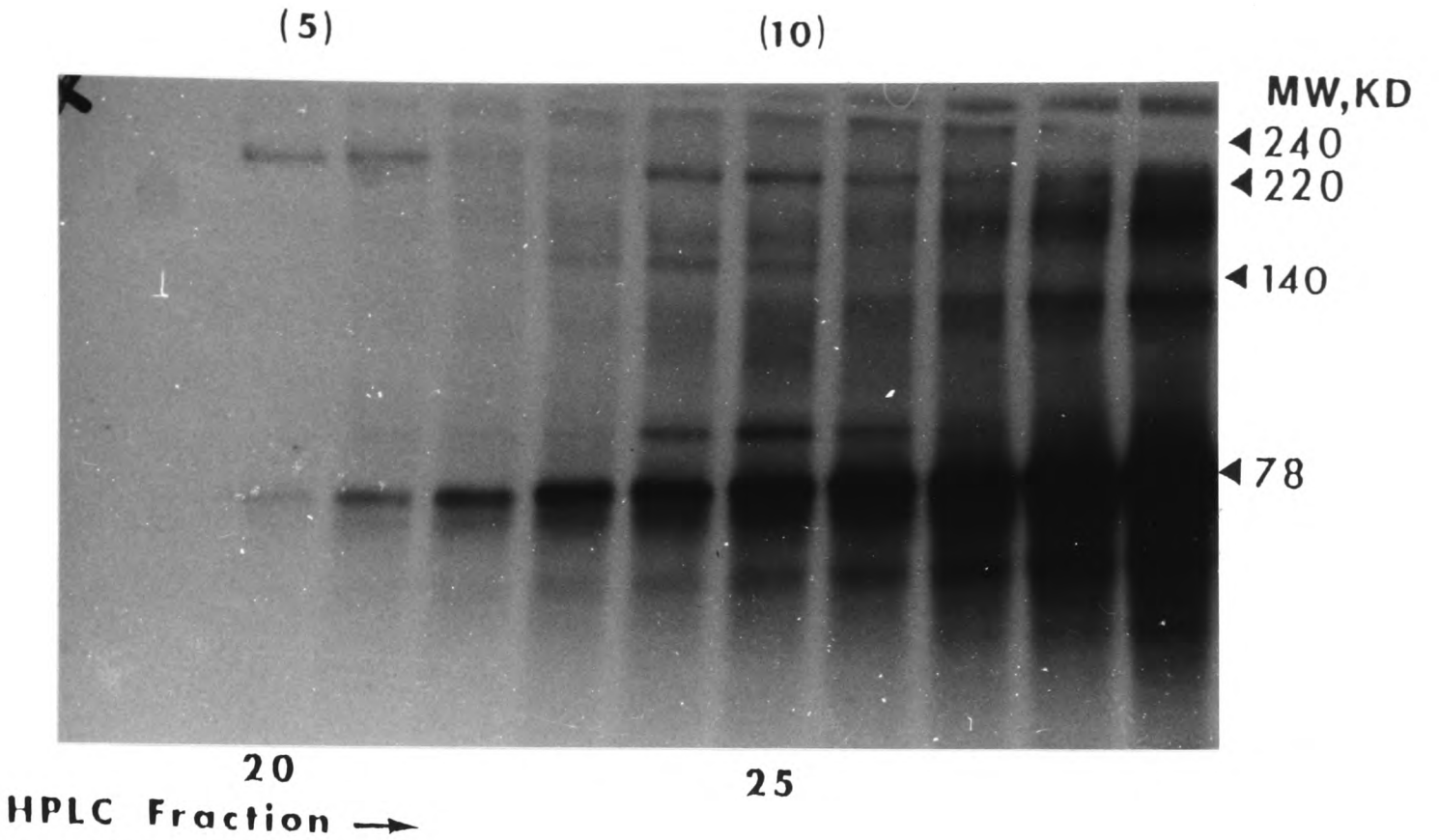

Fig 1(a)

Fig 1. The cap formation and the co-capping induced by mAb PD7/26 on CLL B lymphocytes. (a) control (b) the two-dimensional matrix of the Triton-X-100 extract of the PD7/26-capped B-CLL cells. A significant amount of materials was shifted to the front of the elution. The observation was discussed in section 2.2. Groups 1 and 2 were distinguished by their peak distributions and marked here by an arrow. The 'Time Mode' parameters were set at 'wait' 4 min., 'collection' 40 fractions. The fractions No 20-30 for the control and fractions No 15-25 for the PD7/26-capped were shown here. The numbers in the brackets indicate the fraction number when 'wait' is 7 min.

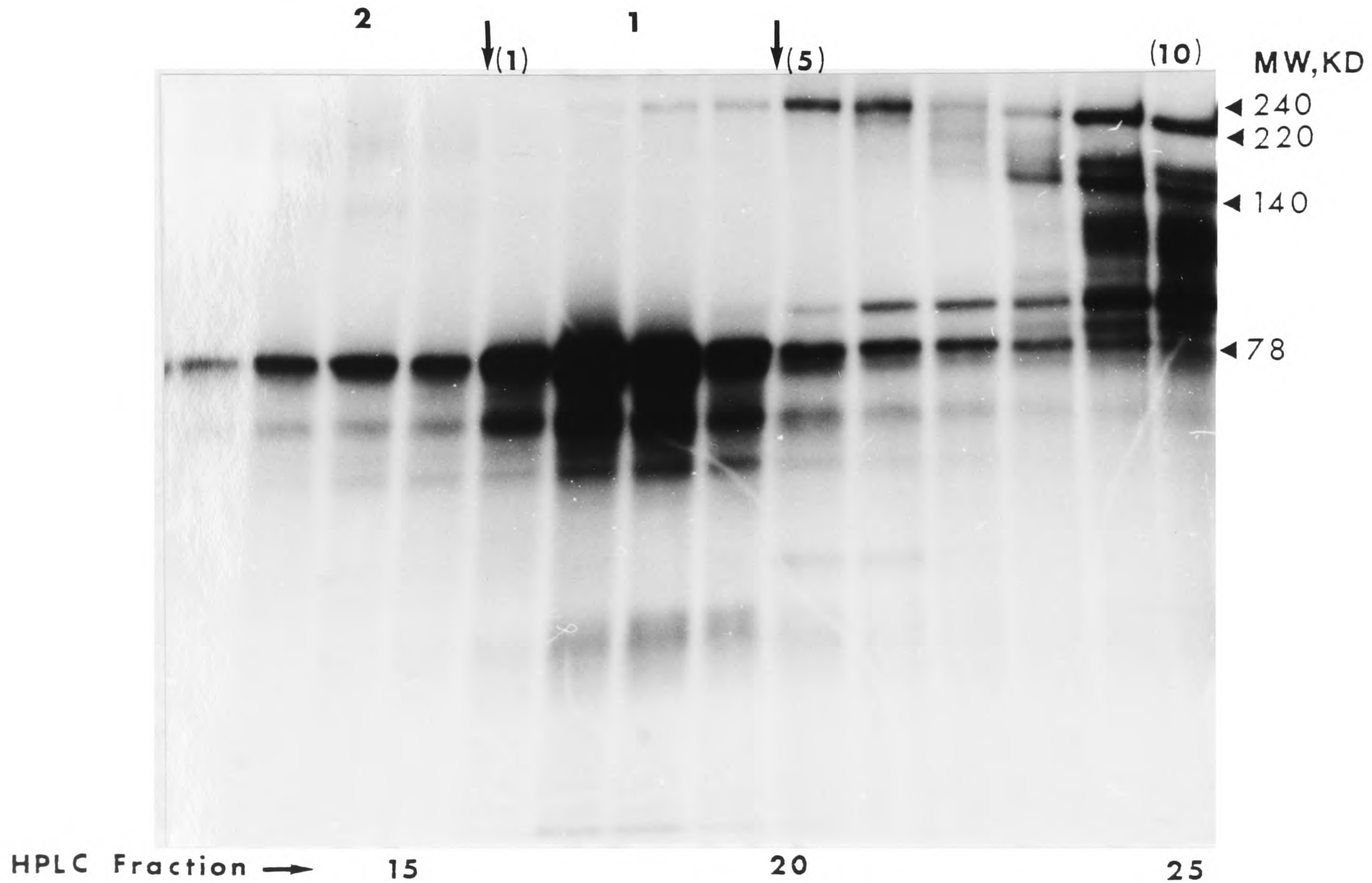

Fig 1(b)

4. Discussion

CD45 antigen is one of the major surface membrane glycoproteins. Previous studies showed that CD45 antigen was induced to form caps in various cells (Bourguignon et.al., 1978; Turner et.al., 1988). *Peanut agglutinin* (PNA) has been reported to cause co-capping of T-200 antigens with PNA receptors on thymocytes (De Petris, 1984). Cap formation of T-200 and co-capping of molecularly independent surface proteins have been reported in thymoma cell lines (Bourguignon et.al., 1978). However, knowledge of CD45 capping and co-capping of other proteins is scarce.

This study demonstrates that the CD45 antigen on B-CLL can be elicited to form caps after prolonged incubation, comfirmed Luciana Bergui et.al., and shows that 15-17 hours are sufficient for the induction of CD45 cap formation with the antibodies tested. Longer incubation is very likely to cause endocytosis or shedding which might account for the decreased percentage of capped cells observed in 20 hours upwards. Another problem caused by long incubation is that the cells are vulnerable to the subsequent washing procedures.

Although the cells cultured in the presence of sodium azide were extensively patched this lateral movement of the receptors seems to be only a physical aggregation which may not be stabilized enough to sustain the subsequent detergent treatment as implicated by the HPLC results. In this case, only a minor band of 60KD and a pair of bands of 33KD and 28KD, respectively, were shifted with the CD45 antigen-antibody complex. In considering of the high percentage of patching, such a small quantity of complexing might not be significant.

It is surprising that the PD7/26-cross-linked CD45 (as judged by immunofluorescent microscopy) is moved forward for only 3 fractions, similar to the treatment under non-capping conditions (see **Chapter 6**). It might be possible that the complex is actually disrupted by Triton X-100. On the other hand, the complex of the co-capped proteins apparently resists the detergent treatment. Another ambiguity is that the co-capped proteins appear not to be co-eluted with the capped CD45. It is not possible to explain these phenomena at this stage, but the co-capping

of proteins is undoubtly caused by the capping treatment although their relationships with the shifted CD45 are unknown.

In **Chapter 6** it was shown that CD45 antigen, shifted by specific monoclonal antibodies on HPLC column, did not show any components co-shifted with it and the antigen was absent from Triton X-100 insoluble fractions, suggesting that the CD45 molecules are not in association with other surface membrane proteins or cytoskeletal filaments. The induction of CD45 cap and the co-capping of other proteins indicate that the association between these proteins is built up during the induction of capping. Stimulation of external ligands and long incubation may initiate the polymerization of actin filaments which plays the key role in the contractile movement of the surface receptors. However, the proteins, which are elicited to co-cap with CD45 antigen (PD7/26), appear not to be associated with the detergent-insoluble fractions, suggesting that they may not be linked or extensively linked to the cytoskeletal filaments during the cap formation. (The analysis of the non-ionic detergent-insoluble material of the capped cells revealed no significant amounts of proteins bound to the cytoskeletal framework.)

So far the knowledge about the interactions between CD45 antigen and the co-capped proteins is limited and it is not yet known how these proteins are elicited to move laterally and co-cap with CD45 antigens. A possible explanation is that binding of external ligands generates signals across the membrane bilayer, which may trigger the polymerization of cytoskeletal filaments. The contractile machinery may be associated only with some membrane proteins in the vicinity of the ligand receptors. These membrane proteins provide anchorage for the underlying cytoskeleton components. Such interactions could change cell topology and the relationships between surface proteins, eliciting the lateral movements of membrane proteins which are molecularly independent from either cytoskeleton or the anchorage proteins. Spacially or functionally related surface proteins, therefore, may be clustered around the ligand receptors but not actually associated with the cytoskeleton. Selectively clustering among heterogeneous membrane proteins has been demonstrated by antibody-blocking (Flaherty and Zimmerman, 1979) and immunocytochemical (Bourguignon, 1979; Raz and Bucana, 1980; Kuby and Wofsy, 1981) studies. In certain phospholipids (Schroit and Pagano, 1981) and glycolipids (Spiegel and

Wilchek, 1981), similar clustering has also been observed, and it can be interrupted by cytochalasins and metabolic inhibitors. The function of such clustering is not clear, but it could, aside from direct transmembrane anchoring and molecular filtering (Bretscher et.al., 1980), implicate the membrane protein interactions without the involvment of cytoskeleton.

The co-capping phenomenon observed in this study implies that molecularly independent proteins in the unperturbated cell membrane can be induced to interact with the antigens bound by specific ligands. This is in conflict with the view that molecularly independent receptors cap independently of one another (Schreiner and Unanue, 1976) but in agreement with Turner et. al. (1988). The data in **Chapter 6** illustrate that the CD45 antigens are not associated with any other membrane proteins. The observed association in this study must be triggered by the signals delivered by the ligand binding. Similar findings have been reported in mouse cell lines (Bourguignon et.al., 1978). Rabbit antibodies to T200 antigen induce co-capping of H-2, TL and Thy-1 antigens with T200 cap whilest it has already been demonstrated that TL, H-2 and Thy-1 antigens are molecularly non-related components in thymus cell membranes (Loor et.al., 1975).

Braun and Unanue (1978) proposed that the attachment of the surface receptors to the cytoskeleton occurs at the patching stage before the actual cap formation. The results presented by this study appear to argue against that point in two aspects: (1) the HPLC-SDS PAGE profiles of the patched cells showed, basically, no differences from the control. If the cytoskeletal filaments formed associations with the membrane receptors, one should expect altered retardation of some proteins. On the other hand, majority of the surface proteins from the patched cells were solubilized by Triton X-100. (2) the HPLC profiles of the patched and the capped cells exhibit distinct differences which can only be generated by irreversible capped complexes. There have been some reports that paching is not a pre-requisit for cap formation (Bourguignon et.al., 1981). A possible mechanism could be as proposed above, i.e. at the patching stage the cytoskeletal filaments are only associated with those anchorage proteins located at the vicinity of the receptors, which can be visualized by immunofluorescent microscopy, but not connected to the receptors.

These explanations are probably premature as several observations in this study are still obscure and contradict with conventional view of capping event. Much future work needs to be done to both clarify and elabrate this preliminary observation of the capping phenomenon by HPLC. The first ambiguity to be clarified is whether the capped structure can remain intact after the detergent treatment. Application of antibodies to other surface markers may also help to elucidate the observations of this study.

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CHAPTER 10. THE STUDY OF BURKITT'S LYMPHOMA CELL LINE NAMALWA

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CHAPTER 10. THE STUDY OF BURKITT'S LYMPHOMA CELL LINE NAMALWA

1. Introduction

The Namalwa cell line is a human B-lymphoma line originated from the tumour cells of an African patient with Burkitt's lymphoma (BL) (Klein et.al., 1972). The Namalwa sublines (KN2, PNT, IPN/45 and 45.43) have been maintained as independent sublines for years (Jeffreys et.al., 1985). The cytogenetic data (Soos et.al., 1981) ascertained that the different sublines were derived from the same individual.

Heterogeneous expression of surface receptors has been demonstrated in Namalwa sublines. These sublines showed marked differences in the expression of surface immunoglobulin and MHC Class II antigens (Guy et.al., 1986; Guy et.al., 1987), suggesting differentiation arrest at different stages of the pathway. Phenotypic variation of the Namalwa lines has also been investigated with specific monoclonal antibodies to surface antigens, such as CD21, CD20 and CD37 et al. (Guy et.al., 1986). Rooney et.al. (1986) have concluded from the changes of cell surface antigens that cells from some freshly established Burkitt's lymphoma cultures progressed towards a more mature (lymphoblastoid-like) state.

The technique established by this study has provided, in principle, an effective methodology for the analysis of surface polypeptide constitution and the detection of differences in receptor expression which might be difficult to observe with conventional methods. The application of HPLC-SDS PAGE technique to the closely related Namalwa sublines presented an opportunity to test the resolution of the method and it has confirmed the considerable potential of the method in distinguishing minor diferences of the cells.

2. Materials and Methods

Namalwa cells were pelleted out of the culture medium, washed and surface radio iodinated as described in **Chapter 2**. A 0.5% Triton X-100 extract was made and analysed by HPLC and SDS-PAGE as in **Chapter 5**.

The nomenclature system for B-CLL lymphocyte surface phenotype is used here but the identity of the nomenclature does not necessarily imply the identity of the molecules or similarity of their functions.

3. Results

The autoradiographs were examined and compared between four sublines KN2, PNT, IPN/45 and 45.43. HPLC fractions containing bands of particular interests from the four sublines were analysed on the same gels to facilitate the comparison. In addition to surface Ig and MHC Class II antigens, several distinct differences were found among the sublines.

45.43 was the only subline which expressed three high molecular weight chains of the CD45 antigen (Fig 1). The lower chain (200KD) was predominant. None of the other three sublines were found to have the third band, they were all predominantly expressing the highest component (230KD) of the CD45, with a weaker 215KD band. The order of the 230KD band of the CD45 expression in ascent was 45.43, KN2, PNT and IPN/45. It was found that, with respect to the autoradiographic level of band 3 (220KD), a consistent component which has been previously used as a frame of reference (see **Chapter 5**), the KN2 subline showed somewhat weaker overall CD45 expression while IPN/45 and PNT were considerably higher.

All four sublines possessed a faint band of an apparent molecular weight of 300KD, which in B-CLL lymphocyte is designated as band 2. By comparing different batches of autoradiographs with each other, it was concluded that 45.43 was the strongest in the expression of 300KD component and KN2 was the weakest while the rest were at intermediate. Because of limited numbers of the sublines available, no coincidental relationship has been established between this component and others.

A diffuse band with an apparent molecular weight of about 150KD appeared in exceptionally early fractions (retention time shorter than CD45), usually 1-2 fractions before that of CD45RA. All four sublines were positive and probably expressed the component at different levels. No equivalent band has been detected in CLL B cells.

Both band 4.1 and 4.2 (CD21) were observed in all Namalwa sublines although the levels varied between the sublines. 45.43 exhibited very strong band 4.2 (CD21), followed by PNT and IPN/45. KN2 was the weakest in band 4.2 expression and the

overall intensity of both band 4.1 and 4.2 was low. The intensity variation of band 4.1 was not as marked as band 4.2 (CD21) and it was difficult to draw a conclusion with respect to its relative level of expression.

A band which was detected only in Namalwa cells was apparent in fractions 7-9 of all four sublines. The component had an apparent molecular weight of 130KD and was strongly expressed in 45.43 and comparatively weak in KN2. IPN/45 and PNT showed a similar level of the band. Another band with similar molecular weight but in fractions 10-12 was found to be considerably higher in 45.43 than other three sublines. It was one of the predominant bands detected by autoradiography in Namalwa cell line. This component was named as band 5 in B-CLL lymphocyte but generally at a much lower level.

Fig 1 shows band 6, a component of 110-120KD highly expressed in 45.43 and its intensity varies in the different cell lines. The four sublines can be placed in the order of 45.43, IPN/45, PNT and KN2 with descending expression of the component.

A 80KD component was found in fractions 10-12 of PNT and IPN/45 sublines but markedly decreased or absent in 45.43 and KN2. A similar component has been identified in B-CLL cells to be the heavy chain of the surface immunoglobulin. A band of 23KD was located in the same fractions and absent from both 45.43 and KN2. This band in B-CLL has been demonstrated to be the light chain of the surface Ig. SDS-PAGE analysis of the non-reduced fractions containing these bands showed a high molecular weight band of about 300KD when both 80KD and 23KD disappeared, confirming that two bands were the heavy and light chains of surface Ig, respectively. In autoradiographs, the expression of the surface Ig was found high in IPN/45 and PNT but undetectable in 45.43. KN2 showed low level expression observable after prolonged exposure.

MHC Class II was highly expressed in KN2 (Fig 3) and very weakly in 45.43 while PNT was higher than IPN/45. The four sublines can be ranked in the order of 45.43, IPN/45, PNT and KN2, according to their increasing expression of Class II MHC antigens. A novel difference was noticed that the autoradiographic intensity of

the ß chain of the molecule in KN2 was much higher than the α chain while in other sublines both were of very similar levels.

Fig 3 shows a 50KD component in fractions 14-16. The component had significantly higher expression in KN2 and PNT than in IPN/45 and 45.43 in which it was almost undetectable. Its expression and molecular weight are reminiscent of the αchain of Class I MHC antigen.

A sharp band at the position of about 75KD was found in all the HPLC fractions except, usually, No 1-3 fractions. All four sublines had such a band and the band was consistently present at the same molecular weight range. Occasionally, similar band was detected in some B-CLL cells but, unlike in Namalwa cells, the band was not reproducible in B-CLL. The nature of the component is not yet understood but it is likely to be an artifact.

In comparison with the profiles of B-CLL lymphocytes, the Namalwa cell lines are very similar to B-CLL but bear some distinct features in their phenotypes and the designation system established in **Chapter 5** can define most of the components in four sublines studied. However, the Namalwa cells tended to generate poorly defined and diffuse bands. At the low molecular weight range of SDS-PAGE, bands were usually less sharpened and resolved. The background density of the autoradiogram in such areas could be high.

Fig 1. HPLC-SDS PAGE profiles of the Namalwa cell lines, showing the progressive decrease of 230KD determinant of CD45 antigen and the changes of other surface components. (a) INP45 (b) PNT (c) KN2 (d) 45.43. The samples were analysed on a 6-11.5% polyacrylamide SDS-PAGE gradient gel.

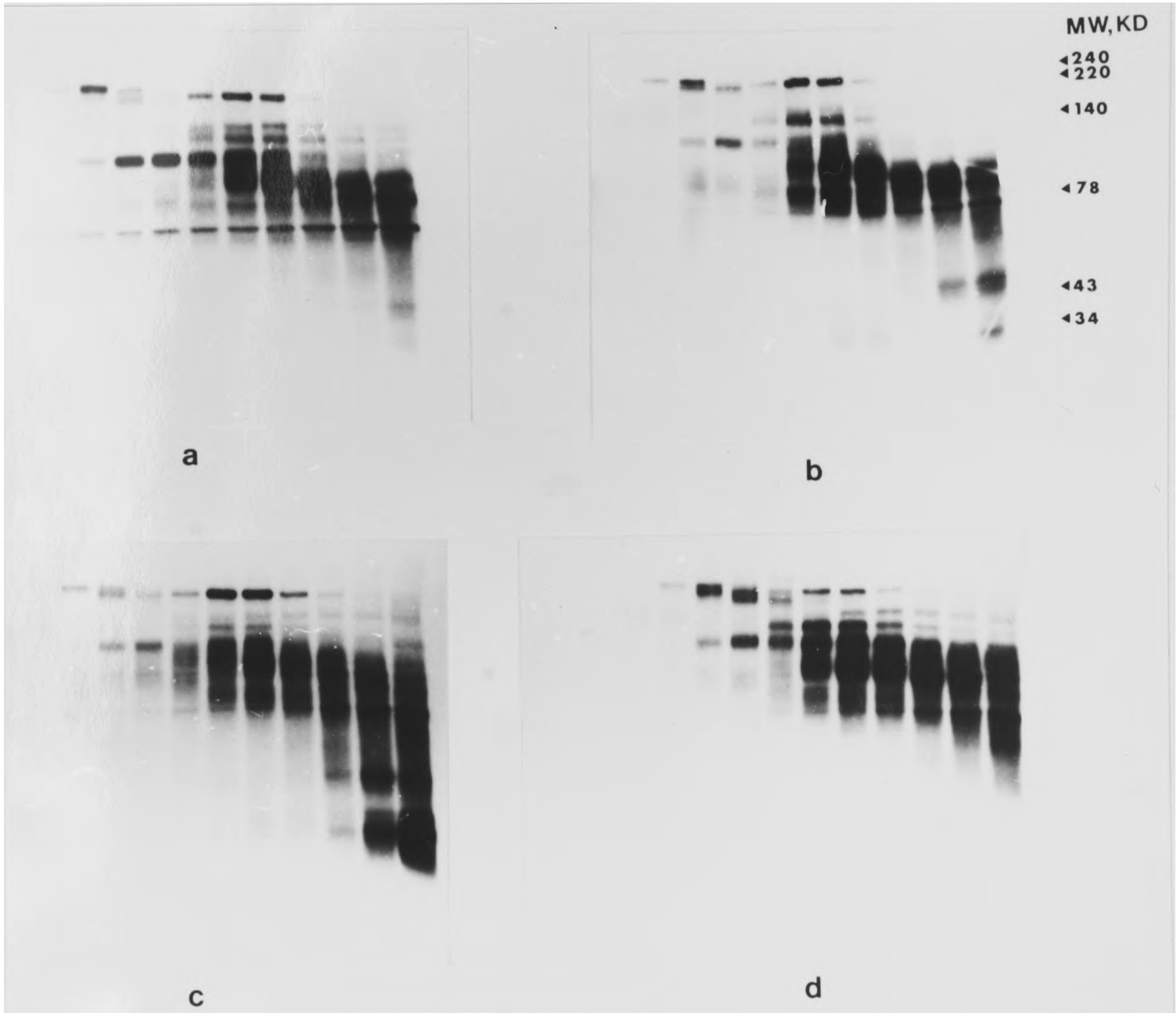
MW, KD
240
220
140
78
43
34
a
b
c
d

Fig 2. Detailed illustration of fractions 7-9 of the cell lines to show the differences in CD45 and 125KD expressions. (1) KN2 (2) IPN45 (3) PNT (4) 45.43.

Fig 3. Detailed examination of fractions 13-15 of the KN2 and 45.43 sublines on their expressions of MHC Class II and a 43KD component. The left panel is KN2 and the right panel is 45.43.

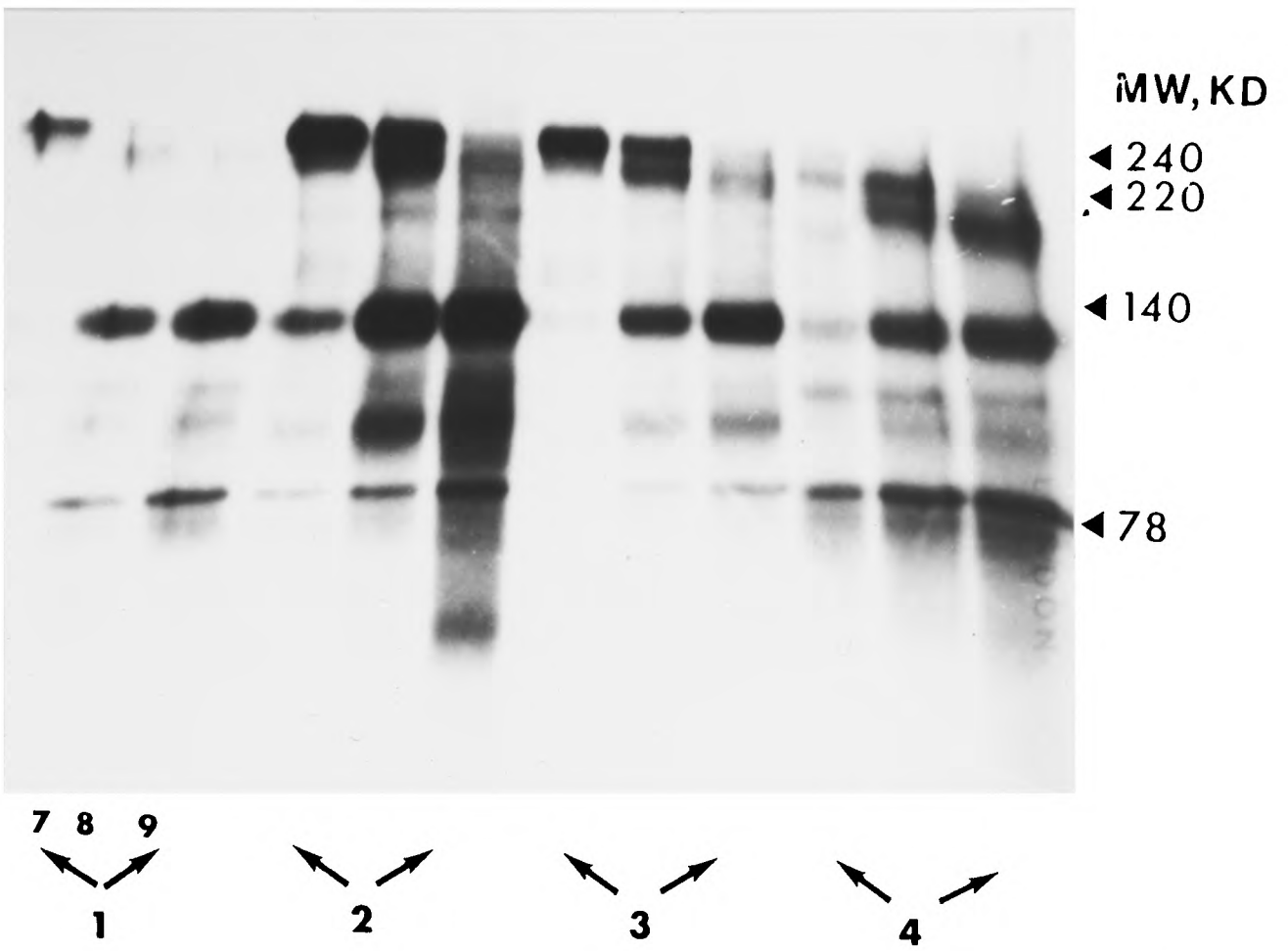

Fig 2

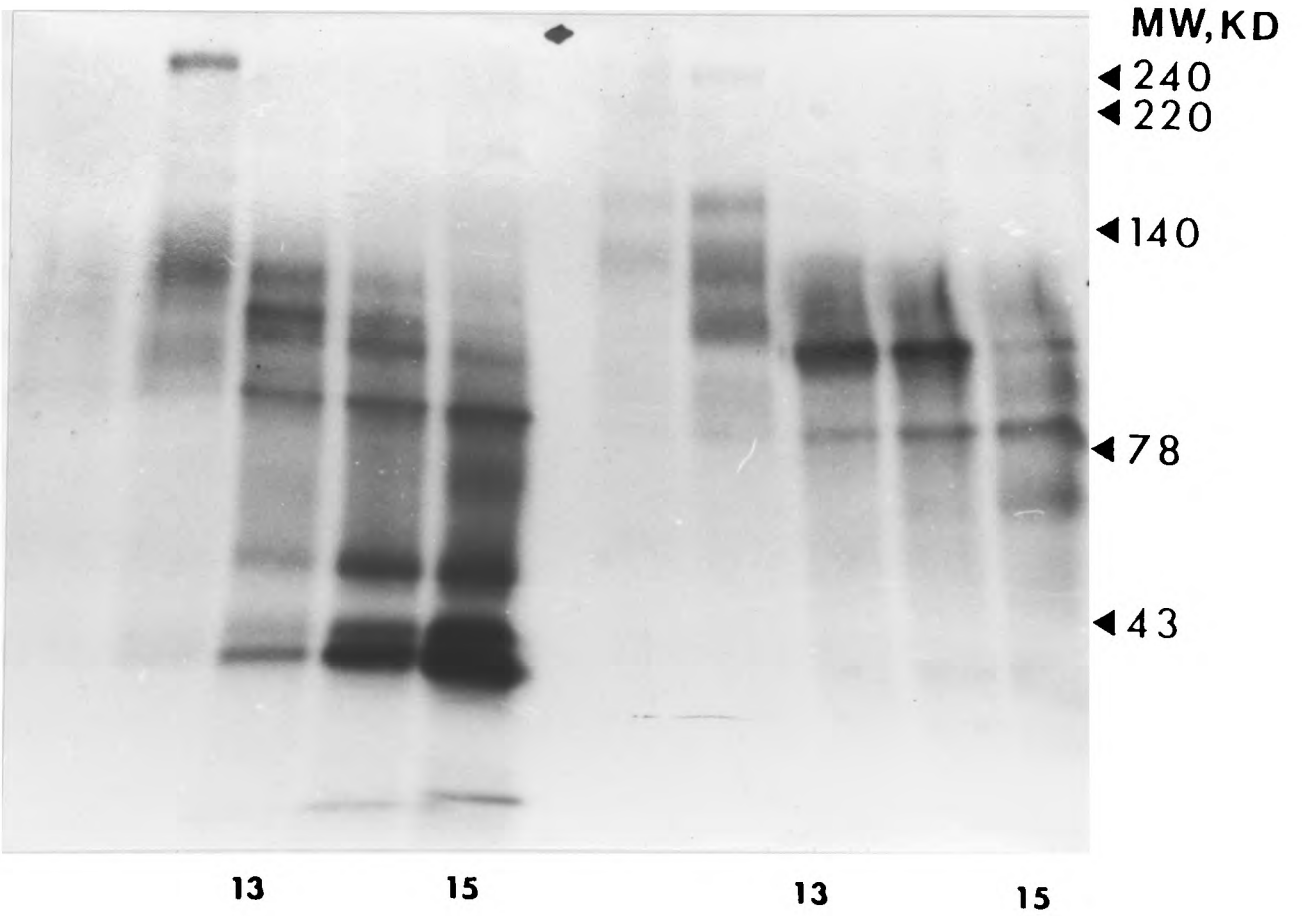

Fig 3

4. Discussion

Limited investigations have been conducted on the phenotypic variation of the Burkitt's lymphoma cell line Namalwa. The four sublines (KN2, PNT, IPN/45 and 45.43) have been demonstrated to have marked differences in the expression of both Ig and MHC Class II antigens and, recently, differing patterns of expression of cell surface differentiation antigens were investigated by using the CD monoclonal antibodies (Guy et.al., 1987).

According to the relative autoradiographic intensity of 230KD chain of CD45, the four Namalwa sublines can be placed in the order of IPN/45, PNT, KN2 and 45.43 with respect to the decreasing levels of the antigen expression. This conclusion is in consistent with specific monoclonal antibody binding data (unpublished results). The order of increasing F8-11-13 binding in KN2, PNT and IPN/45 sublines as shown by FACS is the same as that of the 230KD levels detected by autoradiographs, implying that these three sublines are closely related in differentiation as the reciprocal relationship of the expression of the four CD45 components is suggested by cell activation study (see **Chapter 8**). The levels of an overall CD45 expression were found, by flow cytomerty, to be in the same order. By contrast, although the CD45 binding in 45.43 was indistinguishable from the other three sublines F8-11-13 binding was markedly low (26% cell positive). The FACS data of CD45 and CD45RA binding in 45.43 subline also suggest that the decrease of CD45RA is compensated by the increase of the lower component (the 200KD component) of the antigen, which was clearly exhibited by the HPLC-SDS PAGE autoradiographs. If the abovementioned coincidence of F8-11-13 binding and the 230KD intensity does implicate the distance of the maturational states of the sublines along the maturational pathway, the weak 230KD and strong 200KD of CD45 and low F8-11-13 binding (as detected by FACS) in 45.43 suggest that the subline must be at distinctly different stages of maturity.

The positive correlation between 230KD band of CD45 and the surface immunoglobulin (sIg M) may exist in the Namalwa cells whereas they were found to be strongly correlated in B-CLL. However, this conclusion is of less certainty because the variation of CD45 antigen expression is very limited to a narrow spectrum within

only four sublines and 45.43 did not have any detectable surface and cytoplasmic Ig which indicates its anomalous state.

It is difficult to construct a relationship between the 230KD or the 200KD chains of the CD45 family and the 300KD high molecular weight component as in B-CLLs because of limited observations. One striking difference of Namalwa cells from B-CLL lymphocytes is that in Namalwa cell lines, the expression of this 300KD component is not exclusive to the low CD45RA-high 200KD cells, actually all sublines are 300KD positive, although the higher the 230KD band is the weaker the 300KD component is expressed.

The Ig synthesis has been correlated with Class II MHC expression (Guy et.al., 1986) by flow cytometric studies. Except for 45.43 subline which expressed no detectable cytoplasmic or secreted Ig, it is found that the Class II MHC and cytoplasmic Ig expression have a reciprocal relationship with CD45RA expression although, in B-CLL lymphocyte, no such a correlation was ever confirmed between Class II MHC and CD45RA by autoradiography (see **Chapter** 7). There are some findings suggesting that the expression of Class II MHC antigens and immunoglobulin may be regulated under some common mechanisms. In both normal and malignant B cells, elevated level of Class II MHC was detected on cells activated by antibodies to surface Ig (Kehrl et.al., 1985; Godal et.al., 1985). Dramatic increase of Class II MHC expression has been observed on B-CLL lymphocytes which were treated with phorbol ester and thereby induced to differentiate (Totterman et.al., 1981; also see **Chapter 8**). Biddison et.al. (1983) suggested that the increased levels of Class II MHC antigens on activated B cells may induce the activation of helper T cells and hence the synthesis and secretion of B cell growth and differentiation factors (Kehrl et.al., 1984). Therefore, elevated levels of Class II MHC may reflect more advanced stages of differentiation or activation of the cells. From this point, the ascending expression of Class II MHC in IPN/45, PNT and KN2 is in concert with the order of the CD45A expression, i.e. KN2 may be at a more advanced stage while IPN/45 at an earlier stage with PNT in the middle. With an exception of 45.43 subline which has no detectable MHC Class II antigens, the correlations between the order of MHC Class II expression and the order of the 230KD chain of CD45RA and sIg expression are consistent with the observation conducted in B-CLL with TPA (see **Chapter 8**),

i.e. MHC Class II and surface Ig expression was markedly increased with a reduction of the 230KD component of CD45RA expression when CLL B cells were activated to differentiate by TPA (see **Chapter 8**). Based on this assumption, IPN/45 may represent the least mature stage of differetiation in four sublines while KN2 or less likely 45.43 the most advanced stage, in contrast to the proposal by Guy et.al. (1986).

The variation of 50KD component is of interest with regard of its molecular weight similarity to Class I MHC which was reported to be lowly expressed in 45.43 and comparatively high in KN2, PNT and IPN45 (Guy et.al., 1986). The results with radiobinding assay, by using monoclonal antibody MHM5 (Guy et.al., 1986), is in consistent with HPLC-SDS PAGE data of the 50KD component, showing that increasing Class I MHC and 50KD expressions are in the order of IPN/45, PNT and KN2 and illustrating the deficient expression in 45.43 subline. However, the identity of this component needs to be confirmed.

Studies have identified that there is impaired ability to synthesize either Ig heavy or light chains in Burkitt's lymphoma cell lines (Benjamin et.al., 1982; McIntosh et.al., 1983). Nevertheless, the majority of the Burkitt's cell lines are reported to synthesize both heavy and light chains (Gunven et.al., 1980). The flow cytometric study (Guy et.al., 1986) of the cytoplasmic and secreted Ig in Namalwa cells supports this suggestion. The negative staining of the 45.43 subline in either cytoplasmic or surface Ig is in concert with its very low Class II MHC expression and places itself at an anomalous position in relating to other sublines. This subline was found to be unstable in MHC Class II expression (Guy, personal conmmunication) although it was never high. With HPLC-SDS PAGE analysis, 45.43 was also found to be susceptable for change in some surface glycoproteins (data not shown). Therefore a question is raised whether 45.43 is a monoclonal line or actually a polyclonal or whether it is arrested stably at a certain differentiation stage. Further study is needed to elucidate these points.

Band 4.2 expression is positively correlated with 230KD of CD45 as established in B-CLL lymphocyte, with an exception of 45.43 subline which was strongly band 4.2-positive by autoradiography. Although band 4.2 has been identified to be CD21 in B-CLL lymphocyte the HPLC-SDS PAGE observation in Namalwa cells seems to be not

in conformity with the data by indirect immunofluorescent assay which showed that only 11-24% 45.43/2 (a subline of 45.43) cells were positive while more than 50% PNT cells were stained by CD21 antibodies (BL-13, HB5 and F97-6B3) (Guy et.al., 1987). The data show great contrast to the radiolabeling observation which demonstrated 45.43 subline to have highest band 4.2. The discrepancy is not understood.

Band 4.1 has the same positive relationship with 230KD band of CD45 as band 4.2 does in all sublines apart from 45.43 which does not show the correlation.

There are at least two components expressed only by the Namalwa lines but not other cell types examined. One is the 130KD predominant band which is absent from all B-CLL, T-CLL and PLL. With respect to its apparent molecular weight, this band is reminiscent of band 5 in B-CLL, but is present in much earlier fractions and clearly distinguishes itself from the band 5 (Fig 2) which is also expressed in all Namalwa sublines. The 130KD, thus, is a distinct component of Namalwa cells and shows considerably variable levels of expression. CD22 seems to be a possible candidate for this component and the expression of CD22 in Namalwa cells, detected by FACS (Guy et.al., 1987), was relatively high, in consistent with the results by autoradiography. The anomalously short retention time of this 130KD band may be explained by the possibility of complexing with some other components.

The 150KD band is another distinct component on Namalwa's surface phenotypes. The retention of the molecule by HPLC column (GF-450) is nearly the same as band 9 defined in some B-CLL lymphocytes. But they are very unlikely to be the same molecules because of the markedly differing molecular weights unless they have greatly differing levels of glycosylation. Actually, the band profile is very diffuse and with features of being a highly glycosylated protein. A SDS-electrophoresis with reduced and non-reduced samples would help to elucidate the identity of the component.

As both are derived from the B lymphocyte lineage, the overall profiles of the Burkitt's lymphoma cell line are similar to CLL B lymphocytes. Individual sublines show limited heterogeneity in their surface phenotypes, suggesting their deviation and

differentiational differences of the maturational pathway. From the results discussed above, it appears that the shift of the surface phenotypes in Namalwa line along the differentiation pathway compares well with that of B-CLL and the correlations of surface proteins and maturation established in B-CLL may be applicable to the Burkitt's lymphoma cells. With regard of this, KN2 could be more mature than IPN/45 while PNT can be possibly placed in the middle. 45/43 subline is anomalous with respect to its surface phenotype and this phenomenon is not understood. The identity of of the four sublines has been confirmed by 'DNA fingerprinting' with the 33.15 hypervariable minisatellite probe (Jeffreys et.al., 1985) and cytogenetic research (Soos et.al., 1981). These studies established that the different Namalwa sublines are derived from the same individual. However, the poor expression of what is normally considered to be a pan-B marker on a substantial proportion of Burkitt's cells is of interest, for example, CD20 (B1) whose expression is across the whole span of the B cell differentiation untill the terminal differentiated plasma cells is absent in 45.43 subline. While CD37 is usually highly expressed in more mature cells, such as hairy cell leukaemia, it is negative in 45.43. Caution must, therefore, be excercised in the interpretation of these data with respect to the maturational stages. One point needing to be particularly born in mind is that these cell lines have been cultured for a considerable period. Cells cultured *in vitro* could have down-regulated synthesis of the antigens. (foetal calf serum was reported to have stimulatory effects on cultured cells (Coutinho and Moller, 1973; Okamura et.al., 1982)) and individual proteins may be affected to diferent extent. Therefore, the variability of antigens seen in the Namalwa cell line may differ from that in freshly prepared neoplastic B cells.

The study of Namalwa cell lines by HPLC also shows considerable potentials of the technique, particularly in detecting minor differences which could be difficult extremely valuable for those unknown components and for the study which does not require complete separation of the constituents.

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CHAPTER 11. GENERAL DISCUSSION

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The establishment of the HPLC-SE technique in this study has facilitated the understanding of cell surface protein expression, particularly those of CLL B lymphocytes. The surface protein expression of B-CLL cells is found to be interrelated and this relationship can be extended to other types of malignant cells. A number of new surface proteins have been identified and their expressions are also confined to the relationships existing between other surface markers. The correlation of these surface proteins with the state of cell maturation is discussed here.

1. Differential Expression of CD45

The present investigation has confirmed the previous findings that the expression of the CD45 determinants by different B-CLL patients varies from typical B cell profiles (1A), through a series of intermediate forms, to T cell-like patterns (1B) as characterized by 230KD/180KD inversion (Smith et.al., 1985; Roxburgh and Cooper, 1987). This conclusion is also supported by a direct comparison of overall phenotypic profiles of normal tonsilar B cell, B-CLL and T-CLL, which is made possible by the HPLC method. It is not yet known whether this CD45RA/CD45RO switch is a progressive event for each cell or whether it is changed by altering the proportions of different subpopulations which bear certain determinants. But in this study discrete pattern, such as $230KD^{+}215KD^{-}200KD^{+}$, has been observed, which may implicate that the proportions of cells carrying distinct CD45 expression are changed. The present observation suggests that the diversity of CD45 expression may reflect the heterogeneity of B-CLLs and the differences of their maturational states. Kerray et.al. (1987) and Dominis et.al. (1987) suggest that CLL consists of heterogeneous populations which is in agreement with this study.

2. Correlation of CD45 with sIg, CD21, Band 4.1, Band 4.3 and Band 2

The earlier reports have demonstrated that the average level of surface immunoglobulin expression in B-CLL is lower than normal lymphocytes (Dighiero et.al., 1980; Brown et.al., 1987) and that the expression of CD45 seems to be correlated with that of sIg. The present observation has largely confirmed the above

findings. The fact that the majority of B-CLL bear sIgM, sIgD or both and occasionally cytoplasmic Ig positive indicates that B-CLL may be at a mature B cell stage of normal differentiation. However, this interpretation is jeopardized by the complexity of sIg expression (Ling, 1983) and by the fact that details of the time course of the appearance and the expression levels of sIg and its modulation are not fully understood.

The restricted CD45 (CD45R) is found on cells of most cases of non-T-ALL which is regarded as at pre-B stage from which CD21 is invariably absent (Tedder et.al., 1984). In normal development, the precursor cells appear to proceed through a pre-B cell stage (Cooper et.al., 1983) and develop to mature B-cells by gradually losing CD10 and TdT activity, by orderly acquiring surface CD20, CD21, CD22 antigens and by inserting IgM and subsequently IgD, to the membrane. The CD21 antigen is lost during early stages of terminal B-cell differentiation with the loss of sIg (Boyd et.al., 1985). The CD21 expression appears to be confined to a rather narrow span of the cell differentiation and restricted to mature B cells. The present investigation implies that B-CLL represents a stage equivalent to the mature B lymphocyte and $CD45RA^{+}CD21^{+}$ may represent a less mature state than $CD45RA^{-}$ $CD21^{-}$.

It is of great interest that the expression of band 4.1 and 4.3 are positively correlated with that of the CD45RA, particularly, as no clustered surface antigens have molecular masses come close except CD22 and CD11 antigens both of which have been ruled out by the antigen-binding test (see **Chapter 6**). These two proteins are, therefore, probably unidentified surface markers and appear to be intimately related to cell activation. It is intriguing that the above correlations in the B-CLL panel are also echoed in PLL and normal tonsilar cells.

The 160KD band 4.1 is not only expressed by B cells but also by T cells, as demonstrated by two observations: (1) comparison of band 4.1 expression in pure B cells with B and T cell mixture shows that band 4.1 is increased considerably in B-T mixture. (2) T-CLL express a significant amount of band 4.1 although CD45RA and band 4.3 and CD21 are not detectable. Jurkat cell line, which is derived from T cell type ALL, possesses a strong band 4.1. Further work needs to be done to clarify this

point before the established relationships of abovementioned proteins can be extended to T cell type malignancies.

The discovery of the band 2-CD45RA relationship is probably the most marked merit of the HPLC-SE application. The acquisition of this 300KD component appears to be linked to the inversion of CD45RA determinant to 200KD/180KD determinants. It is not known whether there is a threshold for its appearance or whether there is any relationship between its expression and the ratio of CD45RA to 200KD/180KD. Analysis of normal purified tonsilar B, B and T cell mixtures suggests that band 2 is probably expressed only by malignant cells. Band 2 positivity in T-CLL further confirms the proposal. We have come to the conclusion that band 2 is associated with malignant state of cells rather than cell type. The detection of this component in Burkitt's lymphoma cell line (Namalwa) and particularly the finding of the correlated change of 1A and 1B features further supports this point. However, it might also be possible that band 2 is associated with cell activation and the B-CLL cells of those patients who express this component may be differentiationally blocked at an activated stage. Similar investigation in normal B and T cells should be able to answer the questions.

Together with the conclusions discussed above and those previously described, different cell types have constructed a phenotypically continuous spectrum of surface antigen expression. CLL patients possess heterogeneous phenotypes which are distinguished by the expression of CD45, CD21, band 4.1, band 4.3 and band 7 and band 2.

3. Phenotypic Changes Upon TPA Activation

With HPLC, the changes of surface markers caused by TPA are more pronounced than those detected with FACS. Band 4.1, band 4.3 and band 2 appear to be closely associated with cell activation. As it was reported that CD45 determinants changed in a similar fashion in B-CLL as during T cell development, band 4.1 in T cell may be expected to show similar alteration as in B-CLL. The levels of band 2 after TPA activation seem to be related to the initial cell state.

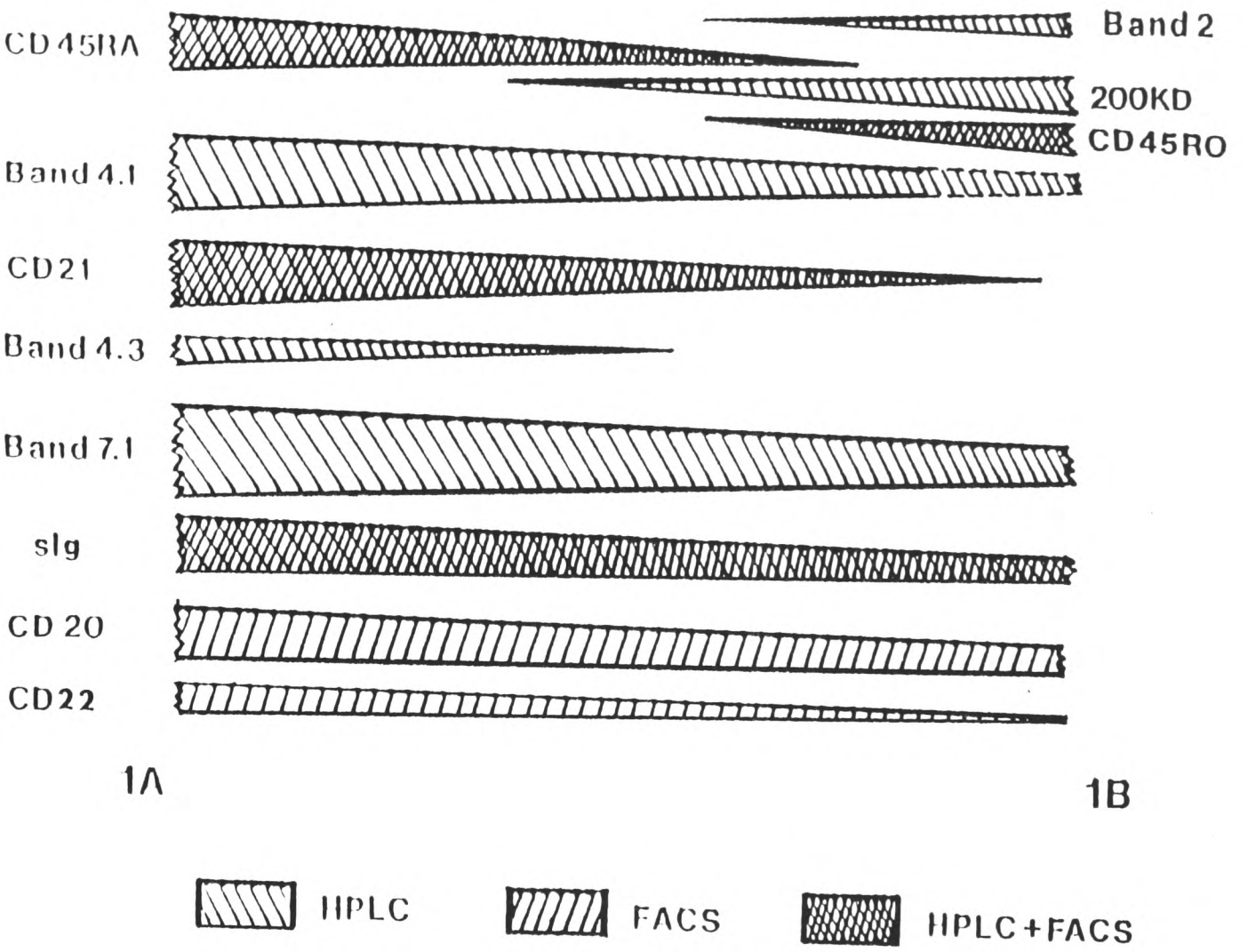

Fig 1. The schematic presentation of the correlations of surface proteins of B-CLL. The HPLC data are based on the levels of radiolabelling of the proteins. The broken lines in band 4.1 indicate the inconsistency of its expression at the 1B end. The 200KD bar refers to the 200KD chain of the CD45 antigen.

Numerous experiments have suggested that TPA activation does induce cells towards to a more advanced stage as reflected by the decrease of CD20, CD21, sIg and induction of cytoplasmic or even secreting Ig. Several authors have also shown similar surface phenotypic alterations during normal lymphocyte development (Freedman et.al., 1987; Akbar et.al., 1988). Comparing the spectrum of phenotypic variations along 1A and 1B cells with that of TPA-induced, it is possible that 1A represents a less mature state while 1B a more mature stage, with intermediate stages in the middle.

4. Relationship Between CLL, PLL and HCL

This is a highly controversial topic as there is a huge body of contradictory observations available. The argument seems to involve mainly two different but related questions: are CLL, PLL and HCL confined to the same differentiation pathway and if they are, what are the relative positions of these leukaemias along the pathway? or do they belong to different lineages? The antigenic profile of the PLL is rather similar to that of B-CLL and consistent, showing $CD10^-$ $CD19^+CD20^+CD21^+CD22^+CD24^+$ but $CD5^-$ and strong sIg^+ which confines it to B-cell lineage. A striking feature of PLL is that it uniformly reacts with an unclustered B cell related mAb FMC-7 (Catovsky et.al., 1981) but does not form rosettes with MRBC. The significance of these differences is still debatable. There are a few reports indicating that CLL cells, upon TPA activation, are induced to express FMC-7 (Robert et.al., 1983). Although most cases of PLL are $CD5^-$ (Gobbi et.al., 1984), CD5 positivity has been observed in a few cases (Stein et.al., 1984). On the other hand, caution must be excercised when MRBC receptors are regarded as a differentiation marker. It has been reported that the MRBC rosetting ability could be lost soon after normal purified $MRBC^+$ cells were cultured in the absence of TPA (Caligaris-Cappio et.al., 1984), as are also the case for malignant cells.

PLL is usually regarded as a variant of B-CLL but its maturation position is not certain. Morphologically, the PLL cells maintain an immature apperance with 1-2 conspicious nucleoli (Galton et.al., 1974; Catovsky, 1977) and may contain intracytoplasmic granules. PLL expresses far more sIg than B-CLL, usually sIgM, and

low MRBC (Koziner et.al., 1980). The reponsiveness of PLL to TPA is variable, 1 of 6 samples became cIg$^+$ (Ziegler-heitblock et.al., 1986).

In this study, the fall of sIg level after B-CLL cell activation and a strong CD45RA expression in HCL both argue against the proposal. The TPA stimulation apparently does not drive CLL to PLL-like phenotype but rather away from it. Phenotypically, PLL is closely related to 1A type, therefore, being at a less mature state. The reponse of PLL to TPA stimulation is variable (Ziegler-Heitbrock et.al., 1986; Yukihiro et.al., 1989) and less consistent when compared with CLLs. It should be conceivable that the responsiveness of a given clone to the stimulant may be related to its stage of differentiation arrest reflecting its 'readiness' to receive appropriate external stimuli, i.e. there may a maturation stage beyond which TPA can not exert its effect. There are studies showing that CLL cells bearing high sIg tend to respond to TPA poorly (Okamura et.al., 1982).

The assignment of HCL to the B cell lineage was largely based on its intrinsic sIg (Rieber et.al., 1979) and the immunoglobulin-gene rearrangement (Korsmeyer et.al., 1983; Cleary et.al., 1984). HCL shares two common feature with B-CLL: (1). the expression of T-cell related features of E-rosette$^+$ (Burns et.al., 1977) and rosette receptor after *in vitro* mitogenic activation (Guglielmi et.al., 1980) (2). the presence of autoimmune manifestations and autoimmune phenomena (Westbrook and Golde, 1985). However, several criteria in addition to the peculiar morphology, discriminate HCL cells from chronic B cell malignancies: strong cytoplasnmic TRAP activity (Yam et.al., 1961), strong Tac$^+$ (CD25) reactivity (Korsmeyer et.al., 1983) and positivity with mAbs such as SHCL3 (CD11b), HC1 and HC2 (Schwarting et.al., 1985). In addition, CD5 is absent from the majority of HCL cases (Stein et.al., 1984).

Caligaris-Cappio et.al. (1984) found that CLL B cells bind CD22 and become strongly TRAP positive and Tac$^+$ (CD25) after TPA stimulation and suggested that cells of CLL and HCL follow the same differentiation pathway as TPA can induce CLL cells to express phenotypic and morphological features of HCL. However, recently, Yukihiro et.al. (1989) argue that the induction of TRAP activity is not related to the chronic B cell leukaemia cell type. The acquisition of hairy cell

morphology in B-CLL after TPA activation is also a highly debatable topic. Some report such acquisitions (Rosver and Golomb, 1980; Caligaris-Cappio et.al., 1984), the others do not (Polliak et.al., 1986; Ziegler-heitblock et.al., 1986; Yukihiro et.al., 1989). Conversely, HCL is not induced to develop B-CLL features although displayed abundant cytoplasmic Ig (Caligaris-Cappio et.al., 1984), which may be in concert with mature features of HCL as indicated by its expression of sIgG (Jansen et.al., 1982) and reactivity with the plasma cell-specific mAbs PCA-1 and PC-1 (Anderson et.al., 1985). These findings may regard HCL as an 'activated' B cell leukaemia.

As the expression of surface proteins, particularly those differentiation markers, is intimately related, any attempt to define cell differentiation stage and pathway by simply using a single marker is oversimplified and jeopardized by this complication, as exemplified by the changes of band 2 and band 4.3. Both components are only present at certain stages but form undissociable parts of the cellular events. Therefore, we here propose that the understanding of the differentiation events and cell lineages should be based on a comprehensive evaluation and assessment of the compilation of those related markers and the overall phenotypic variations.

Taking these observations together, it is hard to definitely conclude the placement of CLL, PLL and HCL along the differentiation pathway. However, a careful analysis of the present data suggests that B-CLL and PLL are closely related disorders. Evaluation of various features of PLL favours its less mature state, so as implicated by its similarities with 1A type CLL. The phenotypic alterations induced by TPA have further confirmed this hypothesis. As we have no opportunity to investigate HCL, a direct comparison of HCL with B-CLL, PLL and other cell types is not possible here. However, HCL appears to be a more mature variant of chronic B leukaemia, compared with B-CLL and PLL. However, the possibility that B-CLL, PLL and HCL originate from distinct lineages can not be excluded as conspicious phenotypic differences between FMC-7$^+$ and CD5$^+$ populations are documented (Melo et.al., 1986). Such exclusive expression of FMC-7 and CD5 may have significant implication that different etiologies of B cell malignancy may depend on the original target B cell subset. Before the normal counterpart of B-CLL can be convincingly addressed, further information is desirable.

According to the surface molecular profiles of HPLC-SDS PAGE two-dimentional matrix, the delineation of PLL, CLL and HCL, along the differentiation pathway, probably is: PLL > 1A CLL > 1B CLL > HCL (?)

5.Putative Normal Counterparts of B-CLL, PLL and HCL

Accumulating evidence suggests that B-CLL lymphocytes represent the expansion of a subpopulation of B cells arrested at a stage between pre-B and mature B lymphocytes. It should be conceivable that a normal counterpart, bearing corresponding features of the malignant cells, can be found. Bofill et.al. (1985) and Caligaris-Cappio and Janossy (1985) described a B cell subpopulation in foetal primary follicles, which bears typical B-markers together with the T-cell-specific antigen CD5 though at a low level (Bofill et.al., 1985). $CD5^+$ B-cells are first detectable in the lymph nodes around the seventeenth week of gestation (Bofill et.al., 1985), becoming prominent at the early phase of the B cell development. The ontogenic investigation shows that the number of $CD5^+$ B cells is inversely related to age (Caligaris-Cappio and Janossy, 1985). About 40% of Ig^+ lymphocytes in fetal spleen and lymph nodes are CD5 positive while in adults the numbers are critically lower--about 5% of lymph node B cells are $CD5^+$ and they are confined to the edge of germinal centres in secondary follicles (Caligaris-Cappio et.al., 1982). These $CD5^+$ B cells also distinguish themselves from other B cells by the property of forming rosettes with mouse red blood cells (MRBC) (Caligaris-Cappio et.al., 1982), resembling CLL B cells. In addition to the phenotypic similarities, $CD5^+$ B lymphocytes of foetal lymph nodes and spleens share a number of other features with CLL B cells such as lectin unresponsiveness and the inability to cap sIg. These lines of observation lead Caligaris-Cappio et.al. (1982) to postulate that this $CD5^+$ B cell subpopulation is the normal counterpart of CLL B lymphocytes.

The murine homologue of CD5 is the Ly-1 antigen (Manohar et.al., 1982). $Ly\text{-}1^+$ cells exhibit many similarities with the $CD5^+$ B cells in human. cDNA clones have been isolated for both Leu-1 (CD5) (Jones et.al., 1986) and Ly-1 (Huang et.al., 1987). Both sequences show 63% identity and 90% homology in their carboxy terminal regions with conservation of a cystein-rich amino-terminal region. In most

mouse strains including neonatal nude mice (Hayakawa et.al., 1983), Ly-1 antigen is co-expressed with a cell population expressing sIgM and sIgD, which is mainly located in the spleen. Ly-1^+ B cells appear early in ontogeny and progressively decrease with age (Hayakawa et.al.,1983). Ly-1^+ B cells can be stimulated, by some T-independent antigens, to polyclonally expand in NZB and (NZB x NZW) F1 mice and to secrete *in vitro* high levels of autoantobodies (Hayakawa et.al., 1983). The progenitors of Ly-1^+ B cells have been demonstrated to be distinct from that of Ly-1^- B (Hayakawa et.al., 1985) and follow a different developmental pathway (Hayakawa et.al., 1985). Several biochemical similarities between Ly-1 and CD5 have been documented (Caligaris-Cappio et.al., 1981). It seems that $CD5^+$ B cells are the human counterpart of the mice Ly-1^+ B subpopulation. Recent studies implicate that the CD5 population may be involved in the production of autoantibodies in man (Casali et.al., 1987; Hardy et.al., 1987).

Although a substantial body of evidence supports the notion that $CD5^+$ B cells may be the normal counterpart of B-CLL, proof that $CD5^-$ B cells may represent a distinct pathway of B-cell development is lacking. There have been reports that TPA (Smith et.al., 1985) can induce the expression of CD5 antigen in normal B cells and that of FMC7 in CLL B cells (Caligaris-Cappio et.al., 1984). Anderson et.al. (1984) show that a small subset of circulating peripheral B lymphocytes bear a similar $CD5^+$ B-CLL phenotype and that they are additionally C_{3b} negative. CD5 expression in B-CLL appears to be an inherent feature as attempts to induce Ly-1 or CD5 expression on already differentiated (sIg^+) B cells have consistently failed (Ritz et.al., 1981; Hayakawa et.al., 1984).

PLL is characterized by its expression of FMC-7 (Catovsky et.al., 1981) and morphorlogically, immature appearance with prominent nucleoli (Galton et.al., 1974; Catovsky, 1977). A tentative normal counterpart of B-PLL has been suggested. Robinson et.al. (1985) defined a subset of B-cells in normal peripheral blood B lymphocytes which were characterized by the presence of a prominent nucleolus and less nulear chromatin condensation and its reactivity with FMC-7. Caligaris-Cappio and Janossy (1985) found a subset of cells in the mantle zone of secondary follicles which show typical features of B-PLL: high sIg, $RFA4^+$ (CD22) and $CD5^-$ and $MRBC^-$.

Very few observations are available for the designation of a normal counterpart for HCL. Robinson et.al. (1985) identified a population of B cells in peripheral B lymphocytes, with morphologically villous outlines and phenotypically being reactive with FMC-7 and aHC1 and aHC2 (Posnett et.al., 1982), both are anti-hairy cell mAbs. The authors claim that this B-cell subpopulation may constitute the normal counterpart of HCL. Normal $MRBC^+$ B cells from tonsils were reported to acquire TRAP positivity and a lymphoblastoid appearance, but no sufficient data are available for an insight of the normal counterpart. It is also suggested that the 'hairy' feature may arise only as a consequence of the neoplasia (Caligaris-Cappio et.al., 1984).

So far, all such hypotheses are tentative and the evidence is not compelling. Although all haematopoietic-originated cells share common precursors the potential 'target' for malignancies is broad which may account for the complexity seen in leukemias. On the other hand, differentiationally arrested cells, though initially homogeneous, may still retain some capacity for further differentiation, therefore, developing phenotypic features distinguished from those of the cells which the disease was initiated. Malignant cells, even derived from the same lineages, should be expected to have different morphological and phenotypical presentations if cells are transformed at different oncogenic sites.

Over the past decades, the surface phenotypic characteristics of malignant cells have been gradually elucidated by the extending availability of monoclonal antibodies. Remarkable progresses have been made in the identification on B-cells of a mosaic of molecules strictly associated to the process of differentiation, maturation and/or activation (Bernard et.al., 1984; einherz et.al., 1986). The understanding of the tissue distribution, traffic characteristics and cell-cell interactions of malignant and normal B-lymphocytes is also updated. The expression of the B-cell-specific CDs (CD19-23) and the B-cell-associated CDs (CD9, 10 and 24) in a coherent fashion during normal B-cell differentiation suggests significant implications of the phenotypic features with respect to the physiological states of the cells. CLL lymphocyte provides an excellent model for this valuable venture as they possess some distinct abnormal features. The relationships between membrane protein reportiore and cellular events will greatly facilitate the understanding of not only the malignancies of the cells, but

also the haemotopoietic events of normal lymphocytes, and, from a general view, the siginificance of the biological membrane functions.

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APPENDIXES

Appendix I. Relative levels of vectorially iodinated surface proteins of B-CLL patients. The assessment is based on a comparative ground by using band 3 as a framework of reference. The band 3 is marked five pluses. Any components of higher level than band 3 also receives five pluses. (1) 1**B** type (2) 1**A** type.

- : undetectable
+(+) : level between + and ++
+/- : faint or not consistently detected
/ : not possible for detection

1. patient Lot. investigated in 1987
2. patient Lot. investigated in 1989
3. PLL
4. patient Par. investigated in 1987
5. patient Par. investigated in 1988
6. T-CLL

APPENDIX I (1) 1**B** type

Patients	Relative Surface Iodination Levels										
	CD45 (KD)				Surface Components						
	230	215	200	180	4.1	4.2	4.3	2	7.1	sIg	CD43
Web	/	/	+	−	−	++	−	/	++(+)	+(+)	+++
Tot	−	+(+)	+++	++++	+(+)	−	−	+++	++	++++	++
Sow	+(+)	++	+++	+	+(+)	++(+)	−	/	++	++	+++
Tho	+	++	++++	/	++	+++	−	+(+)	++(+)	++(+)	++
Wod	+	+++	++++	+	++	−	−	++(+)	++(+)	+(+)	++(+)
Par(87)[4]	+	++	+++(+)	+	−	+(+)	−	+	++	+/−	+/−
For	+	++	++++	+/−	++	+	+/−	+	+	+/−	+/−
And	+	++	++++	+	+/−	+++	−	+(+)	+	+/−	−
Bla	++	++(+)	++++	−	+	++	+	+(+)	+++	++	+
Boy	+/−	++	++++	+	+	+	/	+(+)	/	+	+
Col	+++	+++	++(+)	−	+	++	−	−	/	+/−	−
Par(88)[5]	++(+)	++	++(+)	−	++++	++(+)	++	−	+++	++(+)	++
Sut	+	+++	++++	−	++++	++	−	/	++	++(+)	/
Mat[6]	+	++	+++	+/−	++++	+(+)	+	+/−	+++	+	++(+)
Smr[7]	+/−	+	++++	++++	++++	−	/	++	/	/	/

(2) 1**A** type.

Patients	Relative Surface Iodination Levels										
	CD45 (KD)				Surface Components						
	230	215	200	180	4.1	4.2	4.3	2	7.1	sIg	CD43
Pau	+++++	+(+)	–	–	++	+++	+/–	–	+++(+)	++++	+++
Sca	+++	++	++	–	+++	+	++	–	+++(+)	++(+)	++
McG	+++++	+	+/–	–	+++(+)	+++(+)	++	–	++++	++++	++++
Kay	++++(+)	/	+	–	+++(+)	+++	++	–	+++	++++(+)	+
Lot(87)[1]	++++	–	(++)	–	+++++	++++	++	–	+++++	++++	(+)
Cur	+++++	+++	(+)	–	+++	++++	+	–	+++	+	–
Jac	++++	++	(+)	–	++++	++++	+	–	+++++	++++	+++
Ske	++++	+++	++	–	+(+)	+++	/	–	/	+	/
Gri	+++ (+)	+++	++	–	++	++(+)	+	–	/	+++	/
Bur	+++	+++(+)	++	–	+++	+++	++	–	+++++	++	+++
Lyn	++++	+(+)	–	–	+++(+)	+++(+)	++	–	+++	++++	/
Lot(89)[2]	+++	++	++	–	++(+)	++	–	(+)	+++	++	+/–
Gil	++(+)	++++	++	–	++++	++	/	–	+++	/	/
Wal	+++	+/–	–	–	+++	++++	/	–	/	+	+
Hay[3]	+++++	+/–	–	–	++	+++	++	–	++++	+++++	–

APPENDIX II (1)

Namalwa Sublines	Relative Surface Iodination Levels										
	CD45 (KD)				Surface Components						
	230	215	200	180	4.1	4.2	130 KD	2	50KD	sIg	Class II
IPN/45	++++	+	–	–	++	+++	+++++	+	+	+++	+/–
PNT	++(+)	++	–	–	+	+++	++++	+	+++	++(+)	+ +
KN2	++	++	–	–	+	++	++	+	++++	+	+++++
45.43	+	++(+)	+++(+)	–	+	+++++	+++++	+	+/–	–	+/–

Appendix II (1): The rating of the relative levels of surface iodinated components of Namalwa cell lines. The rating is based on several investigations conducted during the course of the study. Signs are defined in a same way as in Appendix I. The relative levels were also confirmed by FACS study.

(2)

Surface Components	
230KD	45.43 → KN2 → PNT → IPN/45
MHC Class II	KN2 ← PNT ← IPN/45 ← 45.43
surface Ig	KN2 → PNT → IPN/45 ⋯→ 45.43
4.2 (CD21)	KN2 → PNT → IPN/45 → 45.43
50KD	45.43 ← KN2 ← PNT ← IPN/45
130KD	KN2 → PNT → IPN/45 → 45.43
4.1	45.43 → KN2 → PNT → IPN/45

Appendix II (2): The relative positioning of the four sublines of Namalwa with respect to their expression levels of surface markers. The arrow points the increase of the level. '⋯→' means 'not concluded'. 230KD refers to the highest chain of CD45 antigen.

APPENDIX III (1)

Proteins	MW (KD)	Diol 100	Diol 500/with TX-100
		Retention Time (Min)	
α -Lactalbumin	14.2	11.3	12.9/-
Lysozyme	17.5		13.1/13.9
Carbonic Anhydrase	29	11.4	13.2/12.8
Cytochrome C	13		
Albumin	66	10.4	11.8/11.5
EZB 23	150		12.1/12.0
Urease	240	12.0	13.6/13.7
Albumin (Egg)	45	10.6	12.0/12.1
IgM	900		9.9/-
Thyroglobulin	669		9.6/9.4
ß-Lactoglobulin	37.1		13.0/12.6
Ferritin	450		10.6

Appendix III: Retention times (in min) of size-exclusion columns calibrated against standard proteins. The calibration was repeated for at least 2 times, mostly 3 times for each individual columns. The deviaitions of the retention time for individual proteins at different runs were not more than 0.2 min. The data shown here are the averages.

(2)

Proteins	MW(KD)	GF-250	GF-450/with TX-100
		Retention Time (Min)	
BSA	68		10.7/10.8
EZB 23	150	9.7	10.3
Carbonic Anhydrase	29	11	11.4/11.8
Spectrin	230	8.0	
Urease	240		12.0/11.6
Cytochrome C	13	13	12.1/11.8
α -Lactalbumin	14.2	11.3	11.6
IgM	900	7.5	7.6
Lysozyme	17.5		13.4
Thyroglobulin	669	7.7	8.7/8.0
ß-Lactoglobulin	37.1	10.6	11.4/11.4
Albumin (Egg)	45		11.0/11.2
Ferritin	450	8.6	9.1
IgG	150	9.2	

Identification of antigens by high-performance liquid chromatography

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Traditional methods for the identification of antigens usually involve isolation of an antigen/antibody complex by precipitation or binding to a solid-phase preparation of the antibody. Here we introduce an alternative method which identifies antigen by h.p.l.c. An appropriate antibody is added to a mixture of proteins containing the antigen and the elution profile of this mixture compared with the profile of the mixture in the absence of the antibody. The antigen is then recognized as the component whose retention time has been reduced by the addition of the antibody when it is eluted as an antigen/antibody complex.

Cells are washed and suspended in an isotonic Hepes buffer at pH 7.4 and iodinated in the presence of lactoperoxidase (Thompson *et al.*, 1980). After iodination, the cells are washed in the same buffer lacking divalent cations. Cells (5×10^7) are suspended in 100 μl of the buffer and extracted with 100 μl of a 1% Triton X-100 solution in the buffer containing EDTA, phenylmethanesulphanyl fluoride, aprotinin and iodoacetamide, for 10 min on ice. Insoluble material is centrifuged out.

The saturating amount of each antibody is determined by flow cytometry of the intact cells and this amount of antibody added either to the iodinated Triton X-100 extract or to the intact cells before their extraction. In the latter case, excess antibody can be washed away and the appropriate anti-immunoglobulin antibody can be added as a second-step reagent to the mixture.

Extract, with and without antibody, is fractionated on a DuPont GF450 size exclusion column using a mobile phase of 0.2*M*-phosphate buffer (pH 7.4) containing 0.5% (w/v) Triton X-100 and sodium azide as an antibacterial. Samples (100 μl) of the extracts containing the equivalent of 2.5×10^7 cells are used for each run. Fractions are collected on a time mode at 0.2 min intervals. Each fraction is analysed by SDS/polyacrylamide-gel electrophoresis in a Laemmli buffer (Laemmli, 1970).

The method has been tested with antibodies against determinants on the surface of B-lymphocytes ranging from a molecular mass of around 200 kDa (the leucocyte-common antigen) down to class II major histocompatibility complex (MHC) with a mass of around 40 kDa. Fig. 1 illustrates the method using the antibody W6/32 (ex Serotec), a monoclonal antibody against class I MHC. The radioactive 45 kDa of the determinant is found in fractions 16–17 in the sample without antibody. After addition of the antibody to the cells, before Triton X-100 extraction, the determinant is found in fractions 12–13 in the subsequent eluate. When anti-mouse immunoglobulin has been added to the extract a still larger complex is formed and the radioactive MHC molecule is now found in fractions 3–4.

We have drawn attention to the potention of h.p.l.c. as a technique for the identification of antigens. The combination of h.p.l.c with SDS/polyacrylamide-gel electrophoresis is also a powerful method for the analysis of the polypeptide composition of a complex mixture of proteins such as is found in a membrane. The proteins are displayed as a two-dimensional matrix, the position of each band having been determined by several factors. Minor differences between the mixtures, e.g. those found between different clones derived from a tumour, are readily resolved. The method has certain advantages over other methods for the identification of antigens as it is a one-step procedure involving no prolonged washing which could disrupt the binding of antibodies of low affinity. In many cases the antigen can be located on the gel by silver staining without recourse to a radioactive label. The method can be extended in several ways, e.g. the analysis of the epitopic specificity of antibodies. If a determinant is challenged with a mixture of antibodies against the same epitope only one immunoglobulin molecule will be bound, but if the antibodies are directed against different determinants more than one immunoglobulin molecule may bind with a commensurate greater effect on the retention time of the antigen. The method can, in principle,

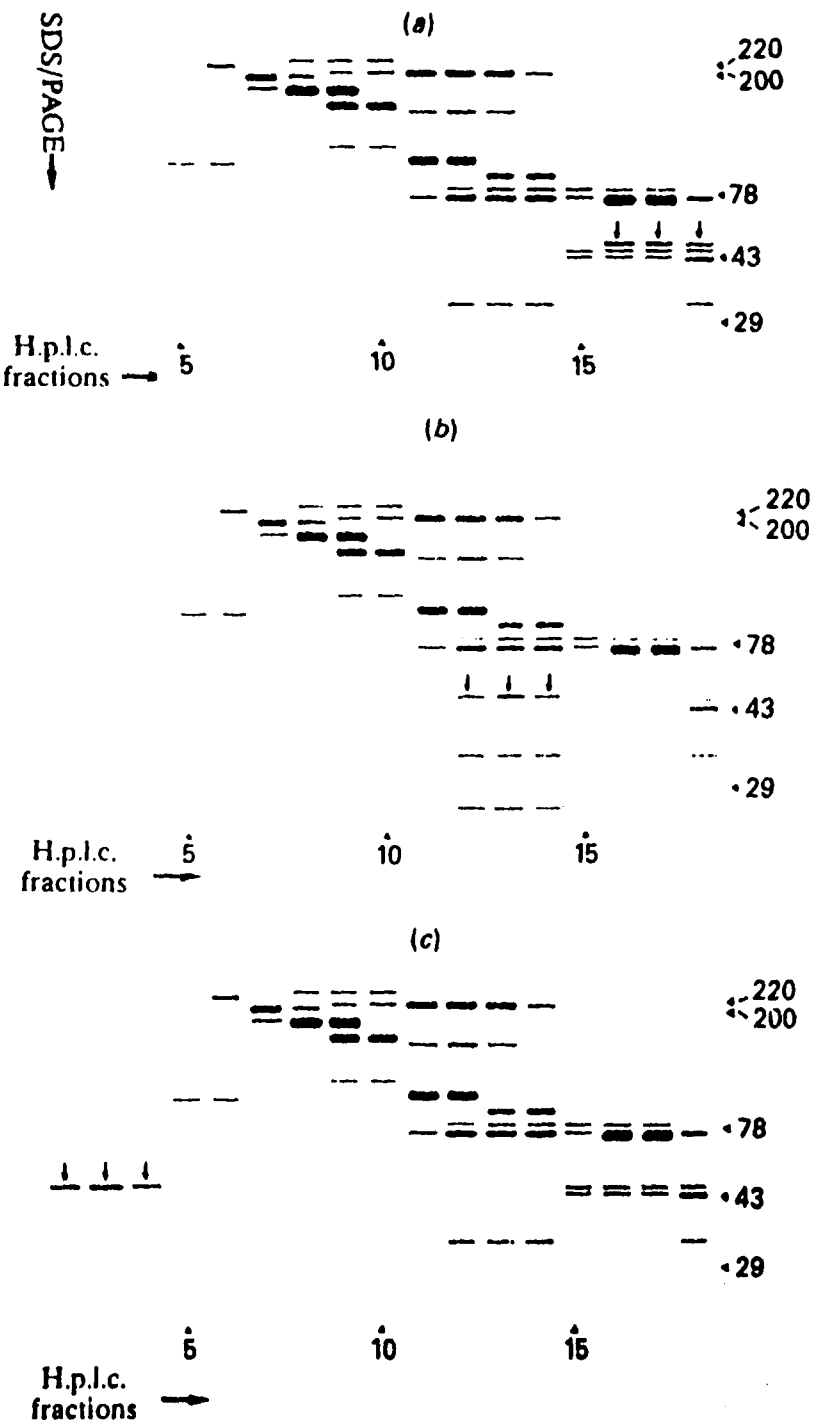

Fig. 1. *Effect of the addition of anti-class I MHC antibody on the elution of the class I determinant of human chronic B-lymphocytic leukaemia lymphocytes*

(*a*) No antibody. (*b*) Antibody added to cells before Triton X-100 extraction. (*c*) Anti-mouse immunoglobulin added to Triton X-100 extract before chromatography. The Figure represents the fluorograph prepared from cells iodinated by lactoperoxidase. Abbreviation: SDS/PAGE, SDS/polyacrylamide-gel electrophoresis.

Abbreviation used: MHC, major histocompatibility complex.

be used for the identification of any ligand–receptor complex where the size of the ligand is appreciable in relation to the size of the receptor.

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The identification of antigens in antibody/antigen complexes using high performance liquid chromatography

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A method for the identification of antigens in a complex mixture of proteins is described. It exploits the effect which addition of a monoclonal antibody and resultant formation of antibody/antigen complexes has on the apparent retention time of the antigen on a size exclusion column. An antigen in the mixture can be identified as that component whose retention time is decreased by addition of its antibody. The principle of the method is demonstrated using an HPLC size exclusion column (DuPont GF450) and three MoAbs, PD7/26, DA6 147 and W6/32 which are directed against three antigens of the lymphocyte surface, the leucocyte-common antigen, class II MHC and class I MHC respectively.

Key words: Chromatography, high performance liquid; Antigen identification

Introduction

The identification of an antigen usually involves its solubilisation and precipitation from solution by its antibody, or adsorption onto its antibody on a solid phase (Goding, 1986). In either case the other proteins in the solution must be separated from the antigen/antibody complex by some washing procedure. The process is complicated in the case of insoluble antigens such as membrane proteins by the requirement for detergents to be present throughout the procedure to retain the antigen in a soluble state. The alternative approach by Western blotting is unsuitable for use with monoclonal antibodies if the relevant epitope is labile during the SDS-PAGE step which precedes the blotting. We here present a technique for the identification of antigens by size exclusion HPLC which obviates the need for a washing procedure and the use of SDS prior to addition of the antibody. The method exploits the increase in the apparent size of an antigen when it is associated with its antibody in the antigen/antibody complex. The antigen can therefore be distinguished from other proteins in the eluate as the component whose retention time is decreased by the addition of the antibody.

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Abbreviations: CLL, chronic lymphocytic leukaemia; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecylsulphate; Hepes, hydroxyethylpiperazine-ethanesulphonic acid; HPLC, high performance liquid chromatography; MoAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulphonyl fluoride.

Methods

Cell preparation

B-CLL lymphocytes isolated from whole blood on Ficoll-Paque (Pharmacia) were washed and suspended in a solution consisting of 133 mM NaCl, 4.5 mM KCl, 5 mM $MgCl_2$ and 2 mM

$CaCl_2$ buffered to pH 7.4 with 10 mM Hepes (buffer 1).

Lactoperoxidase catalysed iodination

1×10^8 cells were suspended in buffer 1 containing 10 mM glucose, 0.05 U glucose oxidase (*Aspergillus niger*, Sigma), 1.5 U lactoperoxidase (bovine milk, Sigma), 0.5 μM $K^{127}I$ and 100 μCi $Na^{125}I$ and incubated for 20 min at 37 °C. Unbound iodine was then removed by repeated washing with ice-cold buffer 1.

Triton extraction

The cells were washed once in buffer 1 lacking divalent cations (buffer 2). 1×10^8 cells were then suspended in 200 μl of buffer 2 and 200 μl of a 1% Triton X-100 solution in buffer 2 (containing EDTA (2 mM), PMSF (1 mM), aprotinin (0.5 TIU/ml) and iodoacetamide (5 mM) to inhibit proteolysis) and the mixture incubated on ice for 10 min. Insoluble material was centrifuged out at $900 \times g$ for 10 min.

Addition of antibody

The requisite concentration of antibody was initially determined by titration of the cells against a series of antibody concentrations, measuring antibody binding by flow cytometry (Brown et al., 1987). Antibody was then added to this saturating concentration, either to the iodinated Triton extract or to the iodinated intact cells, and incubated for 45 min on ice. In the latter case excess antibody was washed away before the Triton extraction and rabbit anti-mouse immunoglobulin (Dako) was added as a second-step to this extract using 80 μg/200 μl extract to enhance the change in apparent retention time of the antigen.

High performance liquid chromatography (HPLC)

The Triton extract was fractionated by a DuPont GF450 size exclusion column (250×9.4 mm) on a Gilson Model 302 HPLC apparatus at a flow rate of 1 ml/min and a pressure of 30 bar. Zorbax PSM 150 DIOL was used as a packing medium for a precolumn. The mobile phase consisted of 0.2 M phosphate buffer (pH 7.4) containing 0.5% Triton and 0.005% sodium azide as an antibacterial agent. The eluate was monitored at 243 nm which was found to be a window in the spectrum of the detergent where proteins could be detected. Fractions were collected on a time mode at 0.2 min intervals. 100 μl of Triton extract containing the equivalent of 2.5×10^7 cells were used for each run.

Gel electrophoresis

100 μl of 125 mM Tris-HCl containing SDS 10%, glycerol 20%, bromophenol blue, 1 mM EDTA and 1 mM dithiothreitol were added to a 200 μl HPLC fraction and the mixture heated for 15 min at 50 °C. The mixture was then fractionated in a discontinuous buffer system (Laemmli, 1970) on a 6–11% polyacrylamide gradient, the gel fixed in methanol/acetic acid/water (2:1:7) and finally stained with silver (Thompson, 1987). Radioactive components were detected by fluorography using an intensifying screen and pre-flashed X ray film.

Results

The efficacy of the method was demonstrated using three MoAbs against antigens covering a wide range of molecular masses which are expressed on the surface of human B lymphocytes from patients with chronic lymphocytic leukaemia (B CLL).

Fig. 1 shows the effect of the antibody PD7/26 (CD45RB) which is bound by three polypeptides of the leucocyte-common antigen (Pulido et al., 1988). In our system these polypeptides have molecular masses of 230, 215 and 200 kDa range (Smith et al., 1985) and it is seen that three chains of this size have been moved from fractions 7–8 in the control to fractions 4–5. Fig. 2 shows a similar shift produced by the addition of DA6 147 which is an antibody against the α chain of class II MHC (Guy et al., 1986). Since the chromatography was performed in mild non-ionic detergent both α and β subunits of the antigen have shifted as a complex (fractions 14–15 to 11–13) although the antibody is specific for the α chain.

Fig. 3 illustrates an extension of the technique using anti-mouse immunoglobulin as a second step reagent. In Figs. 1 and 2 antibody was added to antigen which had been previously solubilised from

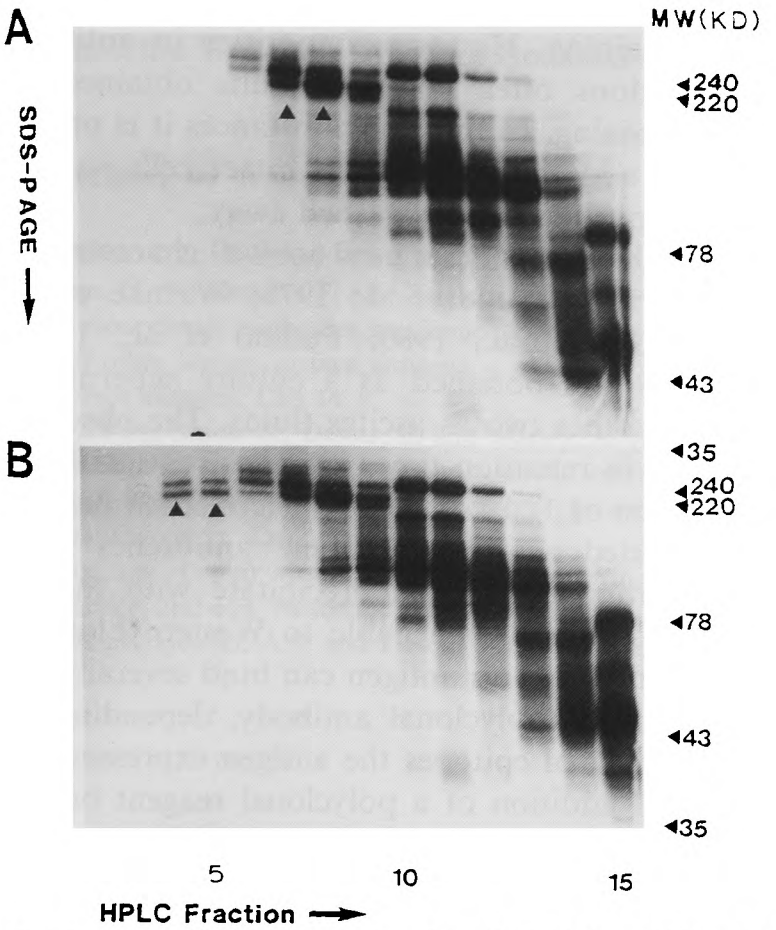

Fig. 1. Autoradiographs showing the effect of the addition of the anti-leucocyte common MoAb PD7/26 on the retention time of the antigen. It is seen that addition of the antibody has shifted the antigen from the HPLC fractions 7–8 in the untreated control (*A*) to fractions 4–5 in the eluate of the extract + antibody (*B*).

the cells by Triton extraction. In Fig. 3 an anti-class I MHC antibody W6/32 (Barnstable et al., 1978) has been added to the intact cells prior to Triton solubilisation and, as shown in Fig. 3B, the effect is very similar to Figs. 1 and 2. In Fig. 3C the shift in the position of the antigen has been amplified by addition of the anti-mouse immunoglobulin to the Triton extract. When a second step reagent is used it is preferable to add the first step to the intact cells, as shown here, since this provides the opportunity to wash away excess first antibody before addition of the second step.

Discussion

The combination of HPLC and SDS-PAGE provides a powerful technique for the analysis of complex mixtures of proteins, particularly those containing high molecular weight proteins. The proteins can be displayed as a two-dimensional matrix and each cell type has a characteristic pattern. The patterns shown in this paper were obtained from human B CLL lymphocytes. The polypeptide composition of the plasma membrane of these cells from different patient differs in detail (Smith et al., 1985) and, since three different patients were used these differences are reflected in the SDS gels in Figs. 1–3. Although the method discussed here arose from the need to identify the individual components of such polypeptide matrices, this technique can be extended as a general method for the identification of antigens recognised by monoclonal antibodies.

The technique of antigen identification can complement other procedures which are generally available. The isolation of the antigen/antibody complex is a simple, rapid, one-step process and involves no washing procedure. This can be an

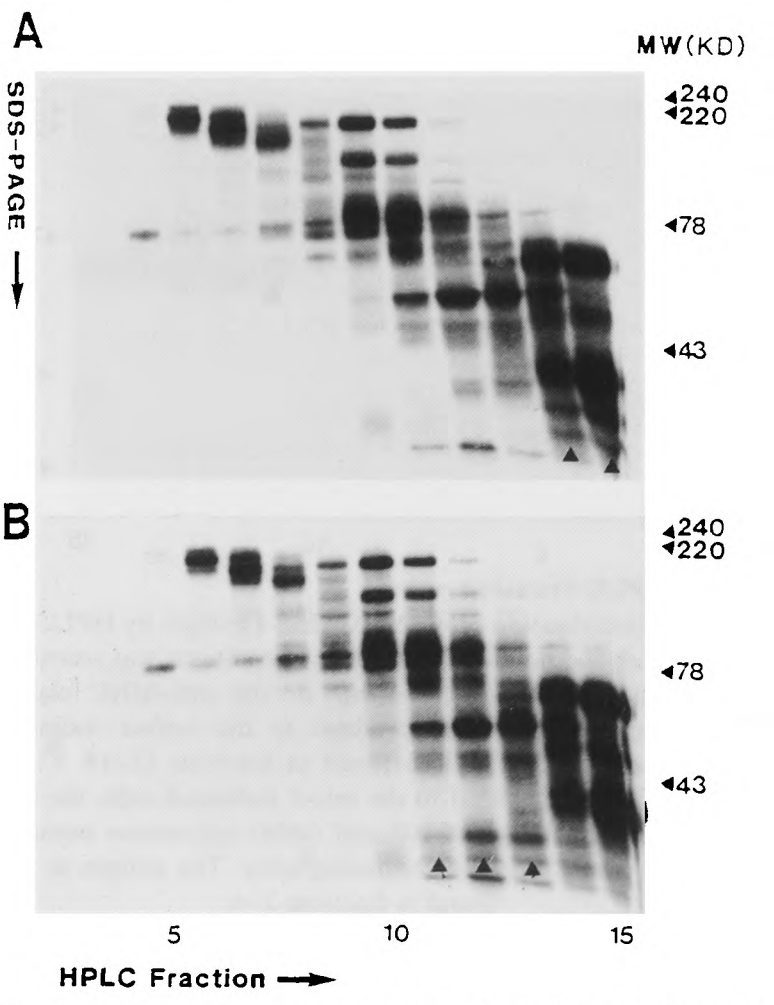

Fig. 2. Autoradiographs showing the effect of the addition of the anti-MHC class II antibody DA6 147 on the retention times of the MHC α and β chains. The antigen is moved from fractions 14–15 in the untreated control (*A*) to fractions 11–13 of the extract + antibody eluate (*B*).

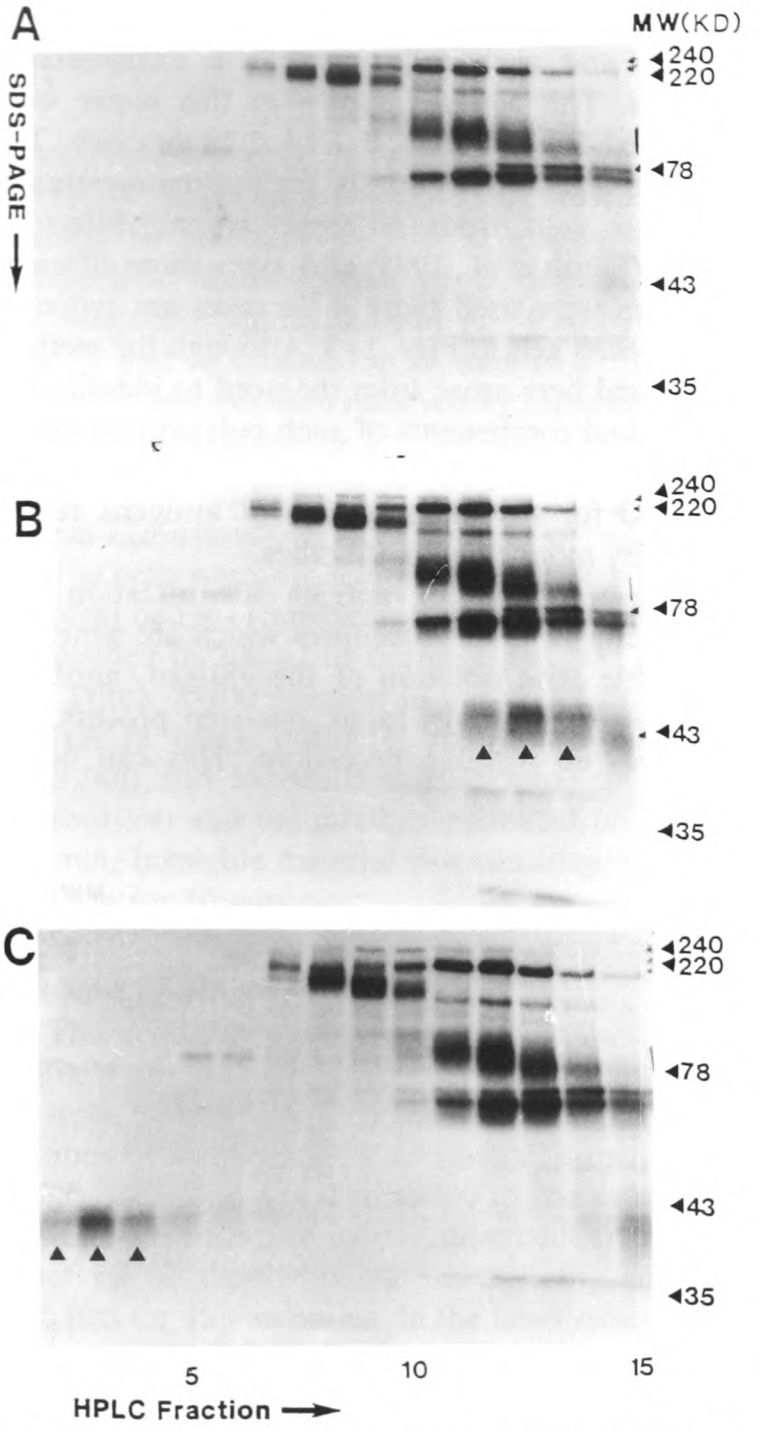

Fig. 3. Identification of the MHC class I antigen by HPLC. *A*: control eluate, no antibody added. The antigen was recovered in fraction 16–18 (not shown). *B*: the anti-MHC class I antibody W6/32 has been added to the surface iodinated intact cells and the antigen found in fractions 12–14. *C*: the W6/32 has been added to the intact iodinated cells, the cells extracted with Triton X-100 and rabbit anti-mouse immunoglobulin added prior to chromatography. The antigen is now found in fractions 2–4.

advantage for antibodies of low affinity. As SDS is not added prior to the interaction of antigen with antibody, the destructive effect of SDS which restricts the use of MoAbs in Western blotting is avoided. The method can dispense with the need for a radioactive label by locating the antigen by silver staining. However, impurities in antibody preparations often confuse results obtained by silver staining. In these circumstances it is preferable to add the antibody to intact cells so that such impurities can be washed away.

The three antibodies used are well characterised MoAbs (Barnstable et al., 1978; Warnke et al., 1983; Guy et al., 1986; Pudido et al., 1988). PD7/26 was obtained as a culture supernatant and the other two as ascites fluids. The observed changes in retention times are consistent with the formation of 1 : 1 complexes. The method has not been tested against polyclonal antibodies since such antibodies immunoprecipitate with facility and are generally amenable to Western blotting. Furthermore, as an antigen can bind several components of a polyclonal antibody, depending on the number of epitopes the antigen expresses, the effect of addition of a polyclonal reagent on the retention time of its antigen would be complex and might produce insoluble precipitates. For this reason addition of a polyclonal second step must be carried out with caution and the second step used at lower concentrations than would be customary for immunoprecipitation. We have been able to identify the determinant of an anti-leucocyte MoAb raised in this laboratory by this method when immunoprecipitation and Western blotting were unsuccessful.

The method may, in principle, be extended in a number of ways. It could be used to explore the epitopic specificity of antibodies. If a pair of antibodies do not compete for the same epitope two immunoglobulin molecules will bind to the antigen, resulting in a greater change in retention time than when either is used alone. Again, the method is not restricted to the detection of antigens but could be used for the identification of any ligand/receptor complex where the size of the ligand is appreciable relative to the size of the receptor and the complex stable in the non-ionic detergent.

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